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**Monotremata, Marsupialia and Placentalia:  
Inferring Phylogenetic Relationships From  
Molecular and Morphological Data**

**A thesis presented in partial fulfillment of the requirements for the  
degree of Doctor of Philosophy in Evolutionary Biology  
at Massey University**

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## Abstract

Living mammals are divided into three groups, Monotremata, Marsupialia and Placentalia. Complete mitochondrial (mt) genome sequencing has provided a large dataset for inferring phylogenetic relationships between these groups and within Placentalia. Marsupials however, are underrepresented, with only mt genomes from an opossum (*Didelphis virginiana*) and a wallaroo (*Macropus robustus*) having been published prior to this study. Herein I report complete mt genome sequences for two further marsupials, a northern brown bandicoot (*Isodon macrourus*) and a common brushtail possum (*Trichosurus vulpecula*). Phylogenetic analysis of the protein-coding and RNA-coding mtDNA sequences provides the first statistically robust support for placing bandicoots within the cohort Australidelphia (Australasian marsupials plus Microbiotheria).

Examination of vertebrate mtDNA sequences revealed two major problems for phylogeny reconstruction, (a) nucleotide (particularly cytosine versus thymine) compositional bias among taxa and (b) differences in substitution processes between data partitions (concatenation bias). Both biases enhanced the signal for Marsupionta, a grouping of marsupials and monotremes. When the DNA sequence was RY-coded (lumping C and T, and lumping A and G) and the maximum-likelihood analysis partitioned over subsets of similarly evolving sites the traditional morphology-based Theria hypothesis (marsupials plus placentals) was recovered.

The relationships of monotremes, marsupials and placentals to Mesozoic (65-250 mybp) fossil taxa that are known from near-complete skeletons were tested with dental, mandibular, vertebral, basicranial and upper and lower appendicular characters. The null hypothesis for the phylogeny of Mesozoic mammals that retained essentially ancestral mammalian niches (morganucodontids, (eutricodonts, (spalacotheriids, (eupantotheres, (eutherians, metatherians)))))) is strongly supported and congruent with respect to the above anatomical region partitions. The inclusion of the fossorial (echidnas) and semi-aquatic (platypuses) monotremes induces extreme and topologically complex incongruence between the anatomical region partitions. A novel incompatibility analysis for examining signal and conflict among characters indicates that homoplasy affecting monotreme placement evolved non-independently among characters, essentially at the level of the anatomical regions. After consideration of monotreme shoulder girdle and forelimb traits, in view of trends among the background phylogeny, it is apparent that correlated reversal of upper appendicular characters has occurred along the monotreme stem lineage. This is consistent with phylogenetic inference from both mtDNA and the other anatomical regions, which together suggest a close relationship between monotremes and therians.

As with the molecular data, the major problem affecting phylogeny reconstruction from morphological data is non-stationarity in evolutionary processes, such as functional convergence. Monotreme affinities appear to be affected by a less recognizable non-stationarity problem, in that niche-related modification results in attraction towards outgroup taxa. Comparison of morphological and molecular trees suggests that outgroup-attraction of “morphological long-branches” may be a common problem. Incorporating information on morphological evolutionary processes will be crucial for inferring the relationships of fossil taxa to extant mammals. Nevertheless, it is encouraging that analysis of RY-coded mtDNA data resulted in a placental tree, that unlike previous mtDNA trees, is entirely consistent with interordinal relationships inferred from long nuclear sequences. Furthermore, divergence dates inferred from the mtDNA analysis suggest that as well as monotremes, at least two marsupial and six placental lineages diverged prior to the Cretaceous-Tertiary boundary. However, it seems likely that the phyletic and ecological diversification of modern mammals was not tightly coupled, with the later diversification being largely restricted to the Tertiary period.

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Oddly enough, my PhD project was born out of an email I sent to David Penny about rates of evolution in plants. That a thesis on the early evolution of mammals resulted is an indication of the eclectic nature of the research interests that David and I share. As well as his encouragement for interdisciplinary studies, many forms of support and ability to evade being hemmed in by orthodoxy, I thank David for putting together and inspiring a stimulating and diverse research group. My other supervisors, Pete Lockhart and Mike Hendy have taught me much about phylogenetics and obediently signed all of the doctoral research committee forms. Cheers!

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After isolating myself during the writing of this thesis, I look forward to re-establishing many friendships and my interests outside of science. I also look forward taking my next steps in attempting to reconstruct evolutionary history.

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### **Papers included in this thesis**

- Phillips, M. J., Lin, Y. -H., Harrison, G. L. and Penny, D. (2001) Mitochondrial genomes of a bandicoot and a brushtail possum confirm the monophyly of australidelphian marsupials. *Proceedings of the Royal Society of London B* **268**, 1533-1538. Reprinted (as Chapter 1) with permission from The Royal society of London.
- Bromham, L., Phillips, M. J. and Penny, D. (1999) Growing up with dinosaurs: molecular dates and the mammalian radiation. *Trends in ecology and Evolution* **14**, 113-118. Reprinted (as Chapter 5.1) with permission from Elsevier Science.

### **Related papers not included in this thesis**

- Phillips, M. J. and Penny, D. (1998) Mass survival of tetrapods across the Cretaceous-Tertiary boundary. In *Geology and Genes* (R. A. Cooper and C. M. Jones, eds.), pp. 63-65. Geological Society of New Zealand, Wellington.
- Lin Y. -H., McLenachan, P. A., Gore, A. R., Phillips, M. J., Ota, R., Hendy, M. and Penny, D. (2002) Four new mitochondrial genomes, and the increased stability of evolutionary trees of mammals from improved taxon sampling. *Molecular Biology and Evolution*. In Press.
- Poole, A. M., Phillips, M.J. and Penny, D. Prokaryote and eukaryote evolvability. *Biosystems*. In Press.

## Abbreviations and symbols

A	adenine	$\times g$	centrifugal force (relative to gravity)
aa	amino acid	$\chi^2$	chi-square
AIC	Akaike information criterion	$\infty$	infinity
C	cytosine	$\Gamma$	gamma-model
CMI	character-map incompatibility	$\alpha$	gamma shape parameter
CMI <sub>O</sub>	observed character-map incompatibility		
CMI <sub>E</sub>	expected character-map incompatibility		
CMI <sub>R</sub>	relative character-map incompatibility		
df	degrees of freedom		
G	guanine		
GTR	general time-reversible (model of nucleotide substitution)		
H <sub>0</sub>	null hypothesis		
H-strand	heavy-strand (mtDNA)		
kb	kilo base(s)		
LB	lower appendicular and basicranial meta-region		
L-strand	light-strand (mtDNA)		
LCA	last common ancestor		
lnL	log-likelihood		
m.	muscle		
mm.	muscles		
ME	minimum-evolution		
ML	maximum-likelihood		
MP	maximum-parsimony		
mt	mitochondrial		
mtDNA	mitochondrial DNA		
mybp	millions of years before present		
MVD	mandibular, vertebral and dental meta-region		
NJ	neighbour-joining		
OTU	operational taxonomic unit		
PHT	partition homogeneity test		
PTN	protein		
R	purine (adenine and guanine)		
RASA	relative apparent synapomorphy analysis		
RCV	relative compositional variability		
RNA	ribonucleic acid		
rRNA	ribosomal RNA		
T	thymine		
ti	transitions		
tRNA	transfer RNA		
tv	transversions		
U	upper appendicular characters		
Y	pyrimidine (cytosine and thymine)		

## Terminology

**abduction** Movement of a body part away from the midline of the body (e.g. the elbow moving laterally, further from the sagittal plane of the body).

**adduction** Movement of a body part toward the midline of the body (e.g. the elbow moving medially, closer to the sagittal plane of the body).

**advanced / primitive** In the context of this work, the words primitive and advanced describe relative placements or character state conditions with respect to the mammalian backbone lineage, rather than suggesting that some character states or taxa are intrinsically more advanced than others. **antebrachium** The lower forelimb, from elbow to hand.

**apparent phylogenetic signal** Character covariance above “noise” in a data matrix that may or may not result from common ancestry (i.e. may be convergence etc.). I often refer to this simply as signal.

**basicranium** The base of the skull

**buccal** In dental morphology, the aspect of teeth that faces the cheek or the outside of the mouth.

**condyle** A rounded projection at the end of a bone, usually as part of a bone to bone joint.

**cursorial** Behaviour of (and adaptation for) running.

**crown group** A taxonomic grouping that includes the last common ancestor (LCA) of a given set of (often extant) taxa and all living and extinct descendents of that LCA.

**crus** The lower hindlimb, from knee to foot.

**distal** In anatomy, away from the centre of the body or away from the end that is attached to the body (e.g. the knee is distal to the hip).

**dorsal** The back, or upper side (in vertebrates, the surface closest to the notochord).

**foramen** A hole, usually for the passage of blood vessels and nerves through bone or cartilage.

**fossorial** Digging adaptation and/ or behavior.

**gamma distribution** In molecular phylogenetics, a continuous or discrete distribution of evolutionary rates across data (DNA or amino acid) sites.

**glenoid** (usually) ossified cup-shaped socket for another bone.

**indel** Insertion or deletion event, leaves a gap in a molecular sequence alignment.

**lateral** Pertains to the side of the body, or as a relative term, the part furthest from the sagittal plane (e.g. the cheeks are lateral to the tongue).

**lingual** In dental morphology, the aspect of teeth that faces the tongue or the inside of the mouth.

**manus** Carpus (=wrist) and forefoot (=hand)

**medial** Pertains to the middle of the body, or as a relative term, the part closest to the sagittal plane (e.g. the tongue is medial to the cheeks).

**NT-coding** Standard coding for DNA (A, C, G, T).

**paradentary bones** Plate-like bones attached to the dentary, these are splenial and coronoid that occur in many Mesozoic mammals

**parasagittal** 1. posture, with feet placed directly under their proximal attachments and the elbow or knee in approximately the same plane as these, 2. locomotion, with limbs swinging through an arc that is approximately parallel to the sagittal plane (the vertical plane through the midline of the body). I consider posture and locomotion that has the manus and pes almost vertically aligned with their proximal attachments, but with the knees and/or elbows somewhat lateral to these (due to humeral/femoral abduction) to be near-parasagittal.

**pes** hind foot (including the ankle)

**phylogenetic signal** Character covariance above “noise” in a data matrix that results or is inferred to result from common ancestry. I refer to character covariance above “noise” in a data matrix that may, or may not, result from common ancestry (i.e. may be convergence etc.) as apparent phylogenetic signal, or simply as signal.

**phylogenetic trend** (or evolutionary trend) The tendency for a character or complex of characters to undergo directional evolution, along a lineage, or within a group of taxa. This relates to gradual (as opposed to punctuated) evolution under selection pressures that remain similar along a lineage, or among members of a clade. As such, in the context of this work, evolutionary trends are not orthogenesis, which implies the maintenance of directional evolution, independently of selection.

**plesiomorphy** An ancestral character state.

**postdentary bones** Three associated bones that are lodged in the postdentary trough on the medial surface of the dentary of mammal-like reptiles and early mammals such as *Morganucodon*, and form the primitive jaw-joint (articular-quadrato). Of these bones, the articular and prearticular were (phylogenetically) transformed into the malleus in modern mammals, while the angular became the tympanic bone, which supports the tympanic membrane of the ear.

**proximal** In anatomy, towards the centre of the body or the end that is attached to the body (e.g. the hip is proximal to the knee).

**purine bias** Nucleotide compositional bias in which the relative frequency of the two purines (adenine versus guanine) differs between taxa. Indicated by variability (or the standard deviation) across taxa in a dataset for the frequency difference between A and G (A–G).

**pyrimidine bias** Nucleotide compositional bias in which the relative frequency of the two pyrimidines (thymine versus cytosine) differs between taxa. Indicated by variability (or the standard deviation) across taxa in a dataset for the frequency difference between T and C (T–C).

**RY-coding** Coding nucleotides as either purines (A and G = R) or pyrimidines (T and C = Y).

**sagittal plane** A vertical plane through the midline of the body, in most bilaterally symmetrical animals (including all tetrapods).

**scansorial** Behaviour including (and adaptation for) both climbing and terrestrial locomotion.

**signal** Character covariance above “noise” in a data matrix that may or may not result from common ancestry (i.e. may result from correlated homoplasy etc.). I also refer to this as apparent phylogenetic signal.

**Stem taxa** Organisms excluded from a specified crown group, but more closely related to that crown group than to another crown group. For example, *Archaeopteryx* is considered to be a stem bird.

**stemminess** A relative measure of phylogenetic structure, defined here as the percentage of uncorrected minimum-evolution tree distance attributed to internal (as opposed to external) branches.

**synapomorphy** A derived character state that is (or is inferred to be) shared between taxa as a result of common ancestry.

**Synapsida** Taxonomic group that includes mammals and taxa more closely related to mammals than are sauropsid reptiles (such as birds, turtles and lizards). The name Synapsida refers to the presence of a single opening in the skull (behind the eye socket).

**synonymous substitution** A nucleotide substitution that does not result in an amino acid replacement. In contrast, a non-synonymous substitution does result in an amino acid replacement.

**transition** In molecular biology, a nucleotide substitution from one purine to another purine (A↔G) or from one pyrimidine to another pyrimidine (T↔C).

**transversion** In molecular biology, a nucleotide substitution from a purine to a pyrimidine (e.g. A→T) or from a pyrimidine to a purine (e.g. T→A).

**trochlea** A pulley-like articular surface.

**ventral** The undersurface (in vertebrates, the surface furthest from the notochord).

## **Candidate's note**

This thesis is a collection of published papers and extensions of manuscripts in preparation for submission, to international journals. Each chapter has an introduction and can be read on its own. The intention for the following introductory notes is to provide additional background information and a rationale for the current examination of relationships among mammals.



## Introductory notes

Living mammals comprise three sub-classes, the egg-laying monotremes, the marsupials (which give birth to altricial live young) and the placentals (which give birth to relatively precocial young). As outlined in Table 1, 26 orders make up these sub-classes. The taxonomic make-up of these orders was determined mostly on the basis of morphological data and has changed little since the classifications of Simpson (1945) for placentals and Aplin and Archer (1987) for marsupials.

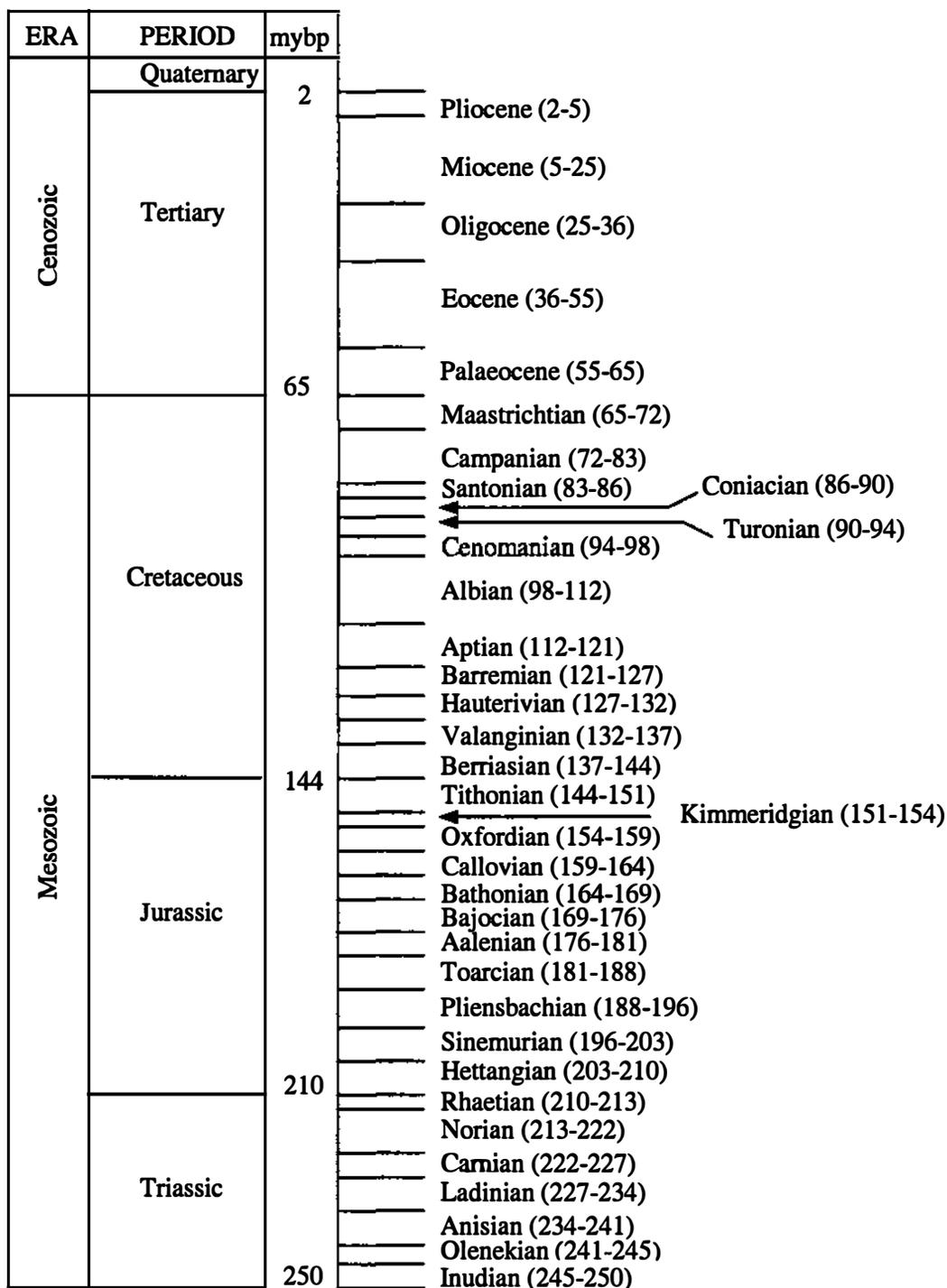
Although morphological studies were very effective in delimiting the mammalian orders, they have not been able to confidently determine the interordinal relationships of mammals, with the exception of a few groups such as Glires and perhaps Tethytheria (Proboscidea plus Sirenia). However, analysis of large molecular datasets, such as those for complete mitochondrial genomes and long nuclear sequences, promises to resolve the interordinal relationships of extant mammals. Important findings have included a clade of mammals with distributions centred in Africa (Afrotheria: Stanhope *et al.* 1998; Springer *et al.* 1999) and a clade including Lagomorpha, Rodentia, Primates, Dermoptera and Scandentia (Supraprimates: Madsen *et al.* 2001; Murphy *et al.* 2001; Waddell *et al.* 2001).

In comparison to Placentalia, less emphasis has been placed on resolving the interordinal relationships of Marsupialia. Perhaps the most significant and certainly the most inclusive proposed grouping of orders within Marsupialia is Australidelphia. This cohort was originally proposed by Szalay (1982) on the basis of ankle morphology and includes (among extant taxa) the Australasian marsupials plus *Dromiciops*, from South America. Almost all recent molecular studies have supported the monophyly of Australidelphia, except for the inclusion of bandicoots (Peramelemorphia), which have often been placed with the ameridelphians, or at the base of the marsupial tree.

Complete mitochondrial genome sequences from a bandicoot (*Isodon macrourus*) and a brushtail possum (*Trichosurus vulpecula*) are reported in Chapter 1. Mitochondrial (mt) genomes are circular and at the cellular level are contained in the mitochondria, so are separate from the chromosomal genes of the nucleus. The mt genomes of vertebrates code for 13 protein-coding genes, their own ribosomal genes (12S and 16S rRNA) and 22 transfer RNA genes (tRNAs). Analysis of the protein and RNA-coding sequences of the above two marsupials, along with those from other complete mt genomes, provides strong support for resolving bandicoot affinities in favour of the Australidelphia hypothesis.

**Table 1** The classification of mammals at the ordinal level (and above). The ordinal classification follows Aplin and Archer (1987) for marsupials and Simpson (1945) for placentals, except for the modifications of Waddell *et al.* (1999c). These modifications include the use of the names: Xenarthra, instead of Edentata (for which a number of fossil taxa of uncertain affinities have also been included, sometimes along with pangolins), Eulipotyphla (instead of Insectivora, which includes fossil taxa of uncertain affinities, as well as tenrecs and golden moles, which together form Afrosoricida) and Cetartiodactyla (which combines Simpson's Artiodactyla and Cetacea). The superordinal groupings are demarcated by the horizontal lines and follow Aplin and Archer (1987) for marsupials and Waddell *et al.* (2001) for placentals. Higher-level placental groupings are: Afrotheria (Paenungulata and Afroinsectiphillia), Supraprimates (Glires and Euarchonta), Laurasiatheria (Fereuungulata, Chiroptera and Eulipotyphla) and Boreoeutheria (Supraprimates and Laurasiatheria). \* McKenna and Bell (1997) place monotremes within the subclass Prototheria, a grouping of mammals of uncertain affinities that has been in constant flux.

Order	Examples	Superordinal groupings
<b>Subclass Prototheria*</b>		
Monotremata	platypus, echidnas	
<b>Subclass Marsupialia</b>		
Didelphimorphia	opossums	Ameridelphia
Paucituberculata	shrew opossums	
Microbiotheria	<i>Dromiciops</i>	Australidelphia
Dasyuromorphia	(marsupial) cats, mice, wolves	
Notoryctemorphia	marsupial moles	
Peramelemorphia	bandicoots	
Diprotodontia	kangaroos, wombats, possums	
<b>Subclass Placentalia</b>		
Xenarthra	sloths, anteaters, armadillos	
Proboscidea	elephants	Paenungulata
Sirenia	sea cows	
Hyracoidea	hyraxes	
Tubulidentata	aardvark	Afroinsectiphillia
Macroscelidea	elephant shrews	
Afrosoricida	tenrecs, golden moles	
Rodentia	rats, porcupines, squirrels	Glires
Lagomorpha	rabbits, pikas	
Scandentia	tree shrews	Euarchonta
Dermoptera	flying lemurs	
Primates	lemurs, monkeys	
Eulipotyphla	hedgehogs, shrews, moles	
Chiroptera	bats	
Carnivora	cats, stoats, seals	Fereuungulata
Pholidota	pangolins	
Perissodactyla	horses, rhinos, tapirs	
Cetartiodactyla	whales, sheep, pigs, camels	



**Figure 1** Geological timescale for the Mesozoic and Cenozoic eras. The stages of the Tertiary, Cretaceous, Jurassic and Triassic Periods are noted to the right, with timing in mybp (millions of years before present) and taken from Swisher (1997). The stages of the Quaternary are the Pleistocene (2-0.01 mybp) and the Holocene or Recent (0.01 mybp to the present).

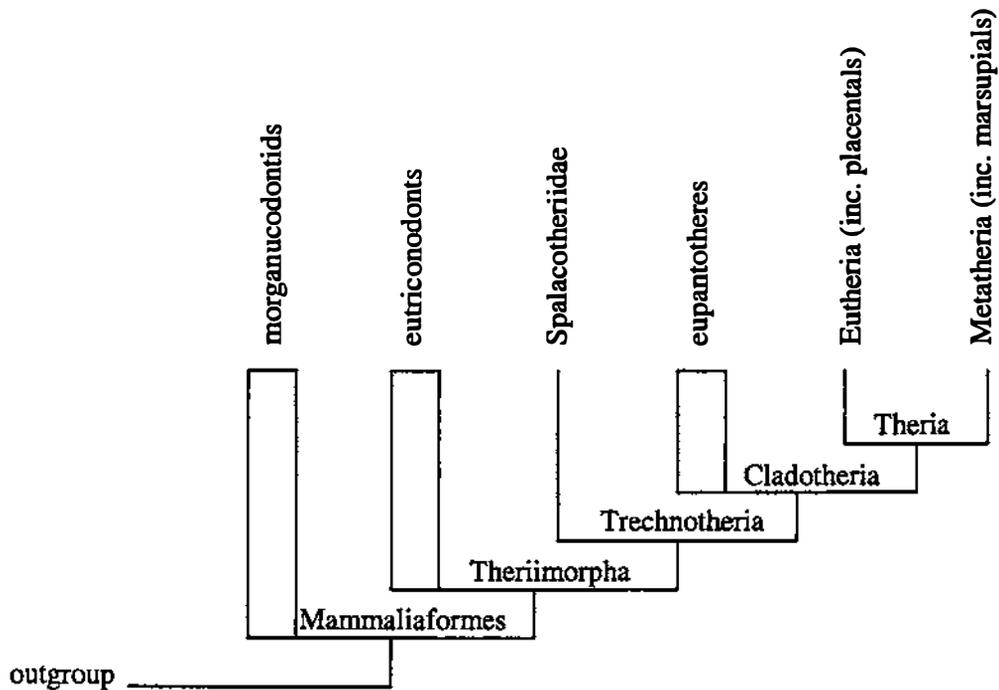
The bandicoot and brushtail possum sequences are also useful for testing the rooting of the mammal tree. The resurrection of the Marsupionta hypothesis of Gregory (1947), which places monotremes and marsupials as sister groups, is one of the most controversial results (Janke *et al.* 1996, 1997, 2002) derived from analysis of mtDNA. Marsupials have traditionally been considered to be the sister group of placentals. This is the Theria hypothesis and it is supported by a multitude of anatomical, physiological and reproductive characters (see Parrington 1974; Marshall 1979; Renfree 1993; Hu *et al.* 1998). In Chapter 2, two possible (non-phylogenetic) explanations for Marsupionta being recovered by analysis of mtDNA sequences are explored. These are nucleotide (and amino acid) compositional bias and heterogeneity of substitution processes between data partitions.

The timing of divergences among the basal groups of mammals is also examined in Chapter 2. For divergence dating based on sequence data, molecular evolutionary distances (or branch-lengths) are translated into time (usually millions of years before present: mybp). Fossil dates on the other hand are typically given by the age of the sedimentary rocks the specimens were found in. The age of the sediments can be determined (at least as upper and lower bounds) by radioisotopic dates for the overlying/underlying/intruding igneous rocks, or by associated faunal (and floral) elements that define chronostratigraphic (*chrono* = time, *strata* = layer) divisions in the geological timescale. The geological timescale for the Mesozoic and Cenozoic eras is shown in Figure 1.

In Chapters 3 and 4, the emphasis for inferring monotreme affinities is moved from their relationship with extant taxa, to their overall placement among mammals, and from DNA sequences to morphological data. Figure 2 shows the phylogeny of the major groups of Mesozoic mammals in which the ancestral (for mammals) small terrestrial (or scansorial) insectivore/carnivore niche was retained. The lineages leading to morganucodontids, eutriconodonts and eupantotheres are drawn as blocks, because these taxa are likely to be paraphyletic (but not polyphyletic).

The clade names given in Figure 2 are for crown-groups and the taxonomy follows McKenna (1975) for clades within Trechnotheria and follows Rowe (1993) for clades at a higher level than Trechnotheria. The differing priorities in taxonomy are necessary because of the different taxa included in the two classifications. The most notable departure in (some of) the literature from the clade name usage in Figure 2 is that “Theria” has often been used to mark the phylogenetic transition from linearly arranged cusps of cheek teeth, to reverse-triangle occluding cusps. Under a crown-group definition (as in Figure 2) Theria represents a clade that only includes metatherians (for which the crown-group is Marsupialia) and eutherians (for which the crown-group is Placentalia). Under the present classification (Figure 2), the transition to reverse-triangle occluding

cusps is marked by the clade Trechnotheria, which includes the spalacotheriid symmetrodonts and the cladotheres.



**Figure 2.** Phylogeny and classification of mammals that retained the ancestral mammalian niche (generalized insectivores), at least during the Mesozoic. Lineages that are boxed are likely to be paraphyletic. The clade names represent crown-groups defined by the last common ancestor nodes that the names are situated above.

In Chapter 3, the placement among the generalized insectivores, of monotremes and another group of uncertain affinities, the herbivorous multituberculates (see Kielan-Jaworowska 1996; Butler 2000), is investigated. It is of particular interest that the apparent phylogenetic signal for the placement of monotremes is affected by the inclusion of multituberculates, and vice versa. Furthermore, highly significant incongruence between anatomical region partitions is inferred for the placement of both monotremes and multituberculates among the generalized insectivores.

In recognition of the dependence on anatomical regions of monotreme affinities among the background phylogeny (the generalized insectivore phylogeny of Figure 2), an alternative method for resolving monotreme affinities is explored in Chapter 4. The level and significance of signal (and conflict) among characters, which relates to the placement of monotremes, is assessed within (and between) anatomical region partitions. This helps to build up an understanding of the nature of the incongruence and in turn is incorporated into a framework for assessing monotreme affinities in terms of the competing homoplasy options that alternative monotreme placements require.

A unifying theme for the molecular and morphological analyses of mammalian phylogeny in this study is the importance of incorporating information on non-stationarity (differences in evolutionary processes across phylogenetic trees). Three factors in particular that differ between taxa (or lineages) and appear to provide misleading “phylogenetic signals” are identified. These are: nucleotide (or amino acid) composition among the mt sequences, ecological niche transitions (for the analysis of morphological data), and branch-length effects for analysis of both the mt and morphological datasets.

Despite the problem of non-stationarity in evolutionary processes, analysis of the mt data was able to resolve the relationships of the modern mammal lineages that arose before the Cretaceous-Tertiary (K-T) boundary (except for the placement of the root on the placental tree, which remains uncertain). The timing of the pre-Tertiary (Mesozoic) diversification of modern mammal groups has been highly controversial. Molecular dating studies (e.g. Hedges *et al.* 1996; Penny *et al.* 1999) have suggested that much of the interordinal diversification of placentals occurred tens of millions of years before the K-T boundary. Palaeontologists (e.g. Benton 1999; Foote *et al.* 1999a) have however pointed out that there is little or no evidence in the fossil record for a Mesozoic diversification of (crown-group) placental mammals. Assumptions made in molecular dating studies and in interpretation of the fossil record are discussed (Chapter 5) as explanations for the apparent discrepancy between the dates inferred from molecular and fossil data for the mammal diversification.

The most likely explanation for the “fossil gap” is that the early phyletic and morphological diversification of modern mammal groups was uncoupled, with the former diversification being initiated well before the K-T boundary and the later being mostly restricted to the Tertiary. This may also explain the lack of synapomorphies that impeded attempts to resolve the interordinal relationships of placental mammals from morphological data. Furthermore, a pre-Tertiary diversification of modern marsupial and placental groups, in the absence of major niche diversification, is discussed in terms of the implications for understanding the ecological interactions of mammals with other vertebrate groups.

# Chapter 1

## **Mitochondrial genomes of a bandicoot and a brushtail possum confirm the monophyly of australidelphian marsupials**

Phillips, M. J., Lin, Y. -H., Harrison, G. L. and Penny, D. (2001)

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## 1.1 Introduction

Aplin and Archer (1987) recognise seven well-defined extant marsupial orders. Of these, Didelphimorphia (opossums), Paucituberculata (shrew opossums, including caenolestids), and the monotypic Microbiotheria (*Dromiciops gliroides*) have an American distribution, while Diprotodontia (kangaroos, possums, koala etc.), Dasyuromorphia (marsupial mice/cats/wolves, numbat), Peramelemorphia (bandicoots) and Notoryctemorphia (marsupial moles) have an Australasian distribution. Morphological studies (e.g. Ride 1962; Szalay 1993a) have offered little consistent resolution of affinities among these seven orders. Nevertheless, epididymal sperm pairing (Temple-Smith 1987) unites didelphimorphians and paucituberculates as the cohort Ameridelphia, and a continuous (as opposed to separate) lower ankle joint unites the remaining taxa (*Dromiciops* and the Australasian marsupials) as the cohort Australidelphia (Szalay 1982). Unfortunately, recent marsupial fossil finds from early Tertiary sites in South America, Antarctica, and Australia (see Woodburne and Case 1996; Goin *et al.* 1999; Godthelp *et al.* 1999) have provided little information for confirming this null hypothesis for a basic split among marsupials. This paper focuses on the monophyly of the Australidelphia.

Our mitochondrial genomes for the northern brown bandicoot (*Isodon macrourus*) and common brushtail possum (*Trichosurus vulpecula*) from the diprotodontian superfamily Phalangeroidea, supplement those for the didelphid, *Didelphis virginiana* (Janke *et al.* 1994), and the wallaroo, *Macropus robustus* (Janke *et al.* 1997) from the diprotodontian superfamily Macropodoidea. Several molecular studies (e.g. Retief *et al.* 1995; Kirsch *et al.* 1997) have singled out bandicoot affinities as the key to resolving the issue of australidelphian monophyly. Using DNA-hybridization distances for the (taxonomically) largest molecular study of marsupial relationships yet, Kirsch *et al.* (1997) found essentially an unresolved polytomy of didelphimorphians, caenolestids, bandicoots, and the remaining Australasian taxa (plus *Dromiciops*). Mitochondrial DNA sequences (Springer *et al.* 1994; Palma and Spotorno 1999; Burk *et al.* 1999) have tended to favour bandicoot associations with one or both ameridelphian groups, or otherwise as the marsupial root. However, nuclear genes (Retief *et al.* 1995: protamine P1; Springer *et al.* 1997a: interphotoreceptor retinoid-binding protein (IRBP); Colgan 1999: phosphoglycerate kinase) have tended to favour a monophyletic Australidelphia. Springer *et al.* (1998) merges IRBP (nuclear) with the mitochondrial genes 12S rRNA, tRNA valine, 16S rRNA and cytochrome *b*, with analyses favouring Australidelphia with 62% to 84% bootstrap support.

Apart from the position of bandicoots, almost all recent molecular and morphological studies are consistent with australidelphian monophyly. Aside from ankle morphology, only a few controversial characters such as reduced lower incisor number have been proposed as

australidelphian morphological synapomorphies (see Marshall *et al.* 1990 and Godthelp *et al.* 1999 for discussion of these characters). Bandicoot affinities being tested in this study are: 1- An association with diprotodontians, essentially confirming **Australidelphia**, 2- Bandicoots as sister to all other extant marsupials (“**Aplacentalia**”), a group characterized by the loss of several characters bandicoots share with placentals. These include a rudimentary chorio-allantoic placenta with umbilicus (Hughes 1974), extended corpus luteum life and progesterone secretion period (Gemmell 1995), and a robust patella (Szalay 1993a), 3- **Opossum-bandicoot** (*Didelphis* with *Isoodon*). Few if any derived morphological characters support bandicoots being associated with ameridelphians, though as noted above, some molecular studies favour this relationship.

## 1.2 Methods

Fresh liver samples from a northern brown bandicoot (*Isoodon macrourus*) and a common brushtail possum (*Trichosurus vulpecula*) were respectively donated by Dr. Robert Gemmell (University of Queensland) and Dr. Phil Cowan (Massey University). Mitochondrial DNA enriched extracts were obtained by preceding standard phenol/chloroform methodology with two initial 1 minute spins (400xg then 1000xg). Roche Molecular Biochemicals Expand<sup>TM</sup> Long Template PCR System was used to amplify fragments of 3-6kb, which overlapped identically, confirming complete mitochondrial genomes. PCR products were directly sequenced on a 377 ABI automated DNA sequencer. Primer details are provided in Appendix H. Sequences were deposited in GenBank under accession numbers AF358864 (bandicoot) and AF357238 (brushtail possum).

The Virginia opossum (Z29573), and wallaroo (Y10524) along with our bandicoot and brushtail possum sequences, form the marsupial ingroup in our analyses. Outgroup taxa include *Ornithorhynchus anatinus* (platypus; X83427), two afrotherians, *Loxodonta africana* (African elephant; AJ 224821), and *Orycteropus afer* (aardvark; Y18475), the xenarthran, *Dasyurus novemcinctus* (nine-banded armadillo; Y11832), and three laurasiatherians, *Balaenoptera physalus* (fin whale; X61145), *Hippopotamus amphibius* (hippopotamus; AJ010957), and *Talpa europaea* (European mole; Y19192).

Taxa such as the hedgehog, primates and rodents were excluded as they inflate both of the following undesirable qualities: 1. The number of phylogenetic unknowns. Excluding taxa that have possibly erroneous associations eliminates their influence on estimating parameters and transformations. 2. Relative loss of phylogenetic signal. In general, the further a taxon is from internal nodes (due to a fast or unusual substitution regime), the less information on ancestry is retained, and the more opportunity there is for substitution bias to accumulate.

Sequences were aligned manually within Se-Al v1.0a1 (Rambaut 1996). Ribosomal RNA (12S and 16S) structure was based on the models of Gutell *et al.* (1993) and Springer and Douzery (1996). We designed putative tRNA secondary structures for each taxon. With gaps and ambiguous sites removed, concatenated data sets for the 11 mammals contain 14365 nucleotide sites, with the 13 protein-coding genes contributing 11010 sites and RNA genes contributing 3355 sites. Unlike the other proteins, NADH6 is not H-strand coded and differs in nucleotide composition. Other than providing extra data, inclusion of an L-strand gene should not affect maximum-parsimony analyses (which treat sites independently). Further, as NADH6 exclusion alters bootstrap support for our maximum-likelihood analyses by only a few percent (less than 1% among marsupials), data shown includes NADH6, reflecting our intention to be data inclusive rather than exclusive. The RNA concatenation included 12S and 16S rRNA and 21 of the 22 tRNA genes. In agreement with Janke *et al.* (1994; 1997), tRNA-Lys is apparently a pseudogene in marsupials, and too variable to align confidently. We refer to the concatenated protein-coding sequences as **PTNaa**, for the amino acid translation, and as **PTN123**, **PTN12**, or **PTN3**, for the nucleotide sequences, with the numbers defining which codon positions are included. The concatenation that includes each rRNA and the 21 tRNA genes is referred to as **RNArt**. RY-coding pools both purines (adenine and guanine: R) and both pyrimidines (cytosine and thymine: Y) into 2-state categories (R, Y).

Many phylogenetic analyses of mitochondrial genomes (including Janke *et al.* 1994; 1997) prefer to exclude PTN3 due to near substitution saturation. Exclusion of PTN3 from phylogenetic analyses here is warranted by their highly significant base composition heterogeneity (Table 1.1, 1), and low signal to noise ratio (inferred from stemminess: Table 1.1). “Stemminess” is a relative measure of phylogenetic structure (Lanyon 1988), defined here as the percentage of uncorrected minimum-evolution tree distance attributed to internal branches. Phylogenetic analyses were performed on individual genes and on PTN12 and RNArt, either separately or together. Maximum-likelihood analysis of PTNaa used ProtML within MOLPHY 2.3 (Adachi and Hasegawa 1996). All maximum-parsimony (MP), minimum-evolution (ME), maximum-likelihood (ML) and partition-homogeneity (5000 replicates) analyses of nucleotide sequences were performed within PAUP\* 4.0b3a (Swofford 1998). Bootstrap % values associated with all trees were calculated from 1000 resampled heuristic searches. ML and MP Kishino-Hasegawa (1989) tests were performed within PAUP\* 4.0b3a.

## 1.3 Results

### 1.3.1 Analysis of composition heterogeneity

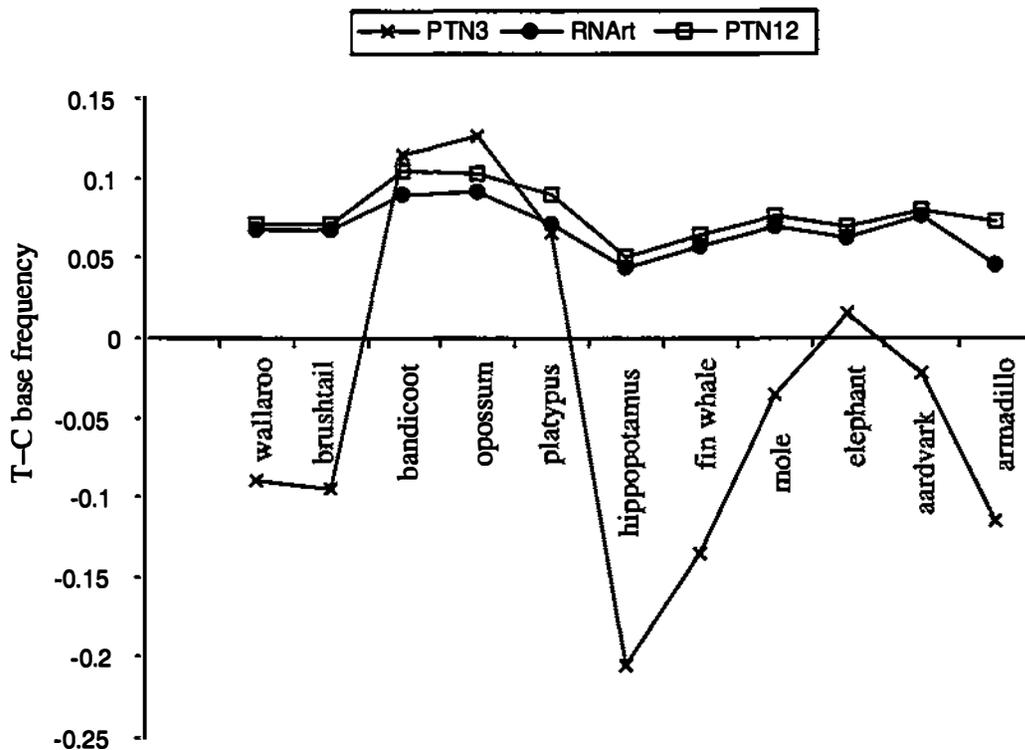
Quantitative analysis reveals differences in nucleotide composition between taxa. Chi-square tests for standard nucleotide (NT-coding: AGCT) base frequency reveal highly significant heterogeneity, even for PTN12 and RNArt (Table 1.1). Conversely, for RY-coding, composition bias is not significant for either PTN12 or RNArt. RY-coding also increases the stemminess of both the protein-coding and RNArt data (Table 1.1). Furthermore, the partition-homogeneity test for the 16 partitions (13 PTN12 genes, 12S rRNA, 16S rRNA, tRNA concatenate) reveals highly significant ( $p=0.0148$ ) incongruence with NT-coding (AGCT), while RY congruence cannot be rejected even at the 10% significance level ( $p=0.1114$ ).

**Table 1.1** Stemminess and composition homogeneity. PAUP\* homogeneity chi-square p-values for the 11 mammals (four marsupials alone), and stemminess values were calculated from variable sites only, for PTN3, PTN12, PTNaa, and RNArt, each coded as NT (AGCT), RY, or amino acids. Chi-square ( $p > 0.05$ , and stemminess  $> 0.20$  are in bold.

	Frequency chi-square		Stemminess
	p-values		
	Mammals	Marsupials	Mammals
PTN3 (NT)	<0.00001	(<0.00001)	0.0392
PTN3 (RY)	<0.00001	(<0.00001)	0.0742
PTN12 (NT)	<0.00001	(<0.00001)	0.1284
PTN12 (RY)	<b>0.1424</b>	<b>(0.99092)</b>	<b>0.2143</b>
PTNaa	<b>0.30287</b>	<b>(0.20936)</b>	0.1383
RNArt (NT)	<0.00001	(0.00012)	0.1436
RNArt (RY)	<b>0.87878</b>	<b>(0.49718)</b>	<b>0.2171</b>

Highly significant nucleotide composition bias, yet low RY composition bias, implies significant bias among purines and/or among pyrimidines. The relative contribution of these purine, and pyrimidine biases are indicated by standard deviations among the 11 taxa for adenine minus guanine (A–G) and thymine minus cytosine (T–C) relative frequencies, which are respectively 205.9 and 546.4 for PTN123+RNArt (14365 sites). Bias among pyrimidines is extreme for marsupials, for the PTN123+RNArt dataset, with relatively low T:C ratios for the wallaroo (1.1095) and brushtail possum (1.1075) and high T:C ratios for the bandicoot (1.4757) and opossum (1.4965). This pyrimidine (T–C) bias among marsupials is greatest for PTN3, but prominent for PTN12 and RNArt (Figure 1.1). Correlation ( $r^2$ ) coefficients for T–C bias among taxa for PTN3 with PTN12 and PTN3 with RNArt are respectively 0.8630 and 0.7786, while these

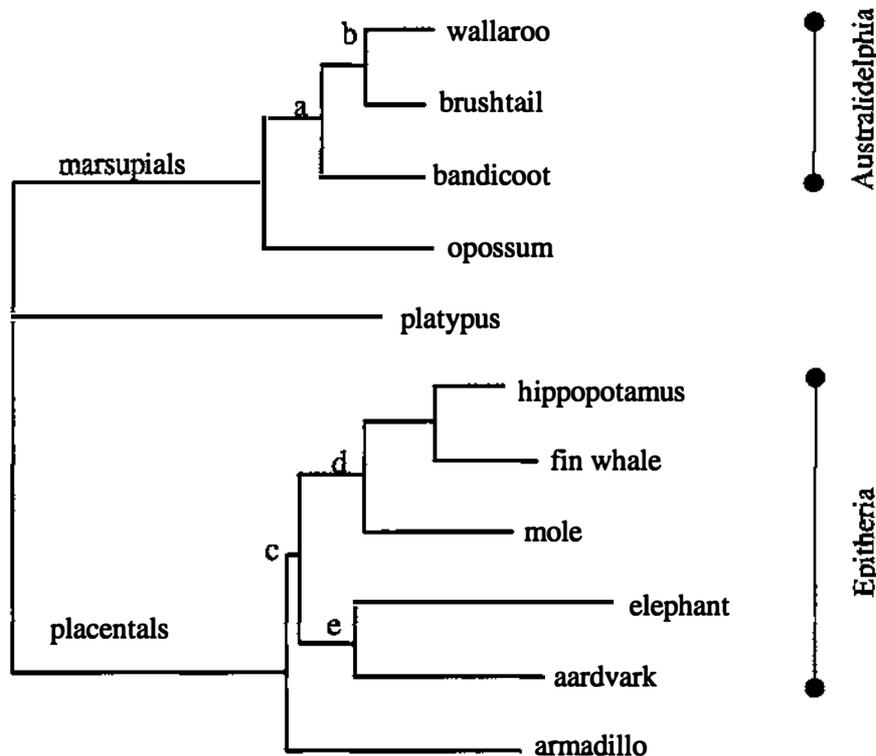
coefficients for A–G are 0.2939 and 0.2629, and for net transversions (Y–R), are 0.2529 and 0.0433. Hence the main effect is from pyrimidine (T–C) bias.



**Figure 1.1** Pyrimidine bias measured as the relative frequency difference between thymine and cytosine (T–C), for RNArt, PTN12 and PTN3.

### 1.3.2 Phylogenetic analysis

Maximum-likelihood analysis of the PTN12+RNArt RY-coded data, incorporating PAUP\* estimates for invariable sites and gamma distribution favours Australidelphia and Epitheria, and as expected, Diprotodontia, Cetartiodactyla, Laurasiatheria and Afrotheria (see Figure 1.2). This tree is also found with ML without assuming any rate heterogeneity among sites, and with MP. Except for Epitheria, for this selection of taxa, the same trees are found for all datasets, analytical methods, and optimality criteria (Figure 1.2). When Epitheria was not favoured, the eutherian root variously associated with Afrotheria or Atlantogenata (grouping Afrotheria and Xenarthra).



	a	b	c	d	e
MP (PTNaa)	100	99	3 <sup>^</sup>	100	47
MP (NT)	100	100	43*	99	91
MP (RY)	<b>100</b>	<b>100</b>	<b>92</b>	<b>99</b>	<b>98</b>
ML (PTNaa) mtREV-24F	100	100	3 <sup>^</sup>	100	53
ML (NT) F81	99	100	31*	100	96
ML (NT) HKY	<b>100</b>	<b>100</b>	<b>49</b>	<b>100</b>	<b>97</b>
ML (RY)	<b>100</b>	<b>100</b>	<b>82</b>	<b>100</b>	<b>99</b>
ME (PTNaa) uncorrected	100	100	1 <sup>^</sup>	100	83
ME (NT) uncorrected	100	100	1 <sup>^</sup>	100	96
ME (NT) GTR	100	100	3 <sup>^</sup>	100	97
ME (NT) LogDet	100	100	3 <sup>^</sup>	100	97
ME (RY) uncorrected	100	100	18 <sup>^</sup>	100	99
ME (RY) LogDet	100	100	19 <sup>^</sup>	100	98

**Figure 1.2** Maximum-likelihood phylogeny for RY-coded PTN12+RNArt, with PAUP\* estimates for invariant sites ( $I=0.5052$ ) and  $\Gamma_8$  ( $\alpha=0.4106$ ). The associated table gives bootstrap support (1000 replications) for nodes a (Australidelphia), b (Diprotodontia), c (Epitheria), d (Laurasiatheria), and e (Afrotheria). MP analyses for PTN12+RNArt (NT and RY-coded), and the translated protein-coding concatenation (PTNaa), are unweighted. ML models include F81 and HKY85 with a 2:1 transition/transversion ratio for nucleotides, CF87 for RY-coding, and mtREV-24F for PTNaa. ME distances are uncorrected, GTR corrected, or LogDet corrected. Unmarked nodes gained 100% bootstrap support in all analyses. Bold indicates Epitheria, \* indicates Atlantogenata (Xenarthra and Afrotheria), and ^ indicates Afrotheria is the basal placental clade.

For RY-coded data, Table 1.2 shows 13 of the 16 gene partitions favouring Australidelphia, with the most significant support tending to be conferred by genes with the largest number of variable sites. Among genes there is a 60% correlation ( $r^2$ ) between the number of variable sites, and  $-\ln L(\text{Australidelphia-Aplacentalia}) + -\ln L(\text{Australidelphia-Opossum-bandicoot})$ . Three genes did

not favour Australidelphia (NADH3, COI and Cytb), though in every case the  $-\ln$  likelihood ( $-\ln L$ ) for Australidelphia differed by less than one standard deviation. Upon concatenation of the 16 partitions, Kishino-Hasegawa tests for MP and ML (with and without invariant sites and gamma distribution estimates) find significant support ( $p < 0.05$ ) for Australidelphia. Epitheria is favoured in each case, though not significantly at  $p \leq 0.05$ .

**Table 1.2** Log-likelihood differences among alternative marsupial roots, for the 16 protein-coding and RNA gene partitions. For each gene partition the difference in RY-coded  $-\ln$  likelihood from the favoured hypothesis (✓), and the number of standard deviations (Kishino-Hasegawa test) below this best score is indicated as \* ( $>1$  s.d.); \*\* ( $>1.5$  s.d.); \*\*\* ( $>2$  s.d.). The number of RY variable sites among the 11 taxa for each partition is given in brackets. *I. macrourus* and *T. vulpecula* protein-coding and RNA-coding genes conform to the standard marsupial order (Janke *et al.* 1994; 1997). Kishino-Hasegawa (KH) test p-values for MP, ML, and ML+I+ $\Gamma$  (invariant sites plus gamma model) are given for PTN12+RNArt.

	Australidelphia	Aplacentalia	Opossum - bandicoot
NADH1 (105)	✓	10.20*	13.37**
NADH2 (227)	✓	7.47*	7.43*
COI (46)	3.53	✓	3.53
COII (48)	✓	3.42	3.53
ATP8 (46)	✓	0.96	0.96
ATP6 (87)	✓	1.63	1.63
COIII (61)	✓	4.85*	4.85*
NADH3 (46)	3.01	1.20	✓
NADH4L (54)	✓	2.77	2.30
NADH4 (245)	✓	11.00**	11.71**
NADH5 (315)	✓	17.13***	13.65**
NADH6 (108)	✓	0.23	2.19
Cytb (99)	1.83	4.51*	✓
12S (158)	✓	4.63	5.32*
16S (246)	✓	2.81	7.78*
tRNA (191)	✓	6.56*	3.91
	PTN12+RNArt Kishino-Hasegawa test p-values		
MP	✓	< 0.0001	< 0.0001
ML	✓	< 0.0001	< 0.0001
ML (I+ $\Gamma$ )	✓	0.0256	0.0310

## 1.4 Discussion

Transversions tend to code for more conservative amino acid substitutions than do transitions (Zhang 2000), and RY-coding eliminates signal from transitions that produce “semi-stable” GT RNA stem pairs (Patel *et al.* 1984). These phenomena may partly explain RY-coded PTN12 and RNArt having more than 50% higher stemminess values than NT-coded (AGCT) or amino acid translated equivalents. This implies RY-coded data possess higher signal to noise ratios, and therefore are less susceptible to phylogenetic signal erosion and systematic biases. Certainly, among this dataset, RY-coding of nucleotides greatly reduces composition bias (Table 1.1) and incongruence among gene partitions. Simply excluding observed transitions may be less effective than RY-coding, as purine and pyrimidine biases may bias the distribution of the four transversion categories. Thus, RY-coding appears to be the most appropriate treatment of this data.

The ML-tree (Figure 1.2) incorporating RY-coding and estimates for an invariant sites plus gamma distribution model, represents our best estimate of the phylogeny. This tree favours Australidelphia and indicates overall substitution rate homogeneity among marsupials. Kishino-Hasegawa tests for maximum-likelihood (Table 1.2) reject Aplacentalia and an opossum-bandicoot grouping for RY-coded PTN12+RNArt, even with the increased variance to signal ratio that Sullivan *et al.* (1999) suggest is imposed by including invariant sites and gamma distribution estimates. Australidelphia is confirmed for each ML model and MP, with two-tailed significance values well below  $p=0.05$ . These results combined with bootstrap resampling support of 99% or greater, regardless of coding (nucleotide, RY, amino acid), substitution model, and optimality criterion (ML, MP, ME) for PTN12+RNArt (Figure 1.2), must be considered as convincing evidence for australidelphian monophyly.

Nucleotide composition heterogeneity is highly significant among taxa (Table 1.1) even with PTN3 excluded, warning against the use of standard nucleotide coding for analysis of mitochondrial sequences. We suspect nucleotide composition heterogeneity is more common than reported for mitochondrial data, as many studies fail to exclude constant sites from chi-square base frequency tests, thus diluting nucleotide composition variability among taxa. With RY composition for PTN12 and RNArt being robust to bias (Table 1.1), highly significant nucleotide (NT-coded) composition bias implies considerable bias among purines and/or among pyrimidines.

Considerably greater standard deviation among taxa for T–C (546.4) than for A–G (205.9) indicates pyrimidines make the greatest contribution to nucleotide composition bias. Greater PTN3 substitution synonymy (including all PTN3 transitions) probably explains T–C variation being far greater for PTN3 than PTN12 or RNArt (Figure 1.1). Synonymous substitutions are expected to be neutral (Maynard Smith 1994) and reflect underlying mutation biases. However, mutation biases

that are small compared to the constraints of non-synonymous substitutions and RNA-folding, will explain little of the base frequency variation among taxa for PTN12 and RNArt. As such, the potential phylogenetic influence of T–C mutation bias for this dataset is illustrated by high correlation ( $r^2$ ) of T–C variation among taxa for PTN3, with that of PTN12 and RNArt (86% and 78%).

The bandicoot and opossum have a greater thymine minus cytosine (T-C) difference than the diprotodontians and outgroup taxa, for PTN3, PTN12 and RNArt (Figure 1.1). This pyrimidine bias will tend to act as a non-independent, across sites “synapomorphy” for Opossum-bandicoot. However, the strength of australidelphian signal is such that it receives at least 99% bootstrap support even for standard nucleotide coding (AGCT) analyses (Figure 1.2). Contrary to this, depending on coding and optimality criteria, Afrotheria, Atlantogenata (Xenarthra, Afrotheria) and Xenarthra are variously favoured as basal among the eutherians (Figure 1.2). Previous mitochondrial genome studies that included laurasiatherians, afrotherians and the armadillo, have tended to associate the armadillo with laurasiatherian taxa (Arnason *et al.* 1999; Mouchaty *et al.* 2000). However, morphological studies almost uniformly support Epitheria, finding Xenarthra (or Edentata, depending on pangolin affinities) to be basal among extant eutherians (e.g. Rose and Emry 1993; Shoshani and McKenna 1998). As such it is interesting that ML and MP favoured Epitheria in every “phylogenetics friendly” RY-coded analysis of PTN12+RNArt (Figure 1.2). Inclusion of primate, rodent, lagomorph and erinaceid sequences is important for testing Epitheria. However, additional sequences from these groups and from Xenarthra and Afrotheria, are required to maintain the sampling strategy of this study (1. minimise the number of phylogenetic unknowns, 2. minimise loss of phylogenetic signal). Increased sampling will help discriminate phylogenetic signal from systematic biases, reduce variance and hence, allow eutherian rooting to be amenable to more sophisticated analysis such as data partitioning.

One obvious drawback of pooling A, G as R and T, C as Y is that it reduces overall genetic variability, and hence increases susceptibility to sampling error. This is not a problem for PTN12+RNArt, as Figure 1.2 shows that RY-coding provides equal or better support than does nucleotide-coding, for “known” relationships such as Diprotodontia, Laurasiatheria and Afrotheria. Cao *et al.* (1998) showed that owing to stochastic variation and systematic differences among individual genes, support for certain affinities only emerges upon summation or concatenation of mitochondrial genes. However, given 100% bootstrap support from the concatenated sequence, and relative congruence (suggested by the homogeneity partitioning test), it is not surprising that for the RY-coded data, 13 of the 16 gene partitions favour Australidelphia (Table 1.2). As alternative hypotheses favoured by individual genes (Opossum-bandicoot: NADH3, Cytb; Aplacentalia: COI) differ in  $-\ln$  likelihood from Australidelphia by less than one standard

deviation, they cannot be said to conflict with Australidelphia. Notably, these three genes are below the 16-partition average for variable site number, and correlation ( $r^2$ ) suggests the number of variable sites describe 60% of the variation in australidelphian signal strength among the gene partitions. As such, a greater relative importance of stochastic variation among small datasets, combined with a pyrimidine bias among marsupials, may explain alternative relationships offered by studies of one or a few mitochondrial genes (e.g. Springer *et al.* 1994; Cao *et al.* 1998; Palma and Spotorno 1999; Burk *et al.* 1999). With many of the more variable gene partitions favouring Australidelphia over one or both alternatives by one or even two standard deviations (Table 1.2), the individual genes taken as a whole, offer overwhelming support for Australidelphia. This corroborates the mostly nuclear molecular studies (Retief *et al.* 1995; Springer *et al.* 1997a; Colgan 1999; Springer *et al.* 1998) that tentatively favour Australidelphia.

Australidelphian monophyly has important biogeographic implications. Unlike Aplacentalia, or Opossum-bandicoot, Australidelphia does not require a complex pattern of trans-Antarctic dispersals, and/or continental extinctions for various marsupial orders. Microbiotheriids were present in the Antarctic Eocene (Goin and Carlini 1995). Determining whether they are sister to or nested within the Australasian marsupials will provide another major constraint for testing hypotheses of marsupial biogeography. However, in the absence of molecular clock / reference date combinations with satisfactory accuracy for examining just the last and first few million years of the Cretaceous and Tertiary respectively, biogeographic inference from molecular sequences is limited. Providing a solid phylogeny is perhaps the most important role for this and other molecular studies, in understanding marsupial biogeography.

Considering the difficulties of placing bandicoots with respect to the marsupial root in previous studies, clear australidelphian affinities shown here with both protein-coding and RNA genes, hints that whole mitochondrial genome analysis should fully resolve marsupial interordinal relationships. For palaeontology, which can pinpoint taxa in space and time, this would provide a morphology independent reference for determining the reliability of specific character transformations. For example, our results increase the diagnostic weight of a continuous lower ankle joint as an australidelphian synapomorphy, while implying that the robust patella and placental-like bandicoot reproductive traits are convergent with eutherians, or were lost more than once by marsupials.

## **Chapter 2**

### **Inferring the root of the mammalian tree from whole mitochondrial genome protein-coding and RNA-coding DNA sequences**



## 2.1 Introduction

Prior to the discoveries during the latter half of the twentieth century of near-complete fossils of Mesozoic mammals, Gregory (1947) provided a most intriguing examination of monotreme relationships. Gregory relied on his palimpsest theory, for which monotreme characters were considered either as caenotelic (adaptive for the current niche) and largely ignored, or as palaeotelic (reflective of phylogenetic and past adaptive history) and examined for indications of relationships with other taxa. From this, he inferred that monotreme affinities lie with marsupials, possibly even more specifically with diprotodontians. Kirsch and Mayer (1998) provide a useful deconstruction of Gregory's Marsupionta hypothesis, while Parrington (1974) argues that most of the similarities between marsupials and monotremes that Gregory cited are, in fact, primitive for Mammalia.

Since Gregory (1947), most morphological studies have supported Theria. Studies of reproduction (e.g. Carrick and Hughes 1982; Renfree 1993; Zeller 1999) and soft tissue/cytology (Griffiths 1978; Tsujii *et al.* 1992; Selwood 1994) have been important in this. However, it has been the comparison of modern mammals with their Mesozoic relatives (see Hopson and Crompton 1969; Kermack and Kielan-Jaworowska 1971; Marshall 1979; Rowe 1988; Hu *et al.* 1997; Lou *et al.* 2002) that has provided the bulk of the evidence for monotreme affinities lying well outside the therian crown group (metatherians, including marsupials and eutherians, including placentals). An exception among morphological studies is Kühne (1973) who believed that like marsupials, platypuses (at least ancestrally) replaced only the third premolars, and considered this a synapomorphy of Marsupionta. However, Lockett and Zeller (1989) showed that Kühne (1973) had misinterpreted the dental series and that there is no evidence for tooth replacement in monotremes at all.

Janke *et al.* (1996) resurrected the Marsupionta hypothesis, finding that the platypus (*Ornithorhynchus anatinus*: Ornithorhynchidae) grouped with the Virginia opossum to the exclusion of eutherians on the basis of analysis of the concatenated sequences from the twelve H-strand encoded mitochondrial (mt) protein-coding genes. This conclusion that monotremes and marsupials are sister taxa has subsequently been strengthened by mt analyses with increased taxon sampling. This has included the addition of a second marsupial sequence (wallaroo: Janke *et al.* 1997) and a short-beaked echidna, *Tachyglossus aculeatus* (Janke *et al.* 2002), a representative from the other extant monotreme family, the Tachyglossidae. Numerous other complete mt genome phylogenetic analyses employing a vast array of analytical methods (e.g. Penny and Hasegawa 1997; Kumazawa *et al.* 1998; Zardoya and Meyer 1998) have also favoured Marsupionta, with support from bootstrap resampling often above 95%.

Janke *et al.* (2002) found additional support for Marsupionta in analysis of the nuclear-encoded 18S rRNA gene. However, statistical support largely relied on indels (putatively, insertions that are shared by marsupials and the platypus). Given that the gaps these leave in the archosaur (outgroup) and placental sequences are of different length (and bordered by bases of uncertain homology), more than one indel is required in each case. Thus the data might be explained equally as well by separate deletion events in archosaurs and placentals, or by insertions in stem mammals and subsequent deletions along the placental stem. Citing their mt protein-coding and 18S rRNA results plus other recent studies that favour Marsupionta, and dismissing those that did not, Janke *et al.* (2002) made the surprising claim that support for the Marsupionta hypothesis from molecular data is “currently unambiguous”.

Far from providing unambiguous support for Marsupionta, molecular phylogenetic studies overall might better be described as providing weak, though highly ambiguous support for the conventional sister-grouping of Metatheria and Eutheria. Most of the nuclear genes for which monotreme sequences are available tend to favour Theria over Marsupionta, although with limited statistical support. These genes include various globins (e.g. McKenna 1987; Lee *et al.* 1999), protamine P1 (Retief *et al.* 1993), the neurotrophins BDNF, NT-3 and NGF (Kullander *et al.* 1997) and immunoglobulin gamma 1 (Belov *et al.* 2002). The latter two analyses claimed to show strong support for Theria, though inspection of their trees reveals bootstrap support values of only 64% and 63% respectively. This is hardly strong support, given that with other relationships fixed, parsimony bootstrap support for Theria will converge on  $\approx 63\%$  if Theria is supported by just one more synapomorphy than is Marsupionta.

Further support for Theria has been attributed (e.g. Lou *et al.* 2002) to the study of Gilbert and Labuda (2000), in which a number of SINEs (short interspersed nuclear elements) were characterized from the three mammalian sub-classes. One of these SINE families (Ther-2) was found in marsupials and placentals, but not in monotremes. However, any implied relationship requires further investigation. Firstly, The presence of the Ther-2 SINEs in the placentals was only indicated by GenBank searches through genomic regions that have not been sequenced in monotremes. Southern analysis only showed substantial hybridization of the Ther-2 probe to the marsupial genomic samples. Hybridization to the placental genomic samples was no more appreciable than to the monotreme (platypus) sample. Secondly, using SINE data to provide reliable synapomorphies requires the flanking regions to be sequenced, in order to show that the insertions are orthologous (homologous through speciation). Nikaido *et al.* (1999) used this technique successfully, to confirm a sister group relationship between whales and hippopotamuses.

Analysis of  $\alpha$ -Lactalbumin (LA) provided the first statistically robust support for Theria based on a molecular sequence (Messer *et al.* 1998). The concerns of Janke *et al.* (2002) regarding Messer *et al.* (1998) rooting the LA dataset with the related lysozyme (LZ) genes may in part be unfounded. The LZ genes diverged from the LA genes after mammals split from diapsid reptiles (Blackburn *et al.* 1989). As such the LZ genes may be a better rooting option than homologous genes from diapsid reptiles (which is not possible in this case), at least in terms of the timing of divergence. Nonetheless, given the nature of the LA and LZ data that is presented, the 100% parsimony bootstrap support that Messer *et al.* (1998) found for Theria does appear somewhat anomalous.

The LA and LZ sequences used by Messer *et al.* (1998) are only a little over 120 amino acid residues long and their maximum-likelihood distances indicated that the LAs average almost one change per site between monotremes and the therians (even before taking into account the effect of constant sites). Such data might not be expected to provide significant support for relationships between taxa that are highly divergent relative to the length of their stem lineage (especially deeper in the tree). Indeed, the bootstrap support values of Messer *et al.* (1998) follow this prediction, with the level of support for Theria being the exception.

Among the LA sequences analysed by Messer *et al.* (1998), the family-level relationships were well resolved, while the deeper-level relationships among the placentals were either unresolved or incorrect (the pig and camel group with the equids, rather than with the other artiodactyls). Monophyly of the placentals, which is strongly supported by analyses of longer sequences, was only more parsimonious than paraphyly (due to marsupial inclusion within Placentalia) in 63% of bootstrap replicates. How then did the deeper still Theria node, which has been notoriously difficult to resolve with much larger datasets, receive 100% bootstrap support? Even the platypus and the echidna, which are closely related relative to the length of their stem lineage, received less support (96%). The possibility that the monotreme and therian LAs are in fact paralogous and diverged during an earlier phase of synapsid evolution deserves attention. Nevertheless, the simplest explanation is that LA underwent a period of adaptive evolution in stem therians, which resulted in a high proportion of changes at generally conservative sites, and that this left a much stronger than expected phylogenetic signal for Theria.

Killian *et al.* (2001) analysed the  $\approx 2250$  amino acid residues of the mannose 6-phosphate/insulin-like growth factor II receptor (MP6/IGF2R) gene for 15 mammals and a chicken. Theria gained  $\geq 97\%$  bootstrap support in each maximum-parsimony (MP), minimum-evolution (ME) and maximum-likelihood (ML) analysis and the interordinal relationships among the placentals were resolved in agreement with recent studies of concatenated nuclear genes (Madsen *et al.* 2001; Murphy *et al.* 2001a). The study of Killian *et al.* (2001) conceivably provides the strongest

molecular evidence for Theria yet. However, in accordance with the misgivings of Janke *et al.* (2002), comparison of MP6/IGF2R between the mammalian sub-classes is likely to provide overconfidence in Theria, due to covariation of functional constraints on evolution. This was signaled by Killian *et al.* (2000) previously showing that in marsupials and placentals (but not in monotremes and the chicken), that MP6/IGF2R binds IGF2 and is imprinted (expression depends on the parent of origin).

The growing number of studies of independent (unlinked) genes that favour Theria has not been matched for Marsupionta. However, Toyosawa *et al.* (1998) analysed the enamel matrix protein amelogenin ( $\approx 200$  amino acid residues) and found that the monotremes and the only marsupial in the study (*Didelphis*) grouped together with 65% bootstrap support in an uncorrected distance NJ (neighbour-joining) analysis. With respect to the monotremes, the most remarkable finding of Toyosawa *et al.* (1998) is that in the echidna, in which teeth never develop, there are no frame-shift indels and most of the conservative amino acid residues are maintained. This implies that quite recent ancestors of echidnas might have possessed functional teeth.

Kirsch and Meyer (1998) found some support for Marsupionta from DNA-DNA hybridization data, but did not appear to place much weight on the finding. Hillis *et al.* (1996) regarded DNA-DNA hybridization as being of limited usefulness for inferring phylogeny for divergences greater than 50 mybp (million years before present). This is evident in the better (taxon) sampled tree of Kirsch and Meyer (1998), in which the sister to Marsupionta is a lizard and the placentals are sister to all other amniotes.

This brings us back to the mitochondrial sequences. These are the only molecular data that provide statistically robust support for Marsupionta and also reliably resolve many deep-level divergences, from mammalian interordinal relationships through to affinities among the amniote classes. In fact, as noted by Janke *et al.* (1996), statistically significant support for Marsupionta is limited to the mt protein-coding genes. Phylogenetic analyses of the mitochondrial transfer RNA (tRNA) and/or ribosomal RNA (rRNA) sequences have tended to find reduced support for Marsupionta (e.g. Waddell *et al.* 1999a; Zardoya and Meyer 2000), or even weak support for Theria (e.g. Gemmill and Westerman 1994).

Whole mt genome data have provided strong support for several spurious relationships, such as a basal position of the hedgehog among placentals (see Lin *et al.* 2002) and the basal chordate "amphioxus" being excluded from a vertebrate-echinoderm clade (Naylor and Brown 1998). In both cases, composition bias was suggested to have contributed to the erroneous placement.

Phillips *et al.* (2001) provided a hint that composition bias may also be contributing to the mt signal (or apparent phylogenetic signal, as character covariance may occur by ways other than shared genetic history) supporting Marsupionta. Bias resulting from differences in the relative frequency of the two pyrimidines (T–C bias, = pyrimidine bias) favoured a sister-relationship between the bandicoot, *Isodon macrourus*, and the opossum, *Didelphis virginiana*. Both of these mt genomes have a predominance of T (as opposed to C) relative to the other mammals. This bias was most extreme at the third codon positions and with these included, a bandicoot-opossum clade is weakly favoured (unpublished data) over the bandicoot associating with the other australidelphian taxa (which was strongly favoured using the more conservative first and second codon positions and the RNA data). The platypus had the next highest thymine content after the two basal marsupials. Thus it is important to determine whether nucleotide composition bias might be contributing to the apparent phylogenetic signal for Marsupionta.

In this chapter I explore the possibility that the distribution of signal among the three hypotheses for monotreme placement (Theria, Marsupionta, and a Monotremata-Eutheria grouping) is affected by composition bias (particularly pyrimidine bias). If such a bias is strong and does not favour the correct phylogeny, most phylogenetic reconstruction methods are not expected to select the correct tree (see Lockhart *et al.* 1994; Foster *et al.* 1997; Mooers and Holmes 2000). In this study, signal for monotreme placement is compared between these standard phylogenetic methods and two methods that are expected to be robust to pyrimidine bias: LogDet (paralinear) distances (Lockhart *et al.* 1994) and RY-coding, which pools purines (adenine and guanine: R) and pyrimidines (cytosine and thymine: Y) into 2-state categories (R, Y).

The most common use of RY-coding has been for transversion parsimony (e.g. Brown *et al.* 1982; Scally *et al.* 2001). I refer to such transversion methods as RY-coded analyses in order to prevent confusion with the transversion parsimony of Lake (1987), and with PAUP\* (Swofford 1998) transversion distances. These latter transversion methods use standard NT-coding (ACGT) with observed transitions simply being ignored. Such methods still differentiate between the four transversion categories.

Base composition non-stationarity aside, whether poor fit between the data and the substitution models may more generally be contributing to support for Marsupionta requires further testing. Numerous studies have shown that poorly-fitting models can result in positively misleading tree support. For example, Sullivan and Swofford (1997) showed that not incorporating rate variation among sites led to rodent monophyly being rejected. Suspicion that support for Marsupionta may have been encouraged by poorly-fitting models of mt sequence evolution is aroused by the observation that no-model (more correctly, no common mechanism among sites: see Steel and

Penny 2000) MP analyses provide far less support for Marsupionta than ML and ME analyses (e.g. Zardoya and Meyer 1998; Janke *et al* 2002). On the other hand, ML and corrected-distance approaches are typically more robust to heterogeneity in substitution processes across sites and across the tree than is parsimony (e.g. Waddell 1995; Sullivan and Swofford 2001).

Models poorly approximate concatenated data when substitution processes differ between the data subsets (Yang 1996). Evolutionary processes (particularly substitution regimes) have been shown to differ substantially across vertebrate mt genomes, such as between protein-coding codon positions (Janke *et al.* 1996; Schmitz 2002), and between RNA loops and stems (Springer and Douzery 1996). The influence of this potential source of phylogenetic inaccuracy has yet to be examined for mitochondrial data, with respect to monotreme affinities. However, DeBry (1999) and Cao *et al.* (2000a) have shown that partitioning likelihood analyses among partitions (such as codon positions or genes) substantially increased the fit between ML models and mammalian mt protein-coding data.

One problem with partitioning analyses, is deciding how to most appropriately subdivide the data. Subsets of data for which evolutionary processes differ are often termed “process partitions”. Bull *et al.* (1993) emphasized incongruence as the criteria for classification of data subsets into different process partitions. Miyamoto and Fitch (1995), on the other hand, argued that *a priori* classification of process partitions (as subsets of characters evolving under similar evolutionary “rules”) provides a more useful framework for phylogenetic studies. In the current study I use partitioned ML analyses. The RNA data is partitioned into loops and stems and five partitions of protein-coding genes with similar observed composition and variable site properties are further partitioned into codon positions. Both partitioning and RY-coding is found to increase the signal for Theria relative to that for Marsupionta.

The finding that complete mt genome protein-coding and RNA-coding data favour the traditional Theria clade has important implications for inferring the timing of the Monotremata/Theria divergence. Consistency with three divergence hypotheses is tested: 1. that monotremes diverged from therians before the Triassic/Jurassic boundary Mammaliaform, *Morganucodon* (Kermack *et al.* 1981), 2. or at least before *Ambondro* (Flynn *et al.* 1999), which is the oldest known member of the Gondwanan radiation of small insectivorous mammals (Australosphenida), within which Lou *et al.* (2001a) proposed monotremes to be nested, and 3. that platypuses and echidnas could have diverged earlier than the oldest reported ornithorhynchid, *Monotrematum* (Pascual 1992a, 1992b).

## 2.2 METHODS

### 2.2.1 Data

Fifteen mammalian ingroup taxa and ten outgroup vertebrates were selected to test hypotheses for relationships between the mammalian sub-classes using whole mt protein-coding and RNA-coding DNA sequences. These taxa and their GenBank accession numbers are listed in Table 2.1. Taxa were chosen in order to maximise the potential for tracking transformations across the node representing the mammalian root (as opposed to maximising the number of taxa used). As such, capturing taxonomic diversity relevant to the mammalian root was balanced with limiting both the number of phylogenetic unknowns and the number of taxa with fast or unusual substitution regimes. The exclusion of taxa such as the hedgehog (*Erinaceus europaeus*), the rat snake (*Dinodon semicarinatus*), the clawed frog (*Xenopus laevis*) and rodents is intended to limit both the potential influence of erroneous relationships on the estimation of parameters and transformations, and the potential for long-branch related problems. Moreover, conservative taxa provide greater certainty of sequence homology, so allow more data to be included for phylogenetic analysis, and might also be expected to retain more phylogenetic signal.

The 25-taxon dataset is used for all maximum-parsimony (MP), minimum-evolution (ME) distance, and protein maximum-likelihood (ML) analyses. This dataset was reduced for the more computationally expensive ML analyses of the DNA data. For this, taxa were excluded under three criteria: 1. Their exclusion reduced phylogenetic uncertainty among the dataset (armadillo, pika, tarsier), 2. Their inclusion contributes little to determining the mammalian root (rook, eastern painted turtle) as they were outgroup taxa for which close sister taxa are retained and 3. They had high taxon variance ratios (Lyons-Weiler and Hoelzer 1997) as determined within RASA 3.0.2 Turbo (Relative Apparent Synapomorphy Analysis: see Lyons-Weiler *et al.* 1996 and Lyons-Weiler 2000). This last criterion indicated that from the 25-taxon dataset, the caecilian and salamander may be having the most misleading influence on phylogenetic inference.

After exclusion of the seven taxa listed above, the taxon variance ratios (which are a measure of the relative contribution of cladistic and phenetic variance) were considerably lower for most of the 18 remaining taxa (see Table 2.1). Recent molecular phylogenetic studies (e.g. Cao *et al.* 2000b; Janke *et al.* 2001; Murphy *et al.* 2001b; Phillips *et al.* 2001) indicate that the only uncertainty among the relationships of these 18 taxa is for the placement of the mammalian root. Consistent with these studies, exclusion of the monotremes yields a 16-taxon phylogeny of vertebrates that is strongly supported (see Appendix A). Hence, the ML analysis of the mtDNA sequences reduces to a comparison between the three hypotheses for the mammalian root, which are referred to by the

alternative sister groupings they support: 1. Theria, 2. Marsupionta, and 3. an unnamed clade comprising monotremes and placentals (referred to in this study as Monotremata-Eutheria).

**Table 2.1** Taxa used in this study, their GenBank accession numbers and variance ratios, calculated using RASA for the PTN12+RNArt dataset with (standard nucleotide: ATCG) NT-coding, for the 25-taxon and the reduced 18-taxon datasets.

	GenBank accession number	Taxon Variance Ratios for PTN12+RNArt (NT)	
		25-taxon dataset	18-taxon dataset
Dogfish ( <i>Mustelus manzano</i> )	AB015962	3.20282	2.39175
Trout ( <i>Oncorhynchus mykiss</i> )	L29771	3.38053	2.49268
Caecilian ( <i>Typhlonectes natans</i> )	AF154051	9.56093	-
Salamander ( <i>Mertensiella luschani</i> )	AF154053	4.55110	-
Mole Skink ( <i>Eumeces egregius</i> )	AB016606	2.95923	2.05102
Iguana ( <i>Iguana iguana</i> )	AJ278511	3.07669	2.15168
Green Turtle ( <i>Chelonia mydas</i> )	AB012104	2.22908	2.02396
Painted Turtle ( <i>Crysemys picta</i> )	AF069423	2.46644	-
Greater Rhea ( <i>Rhea americana</i> )	AF090339	2.52890	2.90120
Rook ( <i>Corvus frugilegus</i> )	Y18522	2.62840	-
Platypus ( <i>Ornithorhynchus anatinus</i> )	X83427	2.25533	1.50787
Echidna ( <i>Tachyglossus aculeatus</i> )	AJ303116	2.25130	1.50978
Wallaroo ( <i>Macropus robustus</i> )	Y10524	2.50690	1.69779
Brush-tail Possum ( <i>Trichosurus vulpecula</i> )	AF357238	2.47295	1.67074
Bandicoot ( <i>Isodon macrourus</i> )	AF358864	2.41658	1.63988
Opossum ( <i>Didelphis virginiana</i> )	Z29573	2.35772	1.59061
Armadillo ( <i>Dasyus novemcinctus</i> )	Y11832	2.43490	-
Pika ( <i>Ochotona collaris</i> )	AF348080	2.43827	-
Hippopotamus ( <i>Hippopotamus amphibius</i> )	AJ010957	2.53385	1.65229
Fin Whale ( <i>Balaenoptera physalus</i> )	X61145	2.46188	1.60505
Horseshoe Bat ( <i>Rhinolophus monoceros</i> )	AF406806	2.51319	1.63715
Flying Fox ( <i>Pteropus scapulatus</i> )	AF321050	2.58287	1.68232
Tarsier ( <i>Tarsius bancanus</i> )	AF348159	2.43197	-
Elephant ( <i>Loxodonta africana</i> )	AJ224821	2.21026	1.48384
Aardvark ( <i>Orycteropus afer</i> )	Y18475	2.46292	1.59787

Sequences were aligned manually within Se-AL v1.0a1 (Rambaut 1996). Ribosomal RNA (12S and 16S) structure was based on the models of Gutell *et al.* (1993) and Springer and Douzery (1996). Putative tRNA secondary structures were designed for each taxon. With gaps and ambiguous sites removed, the concatenated data set contained 13856 nucleotide sites, with the 13 protein-coding genes contributing 10764 sites and RNA-coding genes contributing 3092 sites.

NADH6 and eight of the 22 tRNA genes are coded on the mt L-strand, which has a different nucleotide composition from that of the H-strand (see Reyes *et al.* 1998a). Using the code from the H-strand for all sites avoids these strand-specific differences. However, this disrupts the functional relationship between the DNA code and selection pressures on the protein and RNA products for

the L-strand genes (which affects the substitution regime). It is unclear whether preference should be given to maintaining the strand-specific coding relationship between all genes or to maintenance of the functional relationship between the DNA code and the protein and RNA products or, alternatively, whether the L-strand genes (which provide the minority of sites) should be excluded altogether. Other than providing extra data, inclusion of the L-strand genes will not effect maximum-parsimony (MP) analyses (which treat sites independently). Furthermore, L-strand gene exclusion or inclusion (coded from either the L-strand or H-strand) alters bootstrap support for ME distance and ML analyses by less than 4% (less than 2% for rooting Mammalia). In order to be data-inclusive, the L-strand genes have therefore been included for this study (coded as complements for the DNA sequences).

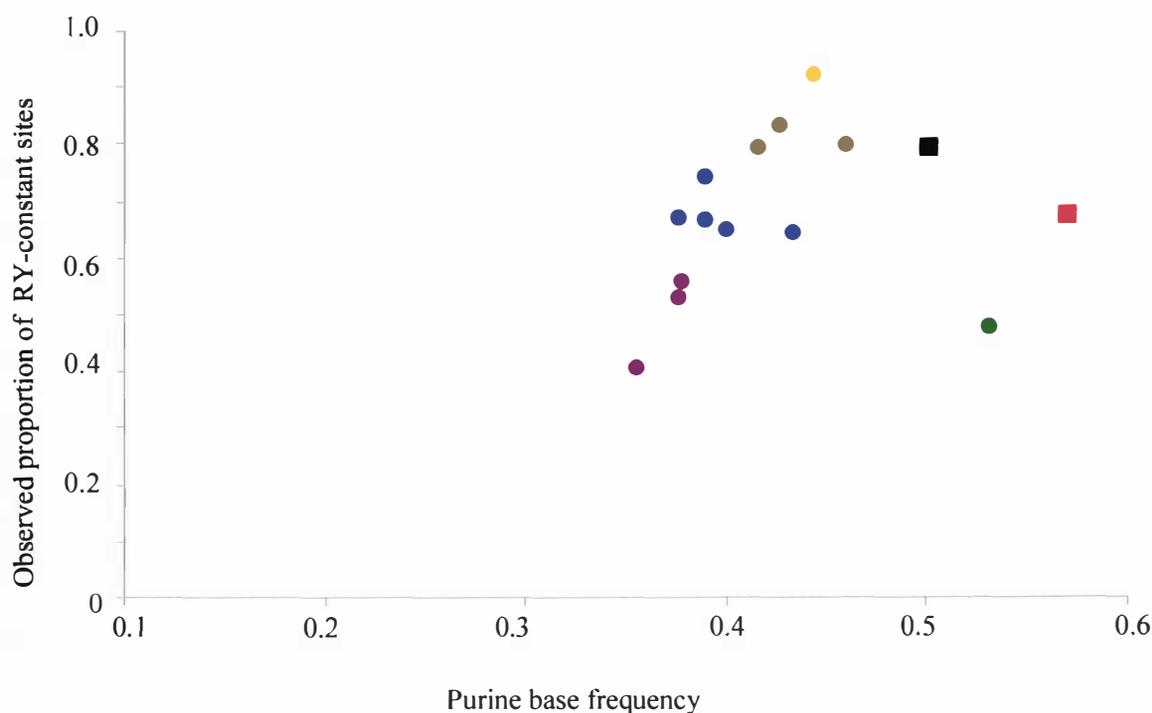
The concatenated protein-coding DNA sequences are referred to as PTN123, PTN12, PTN1, PTN2 or PTN3, with the numbers defining which codon positions are included. The concatenation of the ribosomal and transfer RNA sites is referred to as RNArt. Standard coding of the four DNA nucleotides (A, C, G, T) is referred to as NT-coding. RY-coding pools both purines (adenine and guanine: R) and both pyrimidines (cytosine and thymine: Y) into 2-state categories (R, Y). The amino acid translation of the protein-coding genes is referred to as PTNaa. An alternative treatment (PTNfg) has leucine (Leu), isoleucine (Ile) and valine (Val) lumped as one category, or functional group. This follows suggestions that variation in preference for these often functionally interchangeable amino acids (each being mid-sized, neutral and hydrophobic) accounts for much of the amino acid composition bias among the mt-encoded proteins of vertebrates (Naylor and Brown 1998) and mammals particularly (DeBry 1999).

Concatenating the complete protein-coding and RNA-coding sequence allows for only one model of substitution to govern characters that are clearly not evolving under a homogenous evolutionary process. The aim of dividing these characters into “process partitions” and allowing each to be governed by a different model of substitution is to provide a closer overall fit to the data by circumventing much of this heterogeneity. Partitioning data into increasingly smaller subsets that (apparently) evolve under more similar “rules” is offset by the problem that partitioning increases the number of parameters required to be estimated, which tends to lead to an increased variance-to-signal ratio (Yang *et al.* 1994; Yang 1996; Sullivan *et al.* 1999). More specifically, smaller data subsets will be more susceptible to stochastic error for parameter estimation. This could result in reduced precision for estimating phylogeny and so reduced confidence for supporting, or rejecting, relationships. This appears to be the case (at least mildly) for nodes that are well supported regardless of partitioning or concatenation (e.g. Krajewski *et al.* 1999; Cao *et al.* 2000a; but see Krajewski *et al.* 2000). The hope is that for uncertain nodes, more accurate modelling of evolutionary processes will offset any reduced precision.

With the aim of balancing considerations for heterogeneity of evolutionary processes, with the potential for loss of phylogenetic precision, the RNA-coding data was divided into just two partitions. These are RNAstems and RNAloops, which respectively comprise the stem and loop sites from 12S/16S rRNA and the 22 tRNA genes. Dividing the protein-coding sites into “process partitions” would clearly benefit from good structural models for the proteins (as is the case for the RNA-coding gene products). Unfortunately no such models are currently available. Previous studies that have utilized partitioned ML for mt gene analysis have emphasized differences between the three codon positions and/or between the genes themselves (e.g. Hasegawa *et al.* 1996; Yang 1996; Amrine and Springer 1999; Cao *et al* 2000a, 2000b). These studies have shown that evolutionary processes are very different between the three codon positions. However, as process partitions, the genes are only surrogates for the structural/functional differences between them. Indeed, DeBry (1999) showed that certain partitioning schemes based on amino acid categories reduced likelihood scores (relative to concatenated analyses) more efficiently (with less parameterization) than did gene-based partitions.

For the current study, in order to limit parameter richness and stochastic effects, genes were only partitioned where their observed substitution properties differed markedly. Figure 2.1 illustrates the division of the PTN12+RNArt dataset into RNAloops, RNAstems, and five protein-coding gene partitions. These process partitions are based on the observed proportion of RY-constant sites, and the average frequency of purine bases across the 18 taxa. In Figure 2.1, COI is relatively close to members of the high-constant-sites/moderate-purine-frequency group (COII, COIII, *Cytb*). However, as the observed proportion of RY-constant sites approaches 1.0, the error associated with overestimation of the proportion of variable sites is magnified. Hence, considering that the COI RY-constant site level (0.922) is much closer to 1.0 than are those of COII (0.796), COIII (0.829) and *Cytb* (0.791), COI was treated as a separate partition.

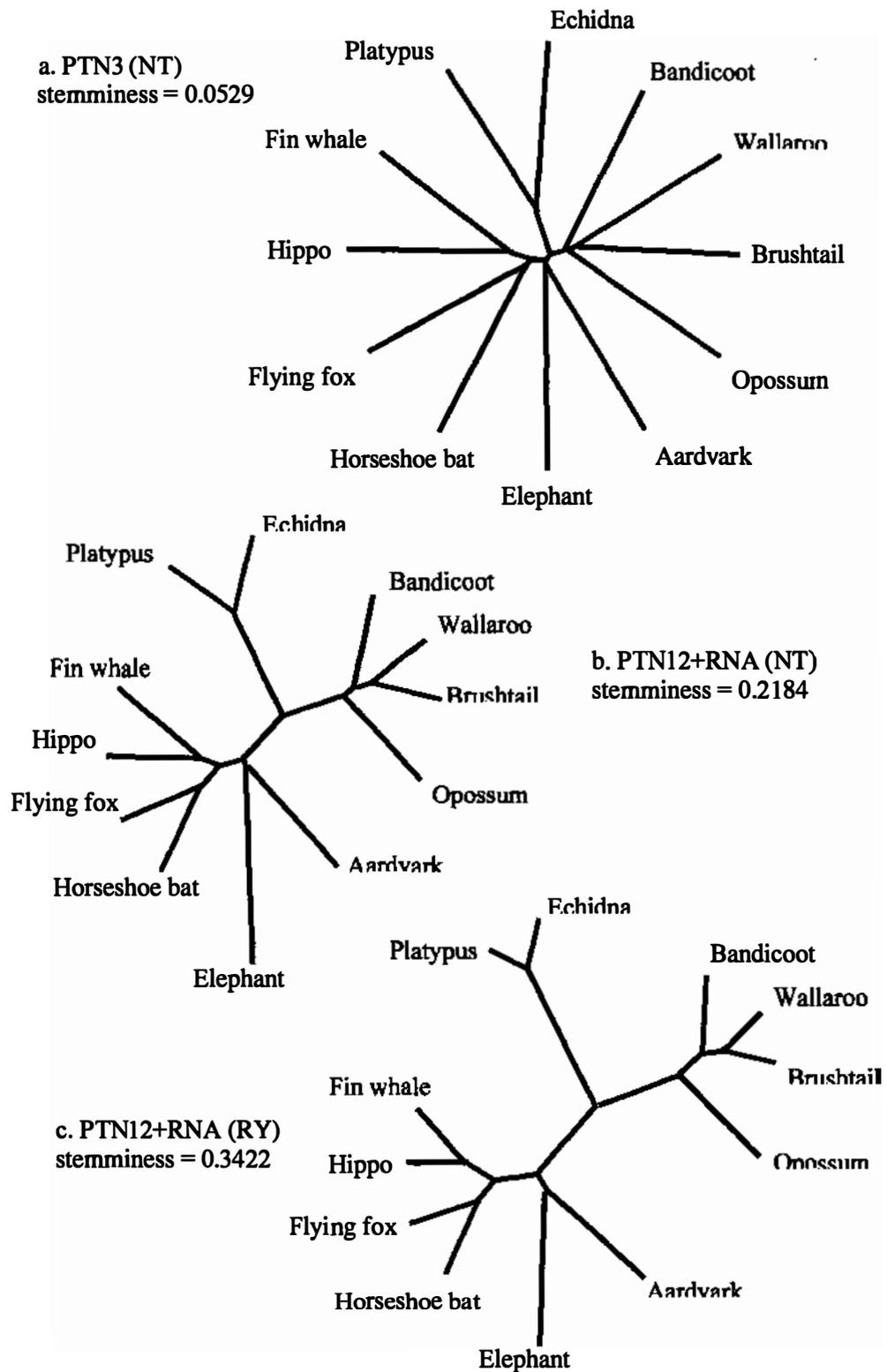
A previous complete mt genome phylogenetic analysis (Phillips *et al.* 2001) and the composition bias and stemminess results for this dataset (Table 2.3) suggest RY-coding to be more reliable than NT-coding for phylogenetic reconstruction involving deep mammalian divergences. It follows that properties of the RY-coding sequences were emphasized for partitioning the mt genes into groups for ML analyses. Nevertheless, pyrimidine versus purine content is also a fundamental property of NT-coded data and observed constant site levels among the 18 taxa are highly correlated between NT and RY-coded data for the PTN12+RNArt dataset ( $r^2 = 0.9054$ ). Thus the partitioning scheme is also expected to improve the fit of ML models to the NT-coded data.



**Figure 2.1** Partitioning of protein-coding (PTN12) and RNA-coding genes for the 18 taxa maximum-likelihood analysis, based on the observed RY-coded constant site proportion and purine base frequency. Groupings are: RNAloops (■); RNAstems (■); COI (●); NADH6 (●); low constant sites / low purine protein-coding genes (ATPase8, NADH2, NADH4L ●); moderate constant sites / low-to-moderate purine protein-coding genes (ATPase6, NADH1, NADH3, NADH4, NADH5 ●); high constant sites / moderate purine protein-coding genes (COII, COIII, Cytb ●). For comparison with the H-strand-coded proteins, the purine base frequency of NADH6 (which is coded on the L-strand) was determined from the H-strand. Data point values are provided in Appendix B.

### 2.2.2 Composition heterogeneity

If there is an essentially homogeneous base and amino acid composition within the ingroup, then alternative positions of the mammalian root will be affected approximately equally by any heterogeneity involving the outgroup taxa. As such, examination of composition heterogeneity focuses on the mammalian ingroup, because the placement of the mammalian root is the main concern of this study. Chi-square tests for composition homogeneity were conducted using PAUP\* 4.0b8 (Swofford 1998) for nucleotide data and Microsoft Excel for amino acid data. These test the null hypothesis that base or amino acid composition is homogenous across taxa, or in other words, that the observed deviation from composition homogeneity can be explained as a sampling effect. Chi-square tests however, are difficult to compare between data treatments, because accepting or rejecting the null hypothesis depends on the number of sites included and the number of character states (e.g. amino acid coding provides only one third as many sites as nucleotide coding, but



**Figure 2.2** Unrooted mammal (ingroup) ME phylograms based on uncorrected distances, illustrating stemminess differences between (a.) NT-coded PTN3, (b.) NT-coded PTN12+RNA and (c.) RY-coded PTN12+RNA.

allows 19, as opposed to three degrees of freedom). Further, even if composition homogeneity is rejected, this gives little or no indication of the potential for any such bias to actually influence phylogeny reconstruction for a given dataset.

In order to compare the relative potential for composition heterogeneity to bias phylogeny reconstruction between different data treatments, I have designed a simple statistic (stemminess/RCV). “Stemminess” is a relative measure of phylogenetic structure (Lanyon 1988), while RCV stands for relative composition variability (see below). Stemminess is defined here as the percentage of uncorrected minimum-evolution tree distance attributed to internal (as opposed to external) branches. Where data treatments are compared for the same tree (taxa and relationships), higher stemminess indicates a higher signal-to-noise ratio. The unrooted phylograms in Figure 2.2 illustrate differences in stemminess between the NT-coded PTN3, NT-coded PTN12+RNArt and RY-coded PTN12+RNArt datasets. The relationships in this tree are supported in each analysis and by recent molecular studies (e.g. Springer *et al.* 1998; Murphy *et al.* 2001b; Phillips *et al.* 2001; Waddell *et al.* 2001; Janke *et al.* 2002). All stemminess values were determined from the same (unrooted) tree topology (shown in Figure 2.2), and thus are comparable.

Relative composition variability (RCV) is defined as the average composition variability among data categories (e.g. A, C, T, G) between taxa, for a given data set. RCV for nucleotides is defined as follows:

$$RCV = \sum_{i=1}^n (|A_i - A^*| + |T_i - T^*| + |C_i - C^*| + |G_i - G^*|) / n.t$$

$A_i$ ,  $T_i$ ,  $C_i$  and  $G_i$  are the numbers of each nucleotide for the  $i$ th taxon.  $A^*$ ,  $T^*$ ,  $C^*$  and  $G^*$  are the average number of each nucleotide for the  $n$  taxa included, while  $t$  is the number of sites included. RCV allows direct comparison of the level (magnitude) of composition bias among given datasets and data treatments. All chi-square tests and RCV values were determined with constant sites excluded, as these sites buffer against inferring composition bias among the sites that contribute directly to apparent phylogenetic signal.

In summary, for data (or data treatments) that are compared for the same tree, lower RCV and higher stemminess values respectively indicate a lower magnitude of composition bias and a lower potential for bias (composition or other non-phylogenetic signals) to influence phylogeny reconstruction. Hence phylogeny estimates from the data treatments (such as coding methods) and partitions that have the highest stemminess/RCV values, are expected to be the least susceptible to composition bias.

The exclusion of PTN3 (due to substitution approaching saturation and/or composition bias) has been preferred in many phylogenetic analyses of mt genomes, including the first molecular studies advocating Marsupionta (Janke *et al.* 1996; Janke *et al.* 1997). The PTN3 data has been excluded from phylogenetic analyses in this study on the basis of its high relative base composition variability (RCV), and low signal-to-noise ratio (inferred from low stemminess), for both NT and RY-coding treatments (see Table 2.3 and Figure 2.5).

### 2.2.3 Phylogenetic analysis

ML analysis of PTNaa was carried out using ProtML within MOLPHY 2.3 (Adachi and Hasegawa 1996). All MP, ME and ML analyses of nucleotide sequences were performed within PAUP\* 4.0b8 (Swofford 1998). Bootstrap percentage values were calculated from 500 resampled heuristic searches. The TN93 (Tamura and Nei 1993) and F81 (Felsenstein 1981) distance corrections were used for ME analyses of the NT and RY-coded data respectively. These methods have the advantage of allowing for unequal equilibrium nucleotide frequencies, unlike models such as Jukes-Cantor (1969) and Kimura 3-parameter (Kimura 1981). The TN93 and F81 analyses were compared with ME analyses that utilize LogDet distances (Lockhart *et al.* 1994). Unlike most distance corrections (including F81 and TN93), the LogDet or paralinear distance (Lake 1994) transformation is intended to be robust to composition heterogeneity among taxa.

For ML analyses, the TN93 (Tamura and Nei 1993) and CF87 (Cavender and Felsenstein 1987) models of sequence evolution were respectively applied to the NT and RY-coded data. The substitution rate matrix estimated for TN93 includes the two transition categories and a single transversion category. This was preferred over the general time reversible (GTR) model (Yang 1994). The GTR model is computationally expensive. In addition, TN93 and CF87 both incorporate a single lumped transversion category. Thus if the NT data are modelled with TN93, the CF87-modelled RY data provide a control for the effect of transitions. Modelling the NT data with GTR does not allow as direct a comparison.

The Kishino-Hasegawa (1989) test was used to examine the three *a priori* hypotheses (Theria, Marsupionta and Monotremata-Eutheria) for each of the ML analyses. Two-tailed probabilities of tree rejection are given. The tests were performed within PAUP\* 4.0b8 for the concatenated nucleotide sequences (PTN12, RNArt and PTN12+RNArt). For partitioned analyses, PAUP\* site  $-\ln L$  values were stored from analysis of each partition and transferred to a Microsoft Excel file that was written to apply the Kishino-Hasegawa (1989) test to the (now) combined site  $-\ln L$  values.

In order to further examine composition bias, ME trees based on pairwise base frequency differences between taxa were constructed. Base frequency data are shown in Table 2.2.

As an example of how the distances were calculated, the distance for A–G (representing purine bias) between the iguana and the rhea is the absolute value  $|(A-G)_{\text{Rhea}} - (A-G)_{\text{Iguana}}|$ , where A–G is the difference between the number of adenine (A) sites and guanine (G) sites for the given taxon.

#### 2.2.4 Molecular dating

With lower base composition variability and relatively less substitution saturation, RY-coding is likely to be more accurate than NT-coding for molecular dating with PTN12+RNArt. Nevertheless, estimating the timing of the divergence between the echidna and the platypus, and between these monotremes and the therian mammals, is still complicated by the lack of rate constancy across the tree. Inspection of Figure 2.9 reveals a general pattern of acceleration in the rate of mt evolution from fish to amphibians and the “reptiles”, through to monotremes and marsupials, which are evolving faster still, and on to the eutherians.

One method (e.g. Takezaki *et al.* 1995) to counter rate differences is to “linearize” the trees by pruning out lineages that differ significantly in their substitution rate. Following this approach, I used simple likelihood ratio tests to assess rate constancy. The test statistic for this is twice the difference in  $-\ln L$ , both with and without a molecular clock constraint. This is compared to a  $\chi^2$  distribution critical value (where the degrees of freedom is the difference in number of branch lengths to be estimated with and without a clock; see Yang 1996). The maximum taxon inclusion along with the monotremes, that is not rejected ( $p < 0.05$ ) by this test is marsupials plus one outgroup taxon. The inclusion of a paraphyletic outgroup (e.g. amphibians and squamates) exposes the rate difference between the mammals and the outgroups, as the root cannot simply be placed at the most convenient position along the mammal stem (to give the illusion of rate constancy).

Without reliable fossil calibration points among the marsupials (or monotremes), the maximum linearized tree is not useful for the purposes of this study. Instead, relative divergences were inferred by using the program TreeEdit (Rambaut and Charleston 2001) to apply non-parametric rate-smoothing (Sanderson 1997) to branch-lengths that were estimated using PAUP\* (see below). The non-parametric rate-smoothing uses a minimum-evolution optimality criterion. Ancestor-descendent rate changes across nodes are minimized for inferring the (ultrameric distance) branch-lengths. Rates on each side of the root (dogfish shark) were inferred independently, and the rate at the root taken as the average of the two lineages. Excepting for the monotremes, taxa of uncertain affinities (Pika, Tarsier, Armadillo) were removed from the 25-taxon dataset for the analyses from which divergence times were inferred.

The input branch-lengths used for the non-parametric rate-smoothing were estimated under ME, with ML (CF87) distances that incorporated a SplitsTree2.4 (Huson 1998) estimate for the proportion of constant sites (0.700). ME was preferred over the ML optimality criterion for reasons that will be discussed later. However, branch-lengths were also estimated with ML in order to gauge the level of sampling error that might be affecting the branch-length estimates. The CF87+I+ $\Gamma_8$  model was used, where I and  $\Gamma_8$  respectively indicate that PAUP\* incorporated an invariable sites estimate and an eight-rate discrete gamma distribution.

Potential divergence time calibration points among the taxa of this study are provided by the occurrence of synapsids and diapsids in the late Carboniferous ( $\approx 310$  mybp; see Benton 1993) and by archaeocetid whales being known from India as early as  $\approx 53$  mybp (Bajpai and Gingerich 1998). I prefer not to use the latter calibration point, which is considered to provide a minimum divergence between whales and hippopotamuses (e.g. Gatesy and O'Leary 2001). Branch-length estimates indicate that the hippopotamus branch has evolved at only a little over 60% as fast as the fin whale branch (see Appendix C). The uncertainty of when and in which lineage(s) this rate change occurred could lead to large errors in estimating other dates. Non-parametric rate-smoothing can incorporate rate variation between the synapsids and diapsids more easily, as phylogenetic inference suggests that acceleration occurred along the mammalian stem and that a similar rate was maintained among monotremes and marsupials at least. Another reason to favour the synapsid/diapsid (S/D) calibration is that branch-length estimation errors are relatively smaller for larger divergences. Furthermore, errors in the calibration date itself are increased when extrapolating to deeper divergences, but decreased when interpolating to smaller divergences.

Most molecular dating studies attempt to define a lower bound for divergences. For example, Cooper and Penny (1997) rejected the null hypothesis that particular interordinal avian divergences occurred more recently than the Cretaceous-Tertiary boundary. Here, however, it is of interest to know how old (rather than young) the monotreme crown group and stem lineage might be, so it is preferable to err on the side of an older, rather than younger, calibration for S/D. As such, I used 320 mybp for S/D, rather than the 310 mybp date that has been accepted in a number of molecular dating studies (e.g. Kumar and Hedges 1998; Penny *et al.* 1999). This seems reasonable, given that the last common ancestor (LCA) of any clade will predate the first recognizable members of the daughter lineages.

## 2.3 RESULTS

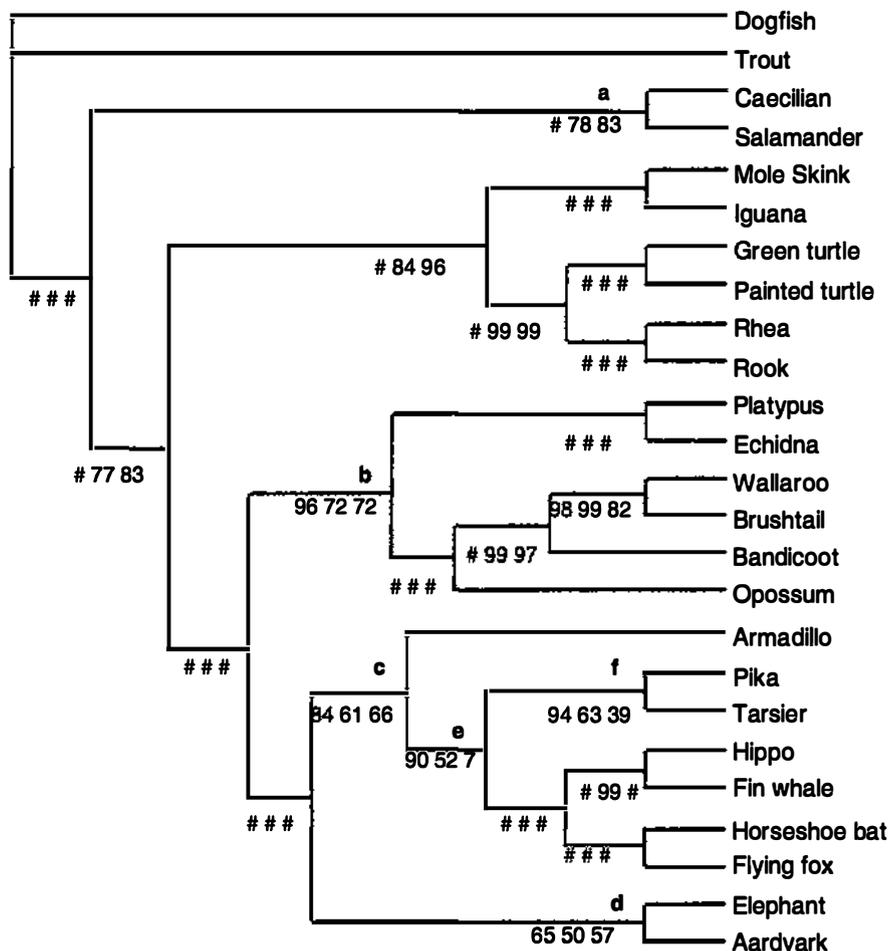
### 2.3.1 *Phylogenetic analysis of the 25-taxon dataset*

In recent phylogenetic studies of mt protein-coding data, ML and MP analyses of the amino acid translation have tended to be favoured over analyses of the corresponding nucleotide data. Saturation at non-synonymous nucleotide sites and a perception that the amino acid translation is less susceptible to biases relating to composition heterogeneity have been the major factors in this. The results of MP and of ML (mtREV-24F model) analysis of the concatenated amino acid data (PTNaa) for the 25-taxon dataset are shown in Figure 2.3. Both analyses favour the same tree, including supporting Marsupionta. MP with PTNfg provides an interesting result. With this treatment (for which Leu, Ile, and Val are lumped together), higher bootstrap support (than with PTNaa) tends to be found for the deep nodes, such as those defining Amphibia, Amniota and Sauropsida (taxa more closely related to squamates and archosaurs than to mammals). Conversely, PTNfg tends to provide lower bootstrap support than PTNaa for shallower nodes such as those defining Diprotodontia, Australidelphia, Supraprimates and Boreoeutheria.

The table associated with Figure 2.3 shows that the tree presented is favoured by each MP and ME analysis of PTN12+RNArt (for both NT and RY-coding treatments), with the exception of the rooting of Mammalia (node b) and Eutheria (node c). In Figure 2.3, each PTNaa (or PTNfg) treatment and three of the four PTN12+RNArt treatments favour Marsupionta (with between 72% and 100% bootstrap support). MP analysis of the RY-coded PTN12+RNArt dataset is the exception. In this case, Monotreme-Eutheria (h) is marginally favoured.

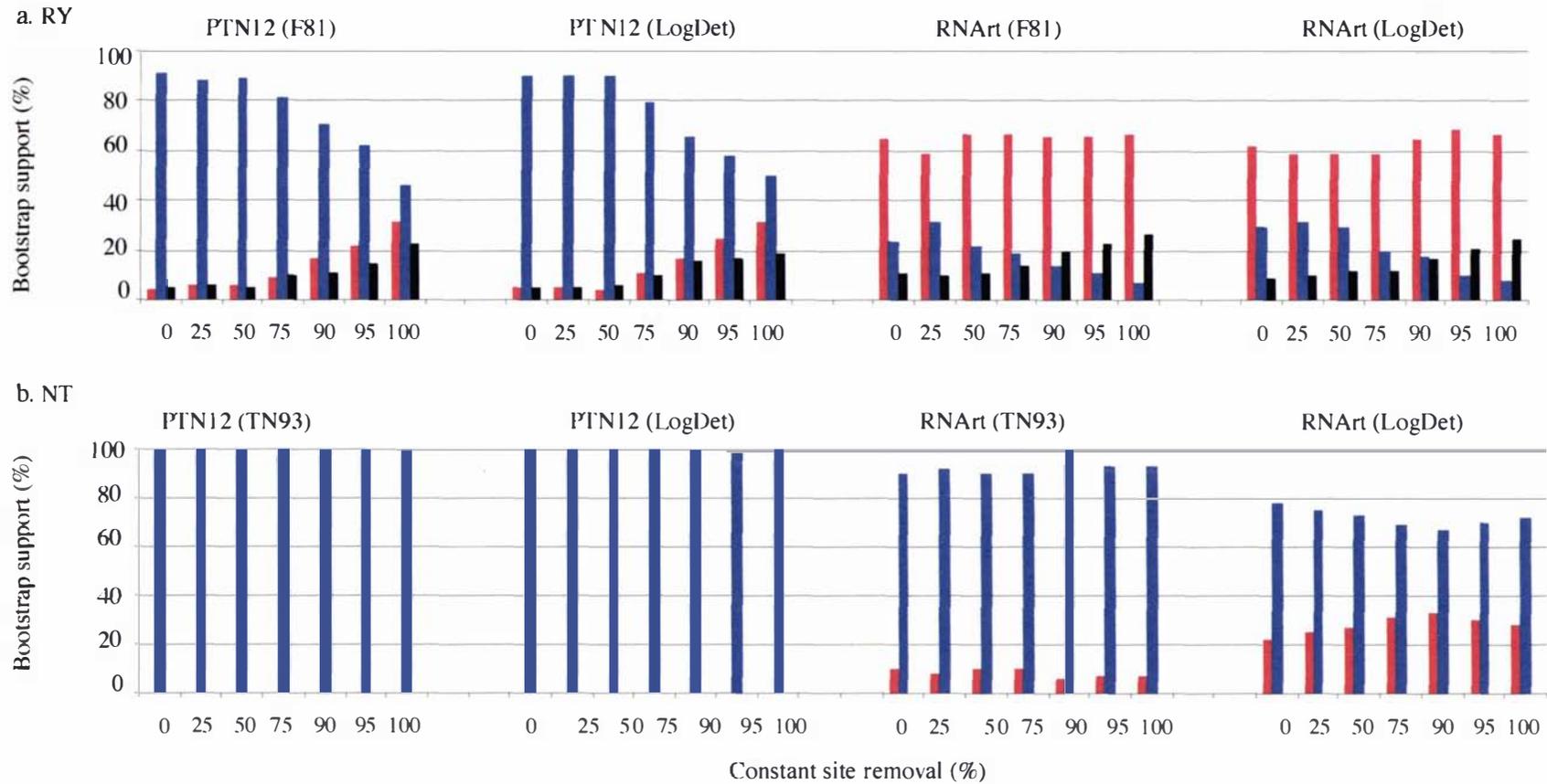
Consecutively higher proportions of constant sites were removed in ME distance analyses of the 25-taxon dataset, for which results are shown in Figure 2.4. The level of support for the alternative mammal rootings shows a number of interesting patterns in these analyses. Regardless of the distance correction or the proportion of constant sites removed, bootstrap support for Theria is higher for RNArt than for PTN12, and higher with RY-coding than with NT-coding. The opposite is true of support for Marsupionta. Some bootstrap support is found for Monotremata-Eutheria, with the RY-coded data (for both PTN12 and RNA), but not with the NT-coded data. This support increases as constant sites are removed, but remains quite low (26% or less).

With the ME distance analyses (Figure 2.4), constant site removal only has a clear (and monotonic) effect for the RY-coded data. As constant sites continue to be removed (above 50%) for these data (PTN12 and RNArt), support for Marsupionta decreases consistently, relative to support for Theria



	a	b	c	d	e	f	g	h
RY-coded MP	100	15	17	94	77	76	19	66
RY-coded ME (F81)	90	87	44	100	90	62	9	3
NT-coded MP	98	80	20	76	71	78	15	5
NT-coded ME (TN93)	99	100	43	89	93	66	0	0

**Figure 2.3** Cladogram showing relationships among 25 vertebrate taxa. The tree is based on analyses of the PTNaa (3588 aa) dataset. Percentage bootstrap support (500 replicates) is given for each node below the associated internode, for ML (MOLPHY mtREV-24F), MP and MP with Leu, Ile, and Val lumped together as one character state. 100% support is denoted by #. Analyses were also carried out on the PTN12+RNArt (10268 bp) dataset, with bootstrap support (500 replicates) for nodes a - f shown in the table for MP and ME analyses of these data coded both as standard nucleotides (NT) and as purines versus pyrimidines (RY). The nodes on the tree that are not listed in the table were recovered in 98% or more of the bootstrap replicates. Nodes g and h (in the table) are not on the given tree, but represent alternative hypotheses to Marsupionta: Theria (g) and Monotremata-Eutheria (h).



**Figure 2.4** Bootstrap support trends with constant site removal (as a percentage of observed constant sites) for three hypotheses of mammalian relationships: Theria (red), Marsupionta (blue) and Monotremata-Eutheria (black). Protein-coding genes (codon positions 1 and 2: PT12) and RNA-coding genes (RNArt) were treated separately. Minimum evolution distances were used in each treatment with F81 and LogDet corrections for RY-coded data (a) and TN93 and LogDet corrections for standard nucleotide data (b).

(and Monotremata-Eutheria). This occurs with both the F81 and LogDet distance corrections. This effect is somewhat limited for RY-coded RNArt, which clearly supports Theria from zero to 100% constant site exclusion. However, for RY-coded PTN12 (for F81 and LogDet respectively), the bootstrap support lead of Marsupionta over Theria is reduced from 87% and 85% without constant site removal, to 15% and 19% with all constant sites removed.

By comparison with the stationary nucleotide composition corrections (F81 for RY-coding, and NT93 for NT-coding), LogDet correction makes very little difference to the pattern of support for the mammalian root, with one exception (see Figure 2.4). Theria gains between 12 and 27 bootstrap percentage points higher support from LogDet than TN93, for NT-coded RNArt (depending on the proportion of constant sites removed). This hints at the possibility of a nucleotide composition bias playing some role in the promotion of Marsupionta. The lack of difference between the TN93 and LogDet corrections for the NT-coded PTN12 analyses might indicate that composition bias does not significantly affect these data. Alternatively, signal for Theria (or Monotremata-Eutheria) might simply be too low, relative to signal for Marsupionta, for any such effect to be observed from bootstrap support. This is possible given that both the Theria and Monotremata-Eutheria hypotheses gain 0% bootstrap support in each ME analysis of the NT-coded PTN12 data (see Figure 2.4).

### 2.3.2 Data examination

Under most circumstances ML is more robust to non-stationarity (variability in the substitution process across the tree) in substitution rate than most parsimony and distance methods (see Huelsenbeck 1995; Swofford *et al.* 2001). However, ML analyses typically assume that base composition is stationary (although the method of Galtier and Gouy 1998 is an exception; see later). Hence it is important to investigate the relative potential among alternative data and treatments for such a bias to affect phylogeny reconstruction in the 18-taxon ML analyses. The importance of this is further underlined by the results of the TN93 and LogDet distance correction analyses of the NT-coded RNArt dataset (Figure 2.4), which revealed the possibility of a composition bias.

**Table 2.2** Base frequencies and differences among pyrimidines (T–C), among purines (A–G) and between purines and pyrimidines (Y–R) for the 18-taxon, 10268 nucleotide PTN12+RNArt concatenate. Note that the average and standard deviations given are for the ingroup (mammalian) taxa only.

	A	C	G	T	T – C	A – G	Y – R
Dogfish	2713	2534	1911	3110	576	802	1020
Trout	2510	2794	2144	2820	26	366	960
MoleSkink	2749	2740	1935	2844	104	814	900
Iguana	2862	2857	1800	2749	-108	1062	944
GrTurtle	2929	2703	1718	2918	215	1211	974
Rhea	2665	2873	1899	2831	-42	766	1140
Platypus	2850	2488	1774	3156	668	1076	1020
Echidna	2849	2513	1783	3123	610	1066	1004
Wallaroo	2879	2560	1760	3069	509	1119	990
Brushtail	2937	2561	1716	3054	493	1221	962
Bandicoot	2909	2404	1736	3219	815	1173	978
Opossum	2987	2384	1689	3208	824	1298	916
Hippo	2879	2652	1789	2948	296	1090	932
FinWhale	2856	2585	1781	3046	461	1075	994
HShoeBat	2822	2621	1816	3009	388	1006	992
FlyingFox	2874	2570	1810	3014	444	1064	900
Elephant	2924	2560	1745	3039	479	1179	930
Aardvark	2888	2529	1730	3121	592	1158	1032
average	2887.8	2535.6	1760.8	3083.8	548.3	1127.1	970.8
std. dev.	45.4	79.4	38.5	82.7	160.8	81.4	42.6

Chi-square tests (Table 2.3) indicate that composition heterogeneity is considerably more significant among the 18-taxon dataset as a whole than for the 12 mammalian (ingroup) taxa. However, RY-composition homogeneity among the 18 taxa cannot be rejected even at  $p < 0.20$  for RNAs stems, RNA loops and PTN2. This contrasts sharply with NT-composition heterogeneity, which is highly significant ( $p < 0.0002$ ) among the 18 taxa for all of the nucleotide datasets. Similarly, amino acid composition heterogeneity is also highly significant ( $p < 0.0002$ ). This is the case regardless of whether or not Leu, Ile, and Val are lumped together.

Lower composition heterogeneity among the ingroup than among the 18-taxon dataset as a whole is noteworthy because composition heterogeneity among the ingroup is required for compositional non-stationarity to affect the rooting of Mammalia in a statistically consistent manner. Composition

heterogeneity among the ingroup is nevertheless significant at  $p < 0.0001$  for PTN3 (with both NT and RY-coding) and for NT-coded PTN1. Many studies exclude PTN3 anyway, though the result for PTN1 is particularly important. It is likely that composition heterogeneity among mammals for NT-coded PTN1 has been masked in many previous analyses, by the inclusion of constant sites and of many very closely related taxa (such as numerous apes).

For the ingroup (mammals), composition homogeneity is not rejected (at  $p < 0.05$ , see Table 2.3) for the amino acid datasets (PTNaa and PTNfg) and the PTN2 and RNArt datasets (both NT and RY-coding), nor for PTN1 with RY-coding. However, the relative composition variability (RCV) results show that considerable variation in the magnitude of composition heterogeneity among the ingroup exists between the NT, RY and amino acid treatments.

**Table 2.3** Relative composition variability (RCV) and stemminess values (see terminology). These are calculated from variable sites only, for PTN1, PTN2, PTN3, RNAs stems and RNAloops (for both NT and RY-coding) and for PTNaa and PTNfg. Homogeneity chi-square p-values are shown for the 18-taxon dataset and for the 12-taxon (mammalian) ingroup dataset. RCV and stemminess values are shown for the ingroup taxa only. Chi-square ( $p$ )  $> 0.05$ , RCV  $< 0.05$  and stemminess values  $> 0.25$  are in bold.

Sites	Data	Chi-square ( $p$ )		RCV	Stemminess
		(18 taxa)	(ingroup)		
PTN1	NT	$<0.0001$	$<0.0001$	0.0734	0.1765
	RY	0.0123	<b>0.2583</b>	<b>0.0269</b>	<b>0.3330</b>
PTN2	NT	$<0.0001$	<b>0.961</b>	<b>0.0342</b>	<b>0.2558</b>
	RY	<b>0.2315</b>	<b>0.579</b>	<b>0.0392</b>	<b>0.3535</b>
PTN3	NT	$<0.0001$	$<0.0001$	0.143	0.0529
	RY	$<0.0001$	$<0.0001$	0.0528	0.1257
RNA stems	NT	$<0.0001$	<b>0.0738</b>	0.0602	<b>0.3169</b>
	RY	<b><math>&gt;0.9999</math></b>	<b>0.9906</b>	<b>0.0302</b>	<b>0.4259</b>
RNA loops	NT	$<0.0001$	<b>0.1684</b>	0.0667	0.187
	RY	<b>0.9797</b>	<b>0.7017</b>	<b>0.0449</b>	<b>0.3116</b>
Amino acids	aa	$<0.0001$	<b>0.3963</b>	0.0819	0.2218
	Fg	$<0.0002$	<b><math>&gt;0.9999</math></b>	0.0612	0.2425

The ingroup RCV values for the amino acid treatments (PTNaa and PTNfg) are higher than for any of the RY-coded data, including PTN3 (Table 2.3). The RCV values for PTNaa and PTNfg were also higher than that for NT-coded PTN2, while the RCV value for PTNaa was higher even than for NT-coded PTN1. Hence the advantage of using the amino acid translation rather than the NT-

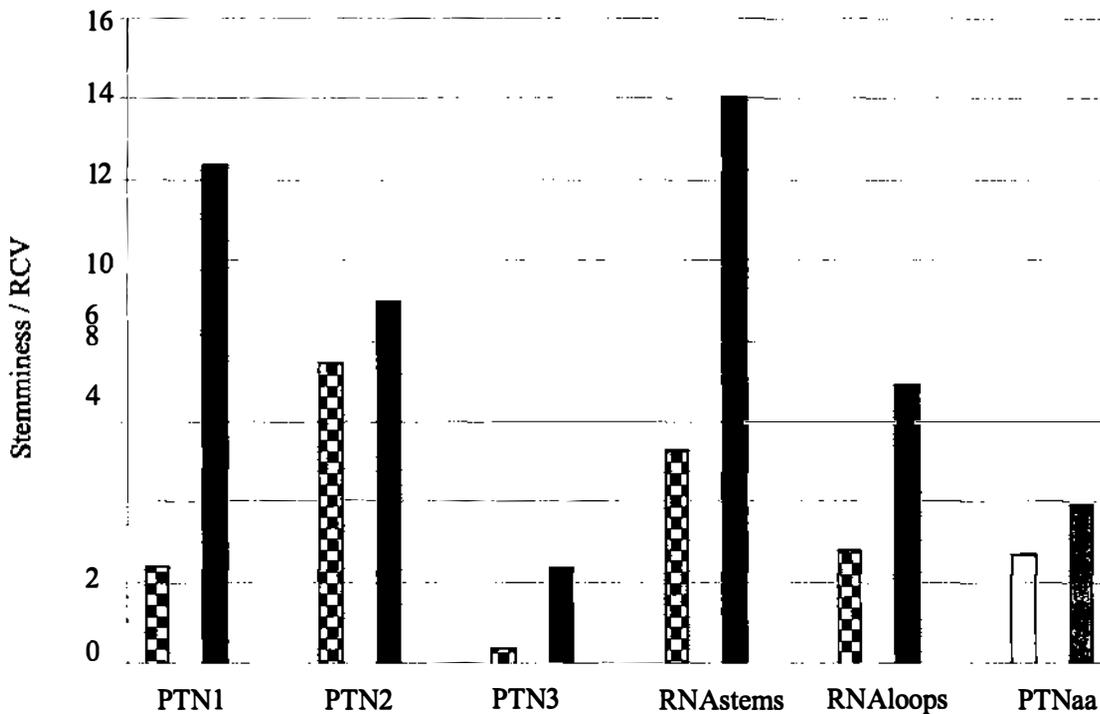
coded data (at least for PTN1) implied by the results of the chi-square test is not apparent in terms of the magnitude of compositional variability. Curiously, RCV is similar for NT and RY-coded PTN2. By contrast, the RCV values with NT-coding are approximately 2.7 times higher than with RY-coding, for PTN1 and PTN3. The situation is similar, although less extreme, for the RNA stems and loops.

Much of the NT-coded nucleotide composition variability derives from variation among taxa for the relative frequencies of the two pyrimidine bases. For the 10268 sites used in the phylogenetic analyses (PTN12+RNArt), the standard deviation among the ingroup taxa for T–C (among pyrimidine frequency difference) is 160.8. This compares with standard deviations of 81.4 for A–G (among purine frequency difference) and 42.6 for Y–R (pyrimidine frequency minus purine frequency bias). Nucleotide frequency data for PTN12+RNArt is provided in Table 2.2 for the 18-taxon ML analysis dataset. Correlation ( $r^2$ -value) between PTN12+RNArt and PTN3 across the ingroup taxa is 0.8815 for T–C, 0.6376 for A–G and 0.5629 for Y–R.

As an indicator of the signal-to-noise ratio among data, the stemminess values shown in Table 2.3 distinguish the RY-coded data as being substantially more likely to have retained phylogenetic signals than the NT-coded data. RY-stemminess values are between 1.34 and 2.38 times higher than the NT-coded equivalents for PTN1, PTN2, PTN3, RNAstems and RNAloops. Furthermore, with the exception of PTN3, the RY-coded data also have considerably higher stemminess than both treatments of the amino acid translation (PTNaa and PTNfg). For the PTN12+RNArt concatenate, RY-coding (Figure 2.2c) provides 1.57 times higher stemminess than NT-coding (Figure 2.2b). In comparing Figures 2.2b and 2.2c, particularly note the lengths of the external branches in relation to the lengths of the internodes that arise from the therian last common ancestor node.

As described in the Methods section, when data (or data treatments) are compared for the same tree (taxa and relationships), lower RCV and higher stemminess respectively indicate a lower magnitude of composition bias and a lower potential for bias (composition or other non-phylogenetic signals) to influence phylogeny reconstruction. Hence the data treatments and partitions that have the highest stemminess/RCV values (stemminess divided by RCV) are expected to provide the phylogeny estimates that are the least susceptible to composition bias. On this basis, inspection of Figure 2.5 shows that the relative potential for compositional non-stationarity to influence phylogenetic inference differs substantially between the different data treatments and between partitions. Particularly prominent is the stemminess/RCV improvement that RY-coding provides by comparison with NT-coding (except for PTN2, where the

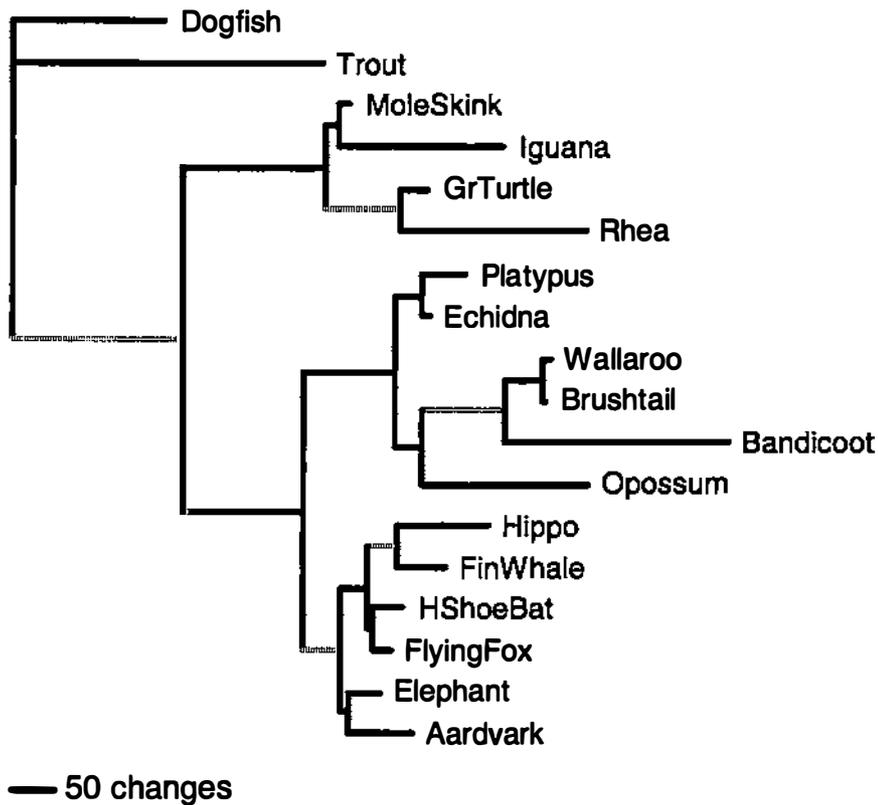
improvement is only slight). Furthermore, stemminess/RCV for PTNaa and PTNfg are low by comparison with the RY-coded PTN1 and PTN2 datasets.



**Figure 2.5** Stemminess / relative composition variability (RCV) for RNAloops loops and RNAstems, as well as the 13 concatenated protein-coding genes, both as nucleotide data (divided into codon positions: PTN1, PTN2 and, PTN3) and as the amino acid sequence (PTNaa). The nucleotide sequences are treated as standard NT (▨) and RY-coded (■). The protein sequence is treated as standard amino acids (□) and with Leu, Ile, and Val lumped together as a functional group (■).

The composition bias results shown in Table 2.3 were derived across the whole data matrix. As such, it is difficult to interpret the relative strength of the various potential compositional biases at the mammalian root, or indeed which rooting they might favour. Such insights however, were gained by constructing minimum-evolution trees from pairwise distances obtained as differences in base frequencies between taxa. Pairwise base frequency distances (see Table 2.2) were determined for A–G (purine bias), T–C (representing pyrimidine bias) and Y–R (representing the frequency bias between purines and pyrimidines). To make these results relevant to the ML analyses, the same 18-taxon PTN12+RNArt data was used, as were the three constraint trees (Marsupionta, Theria, and Monotremata-Eutheria each indicated by arrows on the tree in Appendix A). Allowing variation only in the placement of the mammalian root is important, as it ensures that the distribution of the composition bias across the tree is the same as occurs in the phylogenetic analyses in which the mammalian root is being inferred.

Figure 2.6 shows the T–C pairwise distance ME tree, which favours Marsupionta. With Theria and Monotremata-Eutheria respectively requiring 84.33 and 65.91 more T–C changes than Marsupionta, this pyrimidine bias is by far the largest base compositional bias acting across the mammalian root for PTN12+RNArt. That the T–C pairwise distance tree favours Marsupionta so strongly, could have been predicted from the T–C frequencies shown in Table 2.2. The PTN12+RNArt averages for T–C among the monotremes and marsupials are much higher (639.0 and 660.5 respectively) than among the placentals and outgroup taxa (443.3 and 18.5 respectively).



Distance	Theria	Marsupionta	Mono + Eutheria
T–C	2569.49 (1838.63)	<b>2485.16 (1771.63)</b>	2551.07 (1833.63)
A–G	<b>1923.00 (1452.15)</b>	1936.75 (1455.53)	1927.65 (1455.53)
(T–C)+ (A–G)	3142.97 (2674.77)	<b>3064.79 (2639.61)</b>	3099.38 (2674.77)
Y–R	685.26 (531.96)	684.26 (531.96)	682.82 (531.96)

**Figure 2.6** Minimum evolution (ME) phylogram based on pairwise distances between taxa for T–C base frequencies among the PTN12+RNA dataset. Dotted branches indicate negative branch lengths. The table provides ME scores for the alternative mammalian root hypotheses (with the backbone phylogeny constrained; see Appendix A), for pairwise base-frequency-difference distances among taxa: among pyrimidines (T–C); among purines (A–G); summed among transitions ((T–C) + (A–G)); and between purines and pyrimidines (Y–R). ME scores with negative branch lengths not allowed are shown in brackets. For each analysis the (best) ME score is in bold. Base frequency data is provided in Table 2.2.

Theria is favoured in the A–G pairwise distance ME analysis, though is only better than Marsupionta and Monotremata-Eutheria by 13.75 and 4.65 A–G changes respectively. Differences among the three hypotheses are smaller still for the Y–R pairwise distance data, with Monotremata-Eutheria favoured, but with Theria and Marsupionta requiring only 2.44 and 1.44 further Y–R changes respectively.

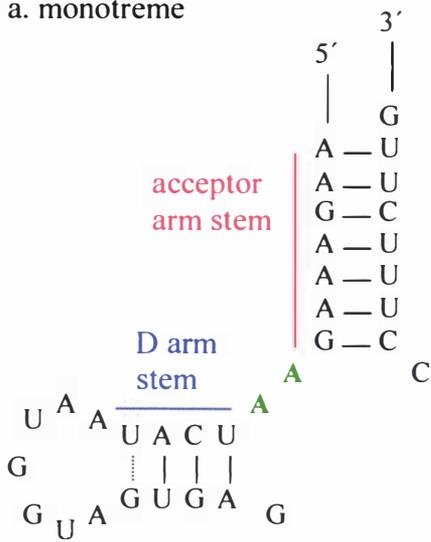
The above results were from trees for which negative branch-lengths were allowed for the constrained internodes. Kuhner and Felsenstein (1994) showed that in some cases, phylogenetic estimation can be improved by forcing all branch-lengths to be non-negative. As shown in the table associated with Figure 2.6, if negative distance branches are not permitted, the results are essentially the same. However, the already small difference between the three hypotheses in the Y–R distance analysis is erased and Marsupionta, Theria, and Monotremata-Eutheria are equally favoured.

### 2.3.3 *tRNA-Serine (UCN)*

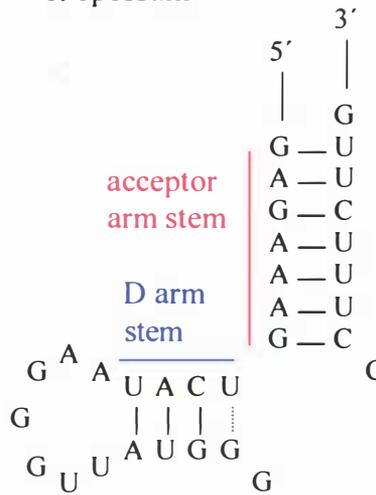
A search of all currently available amniote mt genomes reveals that two base pairs occur between the acceptor-arm and D-arm of tRNA-Serine (UCN) for all non-therians, but not in any marsupials or eutherians (see Figure 2.7). This may represent a therian synapomorphy. The lengths of both the acceptor and D-arm stems are conserved among the 25 taxa in this study. Furthermore, the RY-state of each of the stem pairs is also maintained across the 25 taxa (except for with the first D arm pair). The conservation of the acceptor and D-Arm stems throughout Amniota is complemented by the conservation (among non-therians) of the two bases (not present in therians) that join the stems. These two nucleotides were likely to have both been adenine in the LCA of amniotes. This AA combination occurs in all of the non-therian amniotes included in this study, except for the eastern painted turtle (TA).

When the platypus mt genome was published, Janke *et al.* (1996) noticed that the secondary structure implied by the sequence for the tRNA-Serine (UCN) product was similar to that of non-therians. With the dramatic increase in the number of mt genomes that have since been published, the conserved nature of the deletion (which appears to have occurred along the therian stem lineage) and its potential as a diagnostic feature and synapomorphy of Theria have become more notable.

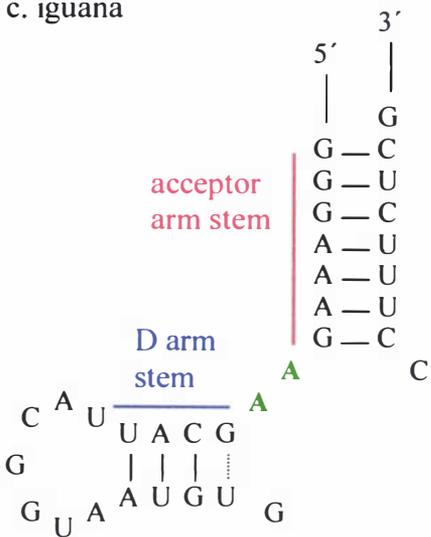
a. monotreme



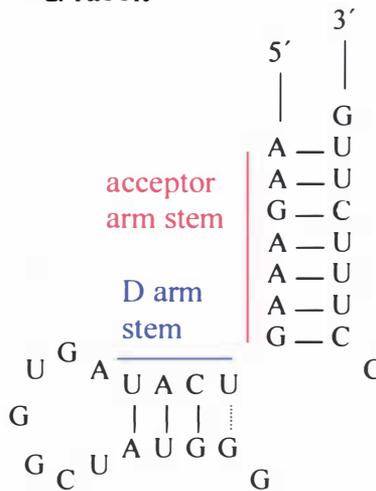
b. opossum



c. iguana



d. rabbit



**Figure 2.7** Putative secondary structure of the tRNA-Serine (UCN) acceptor and D arms for a. monotremes, b. the Virginia opossum, c. the iguana and d. the rabbit. Two nucleotides (probably AA in the last common ancestor of mammals and reptiles) occur between the acceptor and D arm stems in all fish, amphibians, reptiles and monotremes examined in this study, but are not found in therian mammals.

### 2.3.4 Maximum-likelihood analysis of the 18-taxon dataset

The aim of the ML analysis was to examine the effect of three factors on support for the *a priori* hypotheses that define monotreme placement among the 18-taxon tree (Theria, Marsupionta, Monotremata-Eutheria). These factors were: coding (NT versus RY); incorporating among-site rate heterogeneity ( $\pm I$  and  $\Gamma_8$ ); and process partitioning (concatenated versus partitioned analyses). The coding effect was initially examined for PTN12 and RNArt separately.

The ML analyses of NT-coded PTN12 favour Marsupionta over both Theria and Monotremata-Eutheria by approximately 0.9 to 1.4 standard deviations, depending on whether or not among-site rate heterogeneity is incorporated in the TN93 model (Table 2.4). This is in line with previous studies of the mt protein-coding genes, coded either as NT or as the amino acid translation (e.g. Janke *et al.* 1996, 1997, 2002). This favouritism for Marsupionta (with PTN12) is retained with RY-coding, although statistical support is much reduced, with the two alternative hypotheses being only 0.14 to 0.31 standard deviations behind (Table 2.4).

**Table 2.4** Log-likelihood scores ( $-\ln L$ ) for the three mammal rooting hypotheses for both the 18-taxon PTN12 and RNArt (concatenated) datasets. The number of standard deviations ( $t$ ) from the most likely tree (which is denoted ML) and the two-tailed significance ( $p$ ) of this difference is also given. PTN12 and RNArt were analysed separately, both with and without PAUP\* estimates for invariable sites and an 8-category gamma rate distribution ( $I + \Gamma_8$ ). The TN93 model was used for (standard) NT-coding, and the CF model was used for RY-coding. Variance estimates and significance values were determined using the Kishino-Hasegawa (1989) test.

Data	Hypothesis	NT-coding (TN93 model)			RY-coding (CF87 model)		
		$-\ln L$	$t$	$p$	$-\ln L$	$t$	$p$
PTN12	Theria	60884.04	1.0238	0.3059	26402.17	0.3082	0.7580
	Marsupionta	<b>60863.54</b>	<b>ML</b>	<b>ML</b>	<b>26398.24</b>	<b>ML</b>	<b>ML</b>
	Mon+Euth	60891.26	1.4326	0.1520	26400.07	0.1403	0.8884
PTN12 ( $I + \Gamma_8$ )	Theria	55702.29	0.9046	0.3657	24083.79	0.2484	0.8038
	Marsupionta	<b>55694.82</b>	<b>ML</b>	<b>ML</b>	<b>24083.27</b>	<b>ML</b>	<b>ML</b>
	Mon+Euth	55703.93	1.1448	0.2524	24083.77	0.2373	0.8124
RNArt	Theria	<b>26483.47</b>	<b>ML</b>	<b>ML</b>	<b>9546.91</b>	<b>ML</b>	<b>ML</b>
	Marsupionta	26484.87	0.1089	0.9133	9551.77	0.6442	0.5195
	Mon+Euth	26499.51	1.5483	0.1217	9549.91	0.3762	0.7068
RNArt ( $I + \Gamma_8$ )	Theria	24976.44	0.1702	0.8649	<b>8872.90</b>	<b>ML</b>	<b>ML</b>
	Marsupionta	<b>24975.19</b>	<b>ML</b>	<b>ML</b>	8873.05	0.1227	0.9023
	Mon+Euth	24983.52	1.5648	0.1177	8873.29	0.4159	0.6775

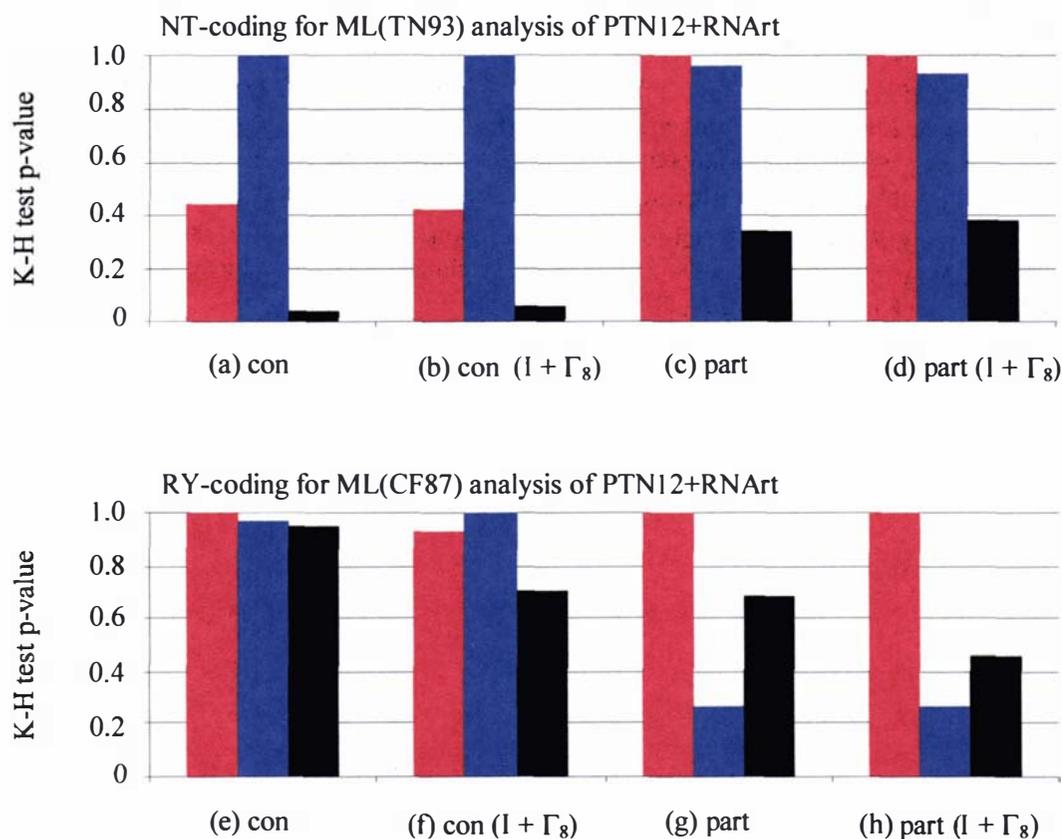
Following the results from the ME analyses (Figure 2.4), ML support for Marsupionta (relative to Theria) is lower for RNArt than for PTN12. Indeed, Theria is the favoured hypothesis in the ML analyses of RNArt, except with NT-coding under the TN93+I+ $\Gamma_8$  model (see Table 2.4). So the trend for RY-coding of the nucleotide data to enhance signal associated with Theria (relative to signal for Marsupionta) is also apparent for RNArt. Nevertheless, under the CF87 and CF87+I+ $\Gamma_8$  models for RY-coded RNArt, Marsupionta is only rejected by 0.1227 and 0.6442 standard deviations respectively.

**Table 2.5** Log-likelihood scores ( $-\ln L$ ) for the three mammal rooting hypotheses for the 18-taxon PTN12+RNArt dataset. The number of standard deviations (t) from the most likely tree (which is denoted ML) and the two-tailed significance (p) of this difference is also given. The treatment column indicates whether the analyses involved concatenated or partitioned PTN12+RNArt data and whether invariable sites and an 8-category gamma rate distribution (I+ $\Gamma_8$ ) were incorporated (these were estimated using PAUP\*). Note that for NT-coding, the treatments correspond to models a-d (of Table 2.6 and Figure 2.8) and are variants of TN93. For RY-coding, the treatments correspond to models e-h and are variants of CF87. For example, model h is partitioned ML(CF87+I+ $\Gamma_8$ ) for RY-coded data. Partitioned analyses involved separate analysis of twelve partitions: RNAsAtems, RNALoops, and (first and second codon sites [separately]) for the five protein-coding gene partitions (see Figure 2.1). Variance estimates and significance values were determined using the Kishino-Hasegawa (1989) test.

Treatment	Hypothesis	NT-coding (TN93 models a-d)			RY-coding (CF87 models e-f)		
		$-\ln L$	t	p	$-\ln L$	t	p
concatenated models a/e	Theria	87741.29	0.7630	0.4455	36108.02	ML	ML
	Marsupionta	87722.80	ML	ML	36108.54	0.0344	0.9726
	Mon+Euth	87767.18	2.0170	0.0437	36108.93	0.0603	0.9519
concatenated (I+ $\Gamma_8$ ) models b/f	Theria	81090.26	0.8029	0.4221	33052.15	0.0866	0.9310
	Marsupionta	81081.24	ML	ML	33051.94	ML	ML
	Mon+Euth	81099.51	1.8819	0.0599	33052.69	0.3764	0.7066
partitioned models c/g	Theria	84002.95	ML	ML	34062.08	ML	ML
	Marsupionta	84004.12	0.0525	0.9582	34078.51	1.1063	0.2686
	Mon+Euth	84022.80	0.9490	0.3426	34068.86	0.4123	0.6801
partitioned (I+ $\Gamma_8$ ) models d/h	Theria	78547.16	ML	ML	31809.89	ML	ML
	Marsupionta	78548.30	0.0949	0.9244	31816.34	1.1082	0.2678
	Mon+Euth	78556.63	0.8660	0.3865	31814.52	0.7329	0.4636

The results of the ML analyses for the combined protein-coding and RNA-coding data (PTN12+RNArt) are illustrated in Figure 2.8, with further details provided in Table 2.5. The Akaike Information Criterion (AIC) was used to examine the appropriateness of adding parameters

to the TN93 (for NT-coding) and CF87 (for RY-coding) ML models. Sakamoto *et al.* (1986) considered that when comparing statistical models, the one that minimizes the AIC is the most appropriate. Comparing ML models in this way has recently become popular (e.g. Adachi *et al* 2000; Cao *et al.* 2000a), largely due to concerns over the  $\chi^2$  distribution being a poor approximation for simple likelihood ratio tests (see Whelan and Goldman 1999). This concern is most acute where parameters are fixed at the boundary of parameter space (Self and Liang 1987), as is the case with the gamma distribution shape parameter, for the null hypothesis of rate homogeneity among variable sites ( $\alpha=\infty$ ).



**Figure 2.8** Kishino-Hasegawa (K-H) test p-values from ML analyses of the PTN12+RNArt dataset. These indicate the significance level for rejecting hypotheses for rooting the mammal tree: Theria ( ■ ), Marsupionta ( ■ ) and Monotremata-Eutheria ( ■ ). The ML (best) tree for each of the four NT-coding treatments (a-d) and four RY-coding treatments (e-h) is nominally allotted a p-value of 1.0. The data were analysed both as the concatenate (con) and separately as twelve partitions (part): RNAs stems, RNAloops, and first and second codon positions for the five groups of protein-coding genes (see figure 5). Inclusion of PAUP\* estimates of invariable sites and an eight category gamma distribution in the likelihood model is denoted (I +  $\Gamma_8$ ).

AIC scores for the ML analyses of PTN12+RNArt are presented in Table 2.6, as pairwise comparisons between the four NT-coding models (a-d) and between the four RY-coding models (e-h). Note that the best (lowest) AIC score between each pair is given in bold, and that models a-h correspond to models a-h in Figure 2.8 and Table 2.5. The AIC score =  $-2\ln L + 2 \times$  (no. free parameters). The  $-\ln L$  scores used in the AIC calculations are shown in Table 2.5. The AIC scores were only calculated for the Theria hypothesis. The choice of Theria, Marsupionta, or Monotremata-Eutheria is unimportant in this context, because the very small  $-\ln L$  differences between the monotreme placement hypotheses relative to the large differences between model treatments. As an example of free parameter determination, TN93+I+ $\Gamma_8$ (con) has 40 free parameters: 33 branch-lengths, three base frequencies (the fourth is not free), two ti/tv rates and two rate heterogeneity parameters (I and  $\alpha$ ). For TN93+I+ $\Gamma_8$ (part), this is multiplied by the number of partitions ( $12 \times 40 = 480$ ).

**Table 2.6** Pairwise comparisons of Akaike Information Criterion (AIC) values for the ML substitution models (a-h) used in analysis of the 18-taxon PTN12+RNArt dataset. For each pairwise model comparison (across the table), the model with the lower value (given in bold) is considered to better approximate the evolutionary process. TN93 models were used for the NT-coded data and CF87 models were used for the RY-coded data. Models for the partitioned analyses are denoted (par), while the models for the concatenated data analyses are denoted (con). The AIC value is  $-2\ln L + 2 \times$  (the number free parameters). The  $-\ln L$  scores for each model can be found in Table 2.5 and are for the Theria hypothesis.

	Model 1		versus	Model 2	
	free parameters	AIC value		free parameters	AIC value
a. TN93-con	38	175558.6	b. TN93+I+ $\Gamma_8$ -con	40	<b>162260.5</b>
a. TN93-con	38	175558.6	c. TN93-par	456	<b>168917.9</b>
a. TN93-con	38	175558.6	d. TN93+I+ $\Gamma_8$ -par	480	<b>158054.3</b>
b. TN93+I+ $\Gamma_8$ -con	40	<b>162260.5</b>	c. TN93-par	456	168917.9
b. TN93+I+ $\Gamma_8$ -con	40	162260.5	d. TN93+I+ $\Gamma_8$ -par	480	<b>158054.3</b>
c. TN93-par	456	168917.9	d. TN93+I+ $\Gamma_8$ -par	480	<b>158054.3</b>
e. CF87-con	34	72284.0	f. CF87+I+ $\Gamma_8$ -con	36	<b>66176.3</b>
e. CF87-con	34	72284.0	g. CF87-par	408	<b>68940.2</b>
e. CF87-con	34	72284.0	h. CF87+I+ $\Gamma_8$ -par	432	<b>64483.8</b>
f. CF87+I+ $\Gamma_8$ -con	36	<b>66176.3</b>	g. CF87-par	408	68940.2
f. CF87+I+ $\Gamma_8$ -con	36	66176.3	h. CF87+I+ $\Gamma_8$ -par	432	<b>64483.8</b>
g. CF87-par	408	68940.2	h. CF87+I+ $\Gamma_8$ -par	432	<b>64483.8</b>

Inspection of Table 2.6 shows that both partitioning and incorporation of I+ $\Gamma_8$  substantially improve the fit of both the TN93 and CF87 models to PTN12+RNArt. Incorporating both partitioning and I+ $\Gamma_8$  further improves the fit of the models. Perhaps the most notable results are that for both NT and RY-coding, incorporating I+ $\Gamma_8$  (which adds two parameters) improves the

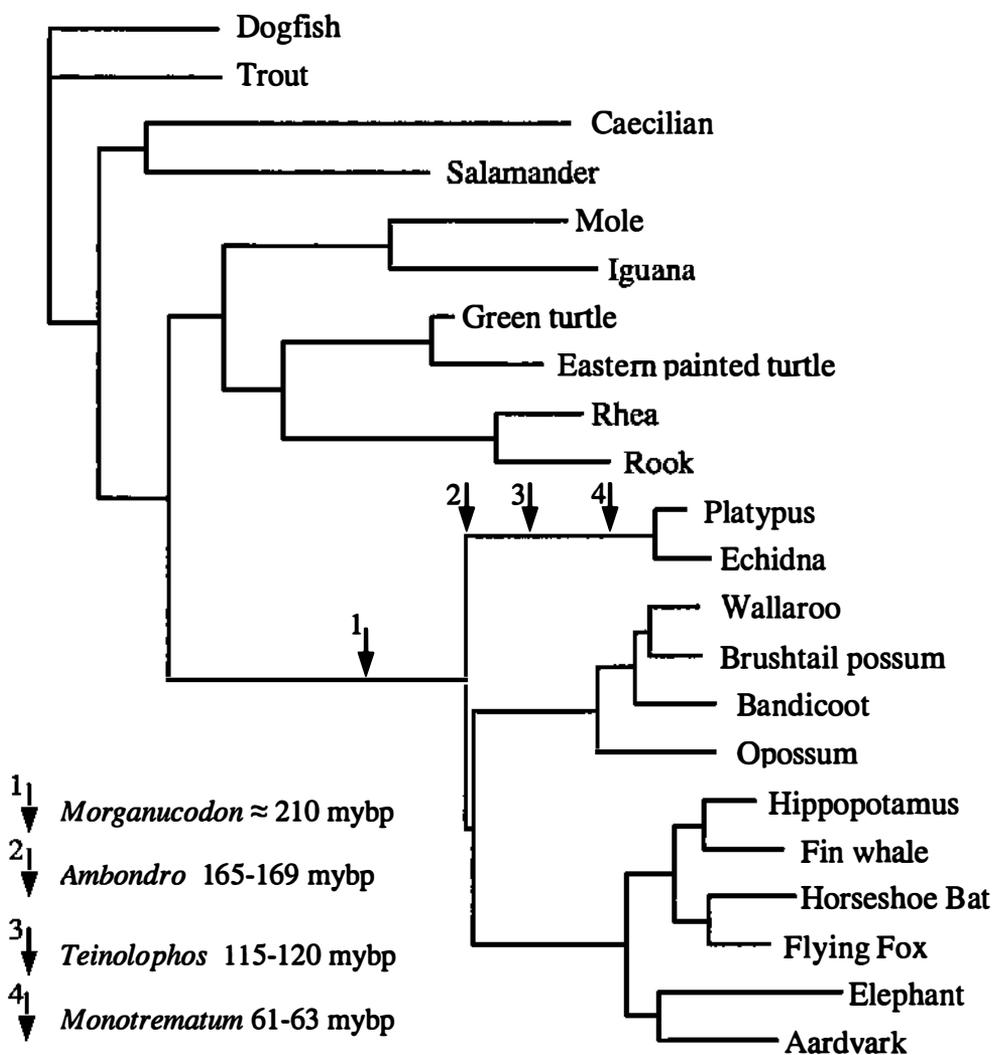
models to a greater extent than does partitioning, which is very parameter-expensive. Whether this is a true indication of the reliability of the respective models will be discussed later.

Returning to the main focus of the ML analyses of PTN12+RNArt, the effects, of coding (NT versus RY), partitioning, and incorporating  $I+\Gamma_8$ , on support for the alternative monotreme placements are illustrated in Figure 2.8. Of the three monotreme placements, only one is consistently more likely among pairwise comparisons. Theria is more likely than Monotremata-Eutheria in each of the eight treatments (a-h). Curiously, the incorporation of  $I+\Gamma_8$  has almost no impact on the relative probability of accepting Theria, Marsupionta, or Monotremata-Eutheria. This is shown in Figure 2.8 by the distribution of Kishino-Hasegawa test p-values among the three hypotheses being almost identical for the following treatments: a and b; c and d; e and f; g and h.

The two factors that do have a substantial (and phylogenetically significant) effect on the distribution of Kishino-Hasegawa test p-values among the three hypotheses are coding and partitioning (see Figure 2.8). The probability of accepting Theria (relative to Marsupionta) is greater with the RY-coding models (e-h) than with the corresponding NT-coding models (a-d). Similarly, partitioning the analyses (models c, d, g, h) increases the probability of accepting Theria (relative to Marsupionta) by comparison with the corresponding concatenated models (a, b, e, f). Considered together, RY-coding and partitioning the sequences (into RNAs stems and RNA loops, plus PTN1 and PTN2 for each of 5 protein groupings) results in a substantial turnaround in preference for the position of the mammalian root compared to standard analyses of mt data. For the concatenated NT-coding models (a and b), Marsupionta is favoured and Theria is rejected by approximately 0.8 standard deviations. In contrast, Theria is favoured by the partitioned RY-coding models (g and h), with Marsupionta being rejected by approximately 1.1 standard deviations.

### 2.3.5 *Molecular dating*

Dating estimates for divergences between and within the three mammalian sub-classes are shown in Table 2.7. These were inferred after non-parametric rate-smoothing was applied to the ME branch-length estimates (shown in Appendix C) from the PTN12+RNArt dataset. Three divergence times are of particular interest here: the monotreme/therian split, the marsupial/placental split and the platypus/echidna split. These were estimated at 166 mybp, 162 mybp, and 34 mybp respectively.



**Figure 2.9** Minimum evolution tree with ML (CF87) distances and a SplitsTree estimate of constant sites (0.700) for the RY-coded PTN12+RNArt dataset. The arrows indicate the temporal and phylogenetic relationships of four fossil taxa. The placement of these taxa (distance along branches) is relative to the divergence dates at the nodes, which were inferred from the branch-length estimates after transformation with non-parametric rate smoothing (see table 2.7).

A higher substitution rate along the echidna lineage than along the platypus lineage (see Figure 2.9) is one potential source of error in estimating the divergence dates. Hence it is of interest to gauge the effect of inferring either an ornithorhynchid rate-decrease, or a tachyglossid rate-increase. These two possibilities were corrected for by re-running the rate-smoothing on the ME tree, firstly with the platypus branch lengthened to equal that of the ME estimate for the echidna, and secondly with the echidna branch shortened to equal that of the ME estimate for the platypus. These

corrections result in inferred dates for the platypus/echidna divergence of 39 mybp and 25 mybp respectively. The corresponding estimates for the monotreme/therian and marsupial/placental divergences differ from those inferred from the un-adjusted ME branch-lengths by less than 3 million years.

As will be discussed later, the dates inferred (after rate-smoothing) from the ML (CF87+I+ $\Gamma_8$ ) branch-lengths appear to be underestimates for the younger splits within Theria. However, the divergences derived from the ML analysis for the monotreme/therian split (167 mybp), the marsupial/placental split (161 mybp) and the platypus/echidna split (32 mybp) are very similar to those inferred from the unadjusted ME branch-lengths (166 mybp, 162 mybp, and 34 mybp respectively).

**Table 2.7** Estimates of divergence times between mammals. These dates are based on non-parametric rate-smoothing of branch-lengths derived from the ME tree (Figure 2.9) and calibrated using a date of 320 mybp for the divergence between synapsids and diapsids.

Taxonomic divergence	Date estimate (mybp)
<u>Between mammalian sub-classes:</u>	
monotremes / therians	166
marsupials / placentals	162
<u>Within Monotremata:</u>	
platypus / echidna	34
<u>Within Marsupialia:</u>	
Australidelphia / Ameridelphia	82
bandicoots / diprotodontians	56
wallaroo / brushtail possum	42
<u>Within Placentalia:</u>	
Afrotheria / Boreoeutheria	88
aardvark / elephant	73
bats / Whippomorpha	62
horseshoe bat / flying fox	43
hippopotamus / fin whale	43

The standard error values associated with the ML (CF87+I+ $\Gamma_8$ ) branch-length estimates allow the effect of sampling error on the divergence dates to be examined. How far back sampling error may allow the monotreme/therian and platypus/echidna divergences to be pushed was examined by adjusting the length of the branches that are adjacent to the Mammalia and Monotremata (crown

group) nodes. As shown in Table 2.8, the divergence estimate for the monotreme/therian split will be increased by (a) shortening the mammal stem, (b) by lengthening the Theria stem and (c) by lengthening the monotreme stem. In each case the specified branch was adjusted to the length at which it is rejected at  $p=0.05$  (as determined by the PAUP\* standard error values for that branch: see Appendix C). Non-parametric rate-smoothing was then applied to the adjusted set of branch-lengths in order to infer the corrected divergence estimates. The same procedure was also applied to the three branches that are adjacent to the Monotremata crown group node, to test how far back sampling error might reasonably account for the platypus/echidna divergence being.

**Table 2.8** The effects of sampling error associated with the ML(CF87+I+ $\Gamma_8$ ) branch-length estimates for PTN12+RNArt on divergence time estimates. Divergence times are given for the original ML(CF87+I+ $\Gamma_8$ ) analysis and for three branch-length adjustments that push back the monotreme/therian divergence (a-c), and three branch-length adjustments that push back the platypus/echidna divergence (d-f). Each alteration involves either shortening or lengthening the specified branch to the length it at which it is rejected at  $p=0.05$  (as determined by the PAUP\* standard error values for that branch: see Appendix C). Non-parametric rate-smoothing was then applied to the adjusted set of branch-lengths in order to infer the corrected divergence estimates.

Branch treatment	Divergence time estimates (mybp)		
	monotremes/therians	marsupials/placentals	platypus/echidna
ML(CF87+I+ $\Gamma_8$ )	167	161	32
<i>Pushing back the monotreme/therian divergence:</i>			
a. mammal stem shorter	177	169	34
b. Theria stem longer	169	155	33
c. monotreme stem longer	169	161	29
d. all of above (a,b,c)	181	165	31
<i>Pushing back the platypus/echidna divergence:</i>			
e. platypus branch longer	167	159	34
f. echidna branch longer	169	160	36
g. monotreme stem shorter	165	157	36
h. all of the above (d,e,f)	166	157	42

Of the three branches adjacent to the Mammalia node, it is adjustment (shortening) of the mammalian stem that pushes monotreme/therian back farthest (177 mybp; Table 2.8a). Even if all three branches are simultaneously adjusted to their  $p=0.05$  error limits (Table 2.8d), the divergence between monotremes and therians is pushed back from the ML (CF87+I+ $\Gamma_8$ )-based date by only 14 million years (to 181 mybp). Perhaps more importantly, regardless of how much the

monotreme/therian dating estimate slides up and down, this basal divergence among extant mammals is closely followed by the marsupial/placental divergence.

Accommodating sampling error for any of the branches adjacent to the Monotremata node (e, f, g in Table 2.8) has relatively little effect on pushing back platypus/echidna. With all three of these branches simultaneously adjusted to their  $p=0.05$  limits, the divergence between platypuses and echidnas is pushed back only as far as 42 mybp (Table 2.8h).

The oldest adjusted dates for the monotreme/therian split (181 mybp) and the platypus/echidna split (42 mybp) depend on all three of the adjacent branches being at their  $p=0.05$  limit for overestimating the divergences, which is perhaps an excessive dependence on the influence of stochastic error. Nevertheless, these results indicate that the divergence times inferred from non-parametric rate-smoothing of the ML branch-length estimates from the RY-coded PTN12+RNArt dataset are quite robust to sampling error. This complements the finding that these estimates are also robust to the apparent rate differences between the platypus and echidna. These results provide confidence in an inferred Middle Jurassic ( $\approx 180$  mybp) cap for the divergence between monotremes and therians, and an inferred Late Eocene ( $\approx 45$  mybp) cap for the divergence between ornithorhynchids and tachyglossids.

## 2.4 Discussion

### 2.4.1 Whole mitochondrial genome sequence data is not inconsistent with the Theria hypothesis

Previous phylogenetic analyses of complete mt genomes have grouped together monotremes and marsupials (Marsupionta) with moderate to high statistical support (e.g. Janke *et al.* 1996, 1997, 2002; Penny and Hasegawa 1997; Waddell *et al.* 1999a). As discussed in the introduction, Marsupionta conflicts with a vast array of morphological data (also see Chapter 4) as well as the majority of studies involving nuclear sequences. I have explored two possible (non-phylogenetic) explanations for analyses of mt genome data supporting Marsupionta.

- Composition bias
- Non-homogenous substitution processes between “process partitions”.

A further source of error for inferring transformations across the root of the mammalian tree is incorrect placement of other taxa. Though generally held to be incorrect (see Madsen *et al.* 2001; Lin *et al.* 2002), most phylogenies derived from mt data place the fast-evolving hedgehog and/or murid rodents at the base of the placental tree. Nevertheless, it appears that these associations, which I also consider to be erroneous (see Chapter 5), do not play a substantial role in the recovery of Marsupionta in mtDNA studies. Neither the hedgehog nor any of the rodents are included in the current study, yet each of the MP, ME, and ML analyses of the concatenated protein-coding sequences (amino acid, or NT-coded DNA) favour Marsupionta (see Figure 2.3 and Table 2.4) with statistical support comparable to that of previous studies.

It is with RY-coding and partitioning of likelihood models that the results of this study depart from those of previous attempts to infer the root of the mammalian tree from whole mt genome data. The main results of these treatments are shown in Figure 2.8. As expected, ML analyses of the NT-coded, concatenated PTN12+RNArt dataset support Marsupionta (Figure 2.8 a, b). Either RY-coding this data (e, f), or allowing the substitution model to vary among the 12 partitions (c, d) erodes this support, such that Marsupionta and Theria effectively become equally likely.

By both partitioning and RY-coding the data (Figure 2.8: g, h), Theria clearly emerges as the favoured hypothesis for rooting the mammalian crown group. However, Marsupionta and Monotremata-Eutheria cannot be rejected at  $p < 0.20$  even for the best-fitting (according to AIC; see Table 2.6) of the models (h: CF87+I+ $\Gamma_8$ ). Nonetheless, the net change that is affected by RY-coding and partitioning, on relative support for the mammal rooting hypotheses, is substantial. As shown in Table 2.5, Marsupionta is approximately 0.8 standard deviations ahead of Theria for the

standard analyses, while Theria is approximately 1.1 standard deviations ahead of Marsupionta with PTN12+RNArt being RY-coded and partitioned.

Waddell *et al.* (1999a) is the only study with substantial sampling of mammals that has previously questioned the support that whole mt genome data give to Marsupionta on the basis of the mt data alone. In that study, support for Marsupionta was shown to be reduced upon removal of many of the outgroup taxa or with the exclusion of many of the highly variable sites. However, both of these treatments might generally be expected to reduce phylogenetic signal at the mammalian root. In any case, Marsupionta remained the favoured hypothesis in all of their analyses.

The most important result of the current study is that rather than unambiguously favouring Marsupionta, under the most conservative (RY-coded) and best fitting model (partitioning, with or without incorporating rate heterogeneity) the whole mt genome data favours Theria. In fact, using these models, ML favours Theria for both the protein-coding (PTN12) and RNA-coding (RNArt) sequences (not shown). The minimum that can be concluded from this is that whole mt genome sequence data are not inconsistent with the data from nuclear genes (e.g.  $\alpha$ -Lactalbumin: Messer *et al.* 1998; MP6/IGF2R: Killian *et al.* 2001). Moreover, analyses of the partitioned, RY-coded mt data do not conflict with the multitude of non-DNA-coding data that has traditionally defined Theria, such as a cochlea that is coiled at least one full turn (Griffiths 1978), the structure of the pectoral girdle (Serenio and McKenna 1995) and hindlimbs (Szalay 1993b; Gambaryan and Averianov 2001), and numerous aspects of male (Carrick and Hughes 1982) and female (Hughes and Carrick 1978) reproduction.

Consistency with other data should not be the criteria by which the alternative treatments of the mt data are assessed. In order to conclude that the whole mt genome data favours Theria, it must reasonably be expected that RY-coding and partitioning the likelihood analysis provides a better estimate of the mammalian root than does the NT-coded, concatenated data. I argue this in the following two sections. Furthermore, I propose that at least two departures from the assumptions of standard substitution models have resulted in biases that have led to Marsupionta being favoured in previous analyses. These are pyrimidine (T–C) bias, and substitution regime heterogeneity between process partitions.

#### 2.4.2 Composition heterogeneity

The current study clearly supports the expectation of Phillips *et al.* (2001) that RY-coding will be more robust to compositional biases among mtDNA sequences than will standard NT-coding. Firstly, NT-coding provides far greater potential for compositional heterogeneity to provide a misleading (apparent) phylogenetic signal. The relative compositional variability (RCV) results (Table 2.3) are testament to this. Secondly, the proportion of (true) phylogenetic signal for deep-level relationships that is overwritten is substantially higher for the NT-coded data than for the RY-coded data. This can be inferred from the stemminess values (Table 2.3).

Given a combination of both high composition variability and low phylogenetic signal retention, any method of phylogenetic inference that assumes compositional stationarity will be unreliable (Waddell 1995). Hence it is noteworthy that RY-coding provides an approximately 2.4-fold advantage over NT-coding in terms of the stemminess/RCV ratio for PTN12+RNArt (see Figure 2.5). Moreover, this advantage is almost 6.5-fold for PTN1, the codon position from which Janke *et al.* (1996) stated that most of the information supporting Marsupionta was derived.

The critical findings from examination of composition variability among taxa, for the NT-coded PTN12+RNArt data, are that non-stationarity favours Marsupionta and is dominated by pyrimidine bias. This is shown by ME trees (Figure 2.6) that are derived from pairwise distances between taxa for T–C frequency difference. The difference between support for Marsupionta and Theria in terms of overall distance (number of changes) is more than four-fold greater for the T–C distance trees than for ME trees based on absolute distances between the NT-coded PTN12+RNArt data (which also favours Marsupionta). This indicates the relative strength of the pyrimidine bias.

By comparison with the pyrimidine bias, the biases among purines (A–G), and between purines and pyrimidines (Y–R) are small. This is true in terms of both contribution to the overall composition variability, and apparent influence on monotreme placement. Indeed, Figure 2.6 shows that if negative branch-lengths are not allowed, ME analysis of the Y–R pairwise distances favours Theria, Marsupionta and Monotremata-Eutheria equally.

Placing the problem of monotreme placement aside for the moment, a number of the findings of this study are relevant to more general issues. These are the relative reliability of NT-coded DNA sequences versus their amino acid translation; the dominant contribution of frequency differences among the two pyrimidines to compositional variability among mt sequences; and the failure of the LogDet transform to recover the putatively correct tree.

Most authors (e.g. Cao *et al.* 2000a; Waddell 2001) consider phylogenetic inference to be more robust to compositional non-stationarity when using the amino acid translation (PTNaa) than when using the (NT-coded) DNA sequence. This is certainly true if the DNA sequence includes PTN3, however, the stemminess/RCV values given in Figure 2.5 suggest that this might be something of a fallacy when PTN3 is excluded.

Chi-square tests for compositional non-stationarity (including those of this study: Table 2.3) do give the impression that PTNaa is less affected by composition variability than NT-coded PTN12. However, Table 2.3 shows that the magnitude of composition variability among the mammals is higher for the amino acid treatment than for NT-coded PTN1. Moreover, NT-coded PTN2 has lower RCV than the amino acid sequence, even when Leu, Ile, and Val are lumped together (PTNfg). The nature of the chi-square test tends to buffer against compositional stationarity being rejected for amino acid data. This is because many of the amino acids contribute only a small proportion of the characters, but elevate the degrees of freedom for the test. Examination of amino acid composition data for vertebrate mt proteins provided in Penny *et al.* (1998) shows this clearly.

Preliminary examination of amino acid frequencies in the dataset used for the ML analyses is consistent with a growing body of evidence (see Foster and Hickey 1999; Singer and Hickey 2000; Schmitz *et al.* 2002) that amino acid compositional variability can often be explained by underlying mutational bias. Among the 18 taxa there is a substantial negative correlation ( $r^2 = 0.85$ ) between the frequency of (MIFVYWC) amino acids with a T (but no C) at either PTN1 or PTN2, and of (TAPRQH) amino acids with a C (but no T) at either PTN1 or PTN2. A strong correlation between PTN3, PTN12, and RNArt for the pyrimidine (T–C) bias among the taxa of this study further supports the hypothesis that the pyrimidine bias largely derives from an underlying mutation bias.

Reyes *et al.* (1998a) found that two major directional mutation biases occurred among mammalian whole mt genomes, G to A, and T to C (with respect to the L-strand). These were respectively suggested to be the result of spontaneous deaminations on the H-strand (during replication) of cytosine to uracil, and of adenine to hypoxanthine. In line with results from human mtDNA (Tanka and Ozawa 1994), the former deamination was shown to occur at a somewhat higher rate than the latter. The higher rate of H-strand cytosine deamination would explain the low representation of guanine on the L-strand, which represents the messenger RNA code for the mt proteins, except for NADH6 (G averages 4% at PTN3 among the mammals included in this study). This in turn helps to explain the relatively low purine bias (for A–G). Most of the guanine sites that are free to vary

have been removed and furthermore, only limited variability can be built upon the low remaining frequency of guanine.

In contrast to the deamination of cytosine, if H-strand deamination of adenine is the major mutation affecting the relative frequency of cytosine and thymine on the L-strand, then this process (or its proofreading correction) must differ considerably across the tree. For example, the T:C frequency ratio at PTN3 is 1.61 for the opossum, compared to 0.37 for the hippopotamus. The finding of this study that variation in the frequencies of the pyrimidines is the dominant bias among mt protein-coding and RNA-coding genes is consistent with previous studies (e.g. Springer and Douzery 1996; Reyes *et al.* 1998b; Phillips *et al.* 2001).

The importance of pyrimidine bias between mt genomes invalidates the dogma that the principle compositional bias is GC content (Sueoka 1962, 1995; see also Mooers and Holmes 2000 for discussion). This assumption has resulted in studies of directional mutation pressure focusing on the relationship between GC content and codon usage, even for mt genomes (e.g. Foster *et al.* 1997; Schmitz *et al.* 2002). Furthermore, the non-homogenous ML (NHML) of Galtier and Gouy (1998) allows only for GC/AT bias and relies on the implicit assumption that the equilibrium frequency of A=T and G=C. Inspection of Table 2.2 shows that this assumption could be very misleading for the taxa used in this analysis. In fact, for PTN12+RNArt there is little evidence that A and T, or G and C even covary among the taxa, with the respective  $r^2$ -values being 0.28 and 0.33. Instead, negative correlations exist between the frequencies of T and C ( $r^2=0.96$ ) and A and G ( $r^2=0.94$ ). These strong negative correlations are consistent with transitions dominating substitution among mt genes (e.g. Springer and Douzery 1996; Yang 1996), as well as with the assertion of Reyes *et al.* (1998a) that adenine and cytosine deaminations are the major point mutations that bias mt nucleotide composition.

Phylogenetic inference from ME using LogDet distances (see Lockhart *et al.* 1994) does not assume compositional stationarity or equity between any of the base frequencies. As such, it is interesting that this method did not recover the putatively correct tree (Theria) for either PTN12 or RNArt when these data were NT-coded (Figure 2.4). For RNArt, the percentage of bootstrap replicates in which Theria was recovered was slightly higher with LogDet than with TN93 (which assumes compositional stationarity). A similar effect was not observable with PTN12.

Lockhart *et al.* (1996) cautioned that removal of invariant sites may be required for LogDet to effectively correct for composition variability. By doing this, Haddrath and Baker (2001) were able to recover a bird phylogeny that was contradicted by a composition bias. However, other authors (e.g. Waddell 1999; Tarrío 2001) have questioned the competence of LogDet for correcting non-

stationarity, in the face of among-site rate variation that is more complex than assuming all variable sites evolve at the same rate. This is because the more slowly-evolving sites will effectively hide the extent of non-stationarity at the faster-evolving sites. This is likely to be relevant for explaining why LogDet was unable to reconstruct Theria from the NT-coded data. ML estimates of the gamma shape parameter ( $\alpha$ ) indicated that, even with invariable sites removed, there is strong rate heterogeneity among sites in both the RNArt and PTN12 datasets.

Unlike methods that attempt to correct for compositional biases, RY-coding is guaranteed to provide immunity to frequency biases among the pyrimidines and among the purines. This is because transitions are “invisible” to RY-coding and the four transversion categories are not distinguished between. In contrast, transversion methods that differentiate between the four transversion categories will not be robust to (for example) convergence in an excess of C to T transitions inflating the apparent proportion of A to T transversions.

Higher stemminess under RY-coding probably relates to the lower rate of transversion mutations (relative to transitions) and the functional importance of the amino acid substitutions that transversions tend to code for. Regardless of the cause, higher stemminess will also help buffer against non-compositional biases. However, if the principle compositional bias among DNA sequences involves the relative frequency of purines (R) and pyrimidines (Y), RY-coding may be no more (if not less) reliable than either NT-coding, or use of the amino acid translation. It is likely that loss of information (on the transitions and not distinguishing transversion categories) will be a more usual drawback of RY-coding. However, this does not seem to be a problem with whole mt genome sequences at deep levels of phylogeny. In fact, RY-coded PTN12 recovers all of the “known” (well accepted) relationships among the 18-taxon dataset with similar or greater efficiency than does NT-coding, or use of PTNaa. However, RY-coding may be less useful where less saturation of transitions occurs, such as at shallower taxonomic levels, or between nuclear sequences.

Further examination of the effect of RY-coding on phylogenetic inference, is warranted for other relationships for which mt data have so far provided anomalous or uncertain results. Examples include the basal placements of the hedgehog among placentals (Mouchaty *et al.* 2000; Cao 2000a), the tarsier among primates (Schmitz *et al.* 2002), Passeriformes among birds (Härlid and Arnason 1999; Mindell *et al.* 1999), and peculiar associations among fish and early chordates (see Naylor and Brown 1998; Rasmussen and Arnason 1999).

Another issue requiring further investigation is the nature of the amino acid compositional bias and its relationship with the nucleotide frequency bias. The present study indicates that the magnitude

of compositional bias relative to phylogenetic signal retention (as inferred from stemminess/RCV) may be no less among the amino acid data than among NT-coded PTN12. Furthermore, the strong negative correlation between the frequency of amino acids coded for by T-rich PTN12 codons and amino acids coded for by C-rich PTN12 codons is evidence for the non-independence of the aa and DNA biases.

Some studies (e.g. Waddell 2001) that espouse the use of the amino acid translation over the DNA sequence suggest that the former may be more informative due to the greater number of character states. This may be true, but could easily be overstated because at most sites only a small subset of the potential aa transformations are possible (viable).

Whether or not PTNaa should be expected to provide for more reliable phylogeny reconstruction than NT-coded PTN12 is unimportant in the context of this study. Importantly though, the combination of lower composition variability and higher phylogenetic signal retention might be expected to result in phylogeny reconstruction with RY-coding being far less susceptible to the misleading effects of compositional non-stationarity. This is confirmed by the ME distance analyses that were based on pairwise base frequency differences between taxa. These showed that nucleotide composition bias (pyrimidine bias) strongly favours a monotreme-marsupial grouping, which should in itself be taken as a warning to be cautious about the NT-coded DNA and PTNaa studies that favour Marsupionta.

#### *2.4.3 Process partitions and comparison of maximum-likelihood models*

Depending on coding (NT or RY) and whether or not rate heterogeneity among sites was incorporated in the models, partitioning (into RNAstems, RNALoops, and 5 protein partitions for PTN1 and PTN2) reduced  $-\ln L$  scores by between 3.1% and 5.7% for ML analyses of PTN12+RNArt. This “partition advantage” is slightly less than that which Amrine and Springer (1999) found when all nucleotide substitution parameters were allowed to vary independently between partitions ( $\approx 5.8$  to 7.2%). This indicates less heterogeneity in substitution processes between the partitions of the current study. This is likely to be explained by the inclusion of third codon positions and both mt and nuclear sequences in the study of Amrine and Springer (1999).

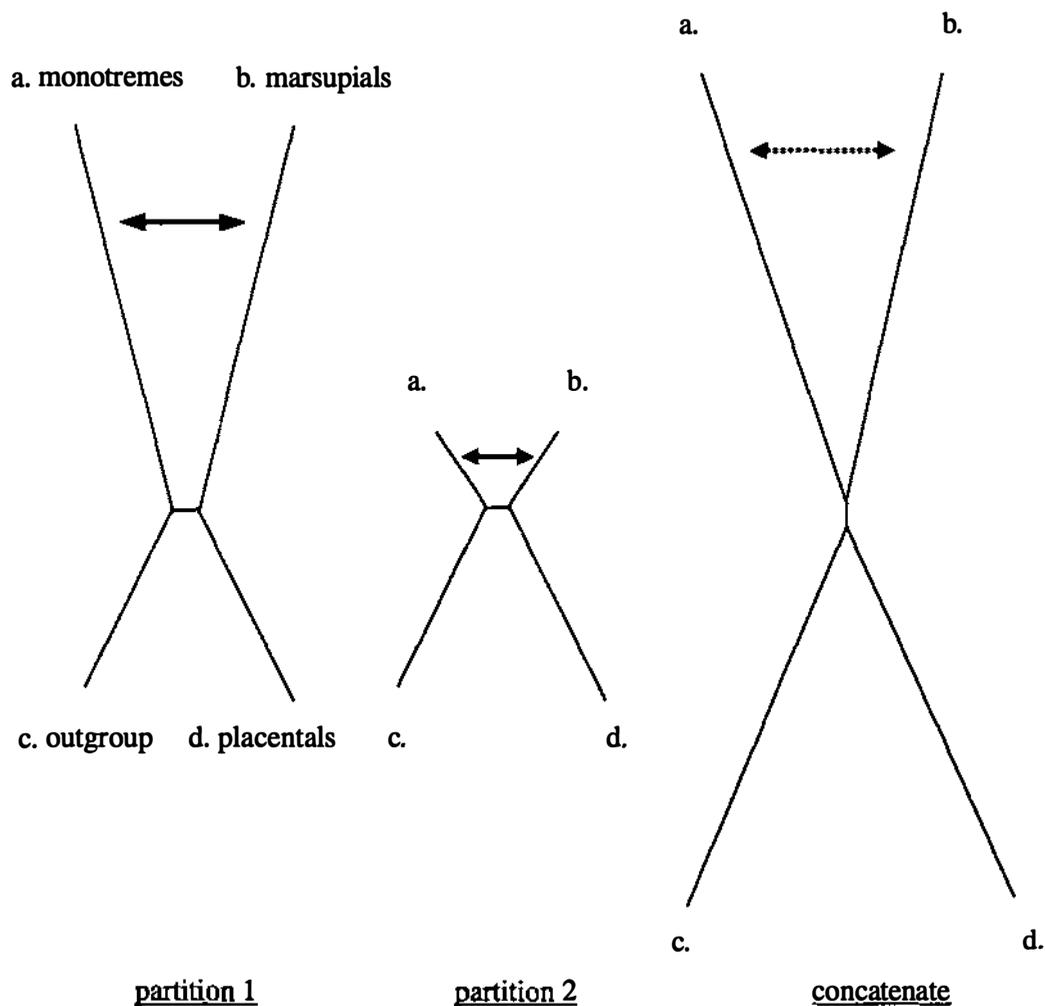
Akaike Information Criterion (AIC) scores indicate that partitioning the likelihood analyses significantly improved the fit between the data and both the TN93 (NT-coding), and CF87 (RY-coding) ML models. One result in particular cautions against the assumption that a better AIC score necessarily means that phylogenetic inference from that model will be more reliable. As shown in Table 2.6, incorporation of rate variation across sites ( $I+\Gamma_8$ ) provides a far better fitting

model than does partitioning, with both TN93 and CF87. However, as shown in Figure 2.8, it is partitioning (c and g), and not the use of  $I+\Gamma_8$  (b and f) that allows recovery of the tree favoured by the models that provide best fit overall (partitioning and  $I+\Gamma_8$ ; d and h).

Inspection of Figure 2.1 reveals considerable variability in the proportion of constant sites and base frequencies between the different proteins and between the RNA stems and loops. Differences between first and second codon positions are even more marked, while relative branch-lengths also differ substantially between partitions (not shown due to the amount of data). Substantial differences in base frequencies, relative branch-length estimation, relative rates across substitution types and rate heterogeneity among sites all justify the partitioning, whether or not the AIC scores are meaningful. Hence, the partitioning analysis adds considerable weight to the Theria hypothesis, which is favoured in all of the partitioned analyses (Figure 2.8: c, d, g, h).

Because partitioning increases the signal for Theria, relative to Marsupionta (with both NT and RY-coding), non-homogeneity of substitution processes between the partitions apparently provides a signal that favours Marsupionta when the data are analysed as concatenated sequences. Whether or not different trees were favoured, partitioning has substantially influenced the distribution of apparent phylogenetic signal between competing hypotheses in a number of previous likelihood and distance analyses (e.g. Cao *et al.* 2000b; Krajewski *et al.* 2000; Caterino *et al.* 2001). However, the mechanisms of the biases involved have not been elaborated on, except to say that analyses of concatenated sequences do not satisfactorily account for heterogeneity in substitution processes and that the partitioning advantage differs according to both the partitioning scheme (DeBry 1999) and the parameters being partitioned (Yang 1996).

As the relationship between the substitution process and the tree is complex even without partitioning, elucidating the mechanism(s) of phylogenetic signal bias uncovered by partitioning is of course difficult. With both the partitioned NT and RY-coded data, most of the relative increase in signal for Theria is attributable to the partitioning of branch-length estimation. Preliminary examination suggests that branch-lengths covary among partitions in a way that is not apparent with the concatenated data. Although this is a complex problem that requires further study, Figure 2.10 provides a simplified model that shows how branch-length covariation between partitions (1 and 2) can mislead ML analysis of the concatenation.



**Figure 2.10** Tree estimates from two hypothetical data partitions for which monotreme and marsupial branch-lengths covary and are complementary, such that they both average out to be more similar to the placental branch-length when the data are concatenated. Methods that correctly estimate branch lengths will be able to account for the branch-length covariation bias (attraction) that is “observable” (full arrows) for the separate partitions, but this will be “hidden” (dashed arrow) by their concatenation, potentially resulting in the incorrect tree being recovered.

Perhaps the most important advantage of ML over standard parsimony methods is that ML incorporates branch-length information (see Swofford *et al.* 1996) and so is less likely to be misled by rate variation across the tree (e.g. Huelsenbeck 1998). Without accounting for rate variation between branches, fast-evolving taxa may be falsely attracted together due to an excess of parallel changes appearing to be synapomorphies (Felsenstein 1978). For partition 1 (Figure 2.10) this will tend to occur for monotremes and marsupials under standard parsimony, or any ML and distance models that underestimate branch lengths (Waddell 1995; Sullivan and Swofford 1997). For partition 2, marsupials and monotremes will also tend to be attracted relative to eutherians and

the outgroup, due to the complementary effect, where the slower-evolving taxa also tend to be attracted. This is due to shared ancestral (plesiomorphic) states in these taxa acting as synapomorphies, having been independently lost by the other (faster-evolving) taxa.

ML models that estimate branch-lengths sufficiently well will be robust to the “observable” branch-length covariation biases and will thus recover the correct tree for the partition 1 data, and for the partition 2 data. However, if the two data partitions are concatenated, the branch lengths will average out. Monotremes and marsupials will still be attracted by the complementary branch-length covariation biases, but these will effectively be “hidden” from current phylogenetic methods, including those that correctly estimate branch-lengths.

For hidden branch-length covariation to mislead phylogenetic inference, the concatenated tree need not have apparently equal rates among taxa (as does the concatenation tree of Figure 2.10). Any biases in the substitution process that are non-independent among characters within partitions, but which are even partially averaged-out among taxa by combining partitions, will reduce the consistency of phylogenetic methods. However, the situation is not better with methods that do not explicitly attempt to model substitution, as these are expected to be inconsistent even when the bias is observable (Steel and Penny 2000; Sullivan and Swofford 2001).

One drawback of partitioning is that fewer characters are used in estimating substitution parameters, so the statistical power of the analysis may be reduced. The obvious counter to this is that assuming an average substitution regime is clearly undesirable if the substitution process is sufficiently heterogeneous between process partitions. This is especially so if complementary biases are present. Caterino *et al.* (1999) emphasized the necessity of developing criteria to help balance the need for partitioning to match data complexity, with the need for statistical practicality. Previous gene-based partitioning of mt proteins have treated each gene separately (e.g. DeBry 1999; Cao *et al.* 2000a, 2000b). It seems likely that separating genes with similar substitution properties will reduce statistical power without further exposing phylogenetic bias. Accordingly, the partitioning scheme of the current study pooled proteins with similar substitution properties (see Figure 2.1).

As noted by DeBry (1999), the development of comprehensive structural models for the mt protein-coding genes is crucial for partitioning the data they provide. The five protein gene-based partitions used in this study probably only act as surrogates for structure-based differences in substitution processes. It would be interesting to investigate whether partitioning schemes that more efficiently incorporate substitution heterogeneity will further increase the statistical significance with which Marsupionta is rejected by whole mt genome data.

#### 2.4.4 Implications of the timing of divergences at the nodes that define Mammalia and Monotremata

The basic aim of estimating monotreme divergence times was to place a cap on the earliest date for which monotremes last shared a common ancestor with Theria (Metatheria and Eutheria), and for when ornithorhynchids and tachyglossids last shared a common ancestor. For this purpose a synapsid/diapsid calibration date (320 mybp) that is 10 million years older than is usually accepted, was used. Non-parametric rate-smoothing of the ME and ML trees that were derived from RY-coded PTN12+RNArt resulted in very similar divergence dates (see Tables 2.7 and 2.8) for Monotremata/Theria, Marsupialia/Placentalia, and Ornithorhynchidae/Tachyglossidae (platypus/echidna).

The lengths of branches adjacent to the Mammalia and Monotremata nodes were adjusted in order to see how much the inferred divergences might be underestimates due to differences between the platypus and echidna evolutionary rates, and due to stochastic error (see Table 2.8). Even in the extreme case of each branch adjacent to the Mammalia node (Table 2.8d) and the Monotremata node (Table 2.8h) being adjusted to its  $p=0.05$  limit (according to sampling error on the ML branch lengths), the divergence times at these nodes were estimated at 181 mybp and 42 mybp respectively. As examination of tables 2.7 and 2.8 show, The RY-coded PTN12+RNArt data are more consistent with a monotreme/therian split at about 170 mybp, and a platypus/echidna split at about 35 mybp. In any case, these Middle Jurassic and early-middle Tertiary dates for the monotreme/therian and platypus/echidna splits, respectively, are consistent with other recent molecular estimates (e.g. Retief *et al.* 1993; Gemmell and Westerman 1994; Messer *et al.* 1998). Janke *et al.* (2002) is an exception in that their estimate for monotremes diverging from marsupials is 110 mybp. This is clearly not correct. The earliest known monotreme (*Teinolophos*: Rich *et al.* 1999; Rich *et al.* 2001a) extends the fossil record for the group back to  $\approx 115$ -120 mybp. The most obvious difference between the divergence time estimation of Janke *et al.* (2002) and the current study is the rooting of Mammalia (Marsupionta versus Theria). The effect of topology is one factor that is often overlooked in molecular dating studies.

Determining upper limits for the monotreme/therian and platypus/echidna divergences allowed testing of three key hypotheses for monotreme placement with respect to fossil taxa. As the arrows on the phylogeny shown in Figure 2.9 indicate, these dating estimates: 1. reject monotremes diverging from therians before (or with) the Triassic/Jurassic boundary ( $\approx 210$  mybp) morganucodontids or putative stem-trechnothere *Kuehneotherium* (Kermack *et al.* 1968); 2. are consistent with monotremes arising from within the Australosphenida (Lou *et al.* 2001a), for which

the Middle Jurassic ( $\approx 167$  mybp) *Ambondro* is the earliest known representative; and 3. reject the first “ornithorhynchid” (*Monotrematum*, from the early Palaeocene ( $\approx 62$  mybp) of Patagonia: Pascual 1992a, 1992b) being a member of the monotreme crown group. The importance of these results is further discussed in Chapter 4.

The inferred date for the marsupial/placental divergence ( $\approx 160$  mybp) is consistent with recent molecular dating studies (e.g. Kumar and Hedges 1998; Penny *et al.* 1999; Cao *et al.* 2000a). However, this date appears anomalous by comparison with fossil evidence. The earliest record for a crown group therian, the eutherian, *Eomaia* (Lou *et al.* 2002), dates to  $\approx 125$  mybp. One possible explanation is that the dates inferred in the current study are intended to be overestimates. However, even reducing the reference date (S/D 320) to 310 mybp and allowing for sampling error in the lengths of branches adjacent to the Theria node (not shown; but performed as for the Mammalia and Monotremata nodes), it would be difficult to reconcile the mt data with the marsupial/placental divergence being more recent than the Jurassic/Cretaceous boundary ( $\approx 145$  mybp).

Benton (1999) is adamant that the fossil record provides a reliable basis for estimating mammal divergence times. However, there is a 15 million year gap between *Eomaia* and the next earliest known eutherian (*Prokennalestes*: see Kielan-Jaworowska and Dashzeveg 1989). Furthermore, unquestioned metatherians are not known from the fossil record until  $\approx 98$  mybp (*Kokopellia*: Cifelli and Muizon 1997), leaving a ghost lineage of at least 27 million years and indicating how poorly sampled the fossil record is for the earliest part of eutherian and metatherian history.

Inspection of Table 2.7 reveals divergence time estimates within Theria that are younger than inferred by many previous molecular studies (e.g. Springer 1997; Waddell *et al.* 1999b; Murphy *et al.* 2001b). The results in this table are, however, consistent with the molecular dates of Waddell *et al.* (2001) that were calculated using a tarsier/human split at 55 mybp and a rabbit/pika split at 42 mybp. The dates inferred in the current study are also more consistent with the mammalian fossil record, which is substantially more complete (see Benton 1993; McKenna and Bell 1997) for the Late Cretaceous and Tertiary, than for when the mammalian sub-classes diverged in the Jurassic/Early Cretaceous.

As the likelihood ratio test was unable to reject rate constancy among the monotremes and marsupials, whole mt data might be useful for estimating divergence times among marsupials. This is of particular interest given the poor early Tertiary (mammal) fossil record from Australia (Archer *et al.* 1999). The estimate obtained in this study for the bandicoot/diprotodontian divergence was 56 mybp. Westerman *et al.* (1999) also estimated the bandicoot/diprotodontid split at  $\approx 56$  mybp, on

the basis of 12S rRNA sequences. Moreover, Godthelp *et al.* (1992) considered the Tingamarra fauna of Eastern Australia (which is dated at  $\approx 54$  mybp) to include stem bandicoots and/or stem dasyuromorphians, and so post-date the basal australidelphian split. Hence, 54-56 mybp would seem to provide a reasonable reference date for the basal split between Australian marsupials.

As archaeocetid whales are known from as early as  $\approx 53$  mybp (Bajpai and Gingerich 1998), the hippopotamus/whale dating estimate of 43 mybp (Table 2.7) is the one split based on ME branch-lengths that appears to be problematic. However, as noted earlier, these two lineages have very different substitution rates. Because substitution rates also differ considerably among other placentals, it is difficult to infer the nature of the rate heterogeneity along the branches that are adjacent to the hippopotamus/whale node. Clearly, molecular dating studies that employ mtDNA should be cautious about estimates extrapolated from this node.

Although the ME and ML (CF87+I+ $\Gamma_8$ ) branch-length estimates resulted in very similar inferred divergence dates for the monotreme/therian, marsupial/placental, and platypus/echidna splits, the concern (signaled in the Methods section) regarding the reliability of the dates derived from the ML branch-lengths, is warranted with respect to the dating of divergences within Theria. As noted above, these appear realistic for the ME estimation (except for the hippopotamus/whale split). However, in addition to the hippopotamus/whale split, using the ML branch-lengths results in three additional divergences being clearly underestimated. These are: wallaroo/brushtail possum at 26 mybp, bats/fereuungulates at 54 mybp, and horseshoe bat/flying fox at 34 mybp. At least one member of the placental pairs is known from before each of these dates (McKenna and Bell 1997). In the case of the two marsupials, there is a large gap in the Australian fossil record before 26 mybp, though representatives of their respective groups were already diverse by this time (Woodburne *et al.* 1993; Archer *et al.* 1999).

The concern with the ML model is that, due to covarion shifts (see Penny *et al.* 2002), rate heterogeneity across sites will depend on how closely related taxa are. This does in fact appear to be the case, as revealed by estimating rate heterogeneity by ML(CF87+I+ $\Gamma_8$ ) analyses of PTN12+RNArt (within PAUP\*). The gamma shape parameter ( $\alpha$ ) for the tree with all of the taxa (as shown in Figure 2.9) was estimated at 0.4156, while this value decreases to 0.2515 without the therians, but is 0.8564 for the placentals alone. This suggests that the  $\alpha$  value for the overall tree may be a best-fit compromise between the small and large divergences (actually the short and long branches). If this is the case, then rate heterogeneity across the tree may be overcorrected for by ML(CF87+I+ $\Gamma_8$ ). Overcorrection for rate heterogeneity among sites will proportionately underestimate smaller branch-lengths (Waddell 1995).

A further problem for ML (and MP) analysis is that proportionately underestimating (or overestimating) smaller branch-lengths may be especially problematic with unbalanced trees. Unbalanced trees have more taxa, and so, more (short) branches, on one side of internal nodes (possibly including the root) than on the other side. Such problems related to tree balance should not be as marked with ME branch-length estimation, as these estimates are derived from distance (between taxa) data, such that if taxa on different sides of a node are the same distance from the outgroup, branch-lengths tend to be constrained accordingly (Rzhetsky and Nei 1992). In contrast, branch-lengths are effectively determined by preferential (or probable) placement of characters on specific branches in MP and ML analyses (see Swofford 1996).

The ME model (with ML-CF87 distances) used to estimate divergence times incorporated a SplitsTree estimate for the proportion of invariable sites, but did not incorporate a gamma distribution. Invariant sites estimation may also be subject to the covarion problem, but without incorporating a gamma distribution, is likely to be a conservative correction for rate heterogeneity across sites, and so will tend to overestimate younger dates when interpolating from an older reference. Furthermore, the reference date errs on the side of overestimation for younger dates and the dating estimates for the monotreme/therian and platypus/echidna splits have been shown to be quite robust to underestimation owing to rate differences between the two monotremes, and to sampling error. Thus it can confidently be inferred from RY-coded PTN12+RNArt that, platypuses and echidnas diverged no earlier than the Eocene, and that unless the therian crown group is much older than the earliest Cretaceous, monotremes split from these no earlier than the Middle Jurassic.

## 2.5 Conclusions

Whole mt sequence data has been the 'jewel in the crown' of the Marsupionta hypothesis. The present finding that more conservative treatment of this data, and the use of a better fitting ML model, result in the traditional Theria hypothesis being favoured further undermines the credibility of the Marsupionta hypothesis. The highly conserved nature of two bases (ancestrally AA) between the acceptor and D arms of the tRNA-Serine (UCN) in non-therian amniotes and the absence of these bases from all known marsupial and placental mt genomes also provides support for Theria.

Springer *et al.* (2001) were critical of the use of mt data for inferring deep-level relationships among mammals. However, mt sequences have a number of valuable properties, such as confidence in orthology, lack of recombination, and ease of collection from a broad range of organisms. Hence it is important to ascertain whether ML model partitioning for RY-coded data

will more generally increase the reliability with which deep-level phylogenies can be reconstructed from whole mitochondrial genome data.

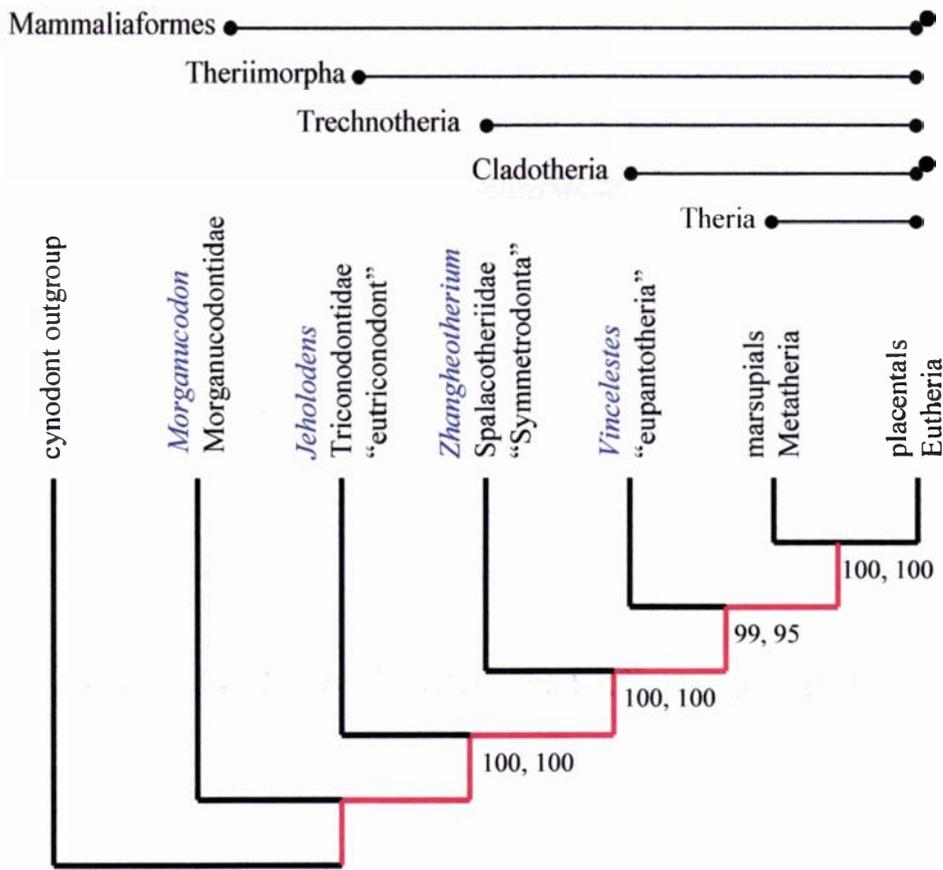
Despite the conclusion that analysis of mt data favours Theria, the current study also provides some vindication for Gregory (1947), Kühne (1973), Janke (1996) and others who argued against the orthodoxy of monotremes being far removed from the therian crown group. Unless a very large covarion bias (perhaps associated with the pyrimidine bias) remains undetected and is not accounted for by the partitioning scheme, it seems unlikely that monotremes diverged from the therian crown group more than 20 million years before marsupials and placentals split. The fact that the partitioned RY-coding analyses provide no greater support for Marsupionta than for a Monotremata-Eutheria grouping tends to argue against the presence of any such substantial undetected bias.

A near-trichotomy at the mammalian root is consistent with recent questioning of some of the “text book” therian synapomorphies, such as oviparity (Zeller 1999), tribosphenic (shearing and grinding) molars (see Lou *et al.* 2001a) and a highly mobile shoulder girdle (Ji *et al.* 1999). It would not be surprising if many of the long-accepted synapomorphies of therians are found to be homoplasious.



## **Chapter 3**

### **Instability of monotreme affinities with Mesozoic mammals**



**Figure 3.1** Maximum parsimony tree (for the primary dataset) for the generalized insectivores (and considered to be the background phylogeny null hypothesis). Bootstrap support values correspond to the clade above them, with the first being for analysis with all characters included (126 steps; HI=0.1270), and the second being for analysis with dental/mandibular characters excluded (77 steps; HI=0.1039). The names of genera used are in blue, while their higher order affinities (as used in the text) are in black, including informal higher order groupings that may not be monophyletic (“ ”). The mammalian backbone lineage is denoted red and the taxonomic ranges of the nested clades are indicated by the spans above the phylogeny.

### 3.1 Introduction

The lineage defining the backbone of mammalian evolution (Figure 3.1, denoted in red) consisted of small terrestrial (or scansorial) forms whose diets consisted primarily of invertebrates, and perhaps small vertebrates (e.g. Lillegraven *et al.* 1979; Kermack and Kermack 1984). The relationships among the major groups of Mesozoic small terrestrial insectivores (which I simply refer to as the generalized insectivores) were originally elucidated on the basis of dental and mandibular evidence (see Simpson 1928; Crompton 1971; McKenna 1975; Prothero 1981). Figure 3.1 indicates these relationships as offshoots from the backbone lineage, which are successively more closely related to modern therians (marsupials and placentals). These are the Late Triassic/Early Jurassic triconodonts (e.g. *Morganucodon*), through to the more advanced “eutricodonts” (e.g. *Jeholodens*), to the spalacotheriid “symmetrodon” (e.g. *Zhangheotherium*), and then the “eupantotheres” (e.g. *Vincelestes*). I will refer to the relationships depicted among these major groups of Mesozoic generalized insectivores simply as the background phylogeny.

Most (possibly all) recent studies are in agreement with the background phylogeny. However, there has been less success in placing among these, highly derived (ecologically and dentally) offshoots such as the multituberculates, which were largely herbivorous (Krause 1982, Kielan-Jaworowska 1996), and monotremes, represented among extant mammals by the semiaquatic platypus and the fossorial echidnas.

George Gaylord Simpson once claimed (quoted from Kermack and Kermack 1984) that the phylogeny of Mesozoic mammals was more a phylogeny of molar cusp patterns. Recent discoveries in China, from Early Cretaceous sites, of near-complete skeletons, including the spalacotheriid, *Zhangheotherium* (Hu *et al.* 1997) and the eutricodont, *Jeholodens* (Ji *et al.* 1999), have allowed the “dental/mandibular” background phylogeny to be tested with characters from a wide range of anatomical regions. It is encouraging that subsequent cladistic analyses (Hu *et al.* 1997, 1998; Ji *et al.* 1999; Lou *et al.* 2001a, 2001b; Wang *et al.* 2001) support the background phylogeny and appear to provide at least “local stability” for monotremes and multituberculates arising from the mammalian backbone lineage within the vicinity of the eutricodonts. However, these analyses have not examined the new character information in the absence of the dental/mandibular characters. Hence, it is uncertain how much the relationships found depend on these dental/mandibular characters, which remain controversial for many of the generalized insectivores (see Bonaparte 1990; Kielan-Jaworowska and Dashzeveg 1998; Sigogneau-Russell 1999), and are perhaps even more so for monotremes (see Archer *et al.* 1992, 1993; Musser 1998).

Regardless of affinities with the generalized insectivores, a close relationship between monotremes and multituberculates has intermittently been suggested, at least since Hopson (1970) and Kermack *et al.* (1981) argued that the structure of the braincase sidewall unites these groups (along with early triconodonts) as the mammalian subclass Prototheria. Presley and Steel (1976) and Presley (1981) showed that the braincase sidewalls of therians and the platypus develop from a homologous ossification, with the differences being as superficial as which other ossifications this fuses with later. Additionally, Griffiths (1978) showed that the development of the echidna braincase sidewall did not fit easily with the simple pattern of evolution argued for the Prototheria hypothesis. Nevertheless, in addition to further arguments regarding braincase structure (Hopson *et al.* 1993), at least mild support has since been found for grouping monotremes with multituberculates on the basis of similarities in ear region anatomy (Wible and Hopson 1993; Meng and Wyss 1995).

A new hypothesis (Lou *et al.* 2001a) proposes that monotremes are highly derived members of a Gondwanan radiation of mammals, that also includes *Ausktribosphenos* (Rich *et al.* 1997) from the Early Cretaceous of Australia, and the Middle Jurassic aged *Ambondro* (Flynn *et al.* 1999) from Madagascar. Lou *et al.* (2001a) named this clade Australosphenida (defining it by a number of dental synapomorphies) and concurred with the original interpretations of *Ausktribosphenos* and *Ambondro*, as far as agreeing that they possessed tribosphenic molars. In tribosphenic teeth, the protocone of the upper molars occlude with the talonid of the lower molars, such that a grinding (*tribein*) function combines with the shearing (*sphen*) and puncturing function of the more anterior reverse triangle cusps (see Crompton 1971; Butler 1990).

Lou *et al.* (2001a) found the australosphenidans to be widely excluded from the radiation of Laurasian tribosphenic mammals (Boreosphenida), which includes therians and their close relatives such as *Kielantherium*. The mandible of the *Ausktribosphenos* holotype has a groove that might have retained postdentary bones (which have been transformed into the middle ear bones of modern mammals). However, this interpretation has been questioned in consideration of the position and/or size of this groove in the second *Ausktribosphenos* specimen (Rich *et al.* 1998) and the related *Bishops* (Rich *et al.* 2001b). It is basicranial and post-cranial data, which are only known from monotremes (among australosphenidans), that supply the bulk of evidence against a close relationship between Australosphenida and Boreosphenida. As such, determining monotreme affinities takes on wider significance for understanding mammalian interrelationships.

The extreme niche-related modification of monotremes is perhaps the most important (at least the most regularly cited, e.g. Jenkins 1970a; Lewis 1983; Kirsch and Mayer 1998) factor contributing to the enigma that is the placement of monotremes among the background phylogeny. The problem

is largely whether monotreme traits are indicative of phylogenetic history, or are a reflection of fossorial/swimming/dietary adaptations. A second contributing factor is the possibility of monotreme traits being the result of parallelism that relates to trends initiated before monotremes split from the mammalian backbone lineage. Of particular note are trends toward: a more parasagittal gait (Jenkins 1973; Blob 2001); ear specialization for improved acoustic insulation and transmittance of airborne sound (Allin 1975; Lou *et al.* 1995; Fox and Meng 1997); and for loss and reduction of postdentary and parodontary elements (Krebs 1971; Allin 1986; Wang *et al.* 2001).

Of particular interest in this study is the examination of anatomical-region effects on phylogenetic reconstruction among the Mesozoic mammals, especially for the affinities of monotremes. Despite the local stability of monotremes in “whole skeletal” analyses, their affinities appear to differ considerably among studies that focus on specific anatomical regions. Monotremes tend to be placed among cladotheres by dental characters (Archer *et al.* 1985; Kielan-Jaworowska *et al.* 1987; Bonaparte 1990; Archer *et al.* 1992, 1993). Alternatively, basicranial studies have typically placed monotremes at a “level” similar to eutriconodonts and multituberculates (Wible and Hopson 1993; Wible *et al.* 1995; Meng and Wyss 1995; Rougier *et al.* 1996;). It is the upper appendicular skeleton that gives monotremes a particularly primitive appearance in comparison to eutriconodonts, multituberculates, and trechnotheres (Rowe 1988; Sereno and McKenna 1995, Ji *et al.* 1999). Note that the words, primitive and advanced, are used here to describe relative placements or character state conditions with respect to the mammalian backbone lineage, rather than suggesting that some character states or taxa are intrinsically more advanced than others. Numerous monotreme shoulder girdle and forelimb traits are shared with cynodonts, though Jenkins (1970a) and Jenkins and Parrington (1976) recognize that some of these may be secondarily derived as fossorial adaptations among monotremes.

In order to examine the general question of whether there is an anatomical-region effect on the background phylogeny, or on the placement of monotremes and multituberculates among these, the data was divided into upper appendicular (U), lower appendicular (L), basicranial (B), mandibular (M), dental (D), and vertebral (V) partitions. The partition homogeneity test (PHT), or incongruence length difference test (first defined in Farris *et al.* 1994) was used to test for incongruence between these partitions. This test has proven useful for detecting incongruence between morphological data partitions, such as between larval characters and female reproductive characters of parasitic wasps (Quickie and Belshaw 1999) and among molecular partitions, such as between 12S ribosomal RNA and cytochrome *b*, for a rodent phylogeny (Cunningham 1997). The PHT has also been used to advise on the feasibility of lumping together molecular and

morphological data, with recent examples being for iguana (Weins and Hollingsworth 2000) and diving duck (McCracken *et al.* 1999) phylogenies.

This study focuses on the effects of taxon sampling and data partitioning (into anatomical regions) on the stability of the background phylogeny of generalized insectivores, and for the placement among these of the ecologically-derived multituberculates and monotremes (particularly). Both the highly modified condition of monotremes and parallelism relating to mammalian phylogenetic trends are contemplated as explanations for the anatomical-region dependence of monotreme affinities.

## 3.2 Methods

### 3.2.1 Data

The primary dataset for analyses is based on the dataset of Ji *et al.* (1999), which has been updated upon consideration of Lou *et al.* (2001a, 2001b). As a further modification, characters were removed where states are: equivalent to, or dependent on, the state of other characters; uncertain for three or more of the taxa; or subject to considerable (especially niche-related) variability in modern mammals (so not expected to be reliable at deep levels of phylogeny). As set out in Appendix D, this leaves 91 characters, which are assigned to the six subsets: vertebral (8), mandibular (9), upper appendicular (22), lower appendicular (20), dental (21) and basicranial (11).

The primary data set for investigating Mesozoic mammal interrelationships includes the generalized insectivores of the background phylogeny (see Figure 3.1). These are: The Late Triassic/Early Jurassic “triconodont” *Morganucodon*; the Early Cretaceous triconodontid eutriconodont, *Jeholodens*; the Early Cretaceous spalacotheriid “symmetrodont” *Zhangheotherium*; the Early Cretaceous eupantothere *Vincelestes*; metatherians (including marsupials); eutherians (including placentals); and an outgroup comprising advanced cynodont characters. For each of these taxa there was 95% or greater character completeness. Expanded analyses included two less complete taxa, the Early Cretaceous gobiconodontid eutriconodont, *Gobiconodon* (76% complete), and the Late Jurassic dryolestoid eupantothere, *Henkelotherium* (66% complete).

The lack of basicranial character completeness for *Jeholodens* (7 of 11) was rectified by replacing the 4 unknown basicranial characters of *Jeholodens* with those states allotted by Rougier *et al.* (1996) to (and conserved across) the triconodontid eutriconodonts: *Trioracodon*, *Triconodon* and *Priacodon*. Ji *et al.* (1999: supp. info.), Lou *et al.* (2001a), and Wang *et al.* (2001) all find

*Jeholodens* to be sister to these triconodontids, to the exclusion of other (gobiconodontid and amphilestid) eutriconodonts. Allotting these triconodontid characters to *Jeholodens* means this OTU (operational taxonomic unit) is in fact a composite that potentially offers a better estimate of triconodontid affinities than does *Jeholodens* alone. Nevertheless, referring to this OTU as *Jeholodens* is perhaps the most informative option, as the vast majority of characters describe this taxon.

Ji *et al.* (1999) included only the extant platypus (*Ornithorhynchus anatinus*: Ornithorhynchidae) to represent monotremes. I have also included a member of the other extant monotreme family (Tachyglossidae), the short-beaked echidna (*Tachyglossus aculeatus*). As tachyglossids lack teeth and only vestigial juvenile teeth are retained by the platypus, monotreme dental characters are represented by those discernible from the Early Cretaceous monotreme, *Steropodon galmani* (Archer *et al.* 1985), or if not discernable in that, then the Oligocene/Miocene platypus genus, *Obdurodon* (Archer *et al.* 1992, 1993; Musser and Archer 1998). This increases the number of dental characters applicable to monotremes from 5 to 16 (of 21). These characters are attributed to an OTU I refer to as Ornithorhynchidae. Molecular dating studies (Retief *et al.* 1993; Gemmell and Westerman 1994; Messer *et al.* 1998; Janke *et al.* 2002) clearly show that the Early Cretaceous *Steropodon* diverged from *Ornithorhynchus* before ornithorhynchids and tachyglossids split. However, allowing ornithorhynchid dental characters to be represented by *Steropodon* simply defines the best current estimate of the ancestral monotreme dental condition within the context of the current study, as all the corresponding tachyglossid characters are defined as unknown.

The dataset described above is the primary dataset for this study and unless otherwise indicated, is the source of all the results. Lou *et al.* (2001a supp. info.) provide a dataset with 125 characters covering a diversity of anatomical regions for 26 mammals (most of which are low in completeness, except for the 56 dental and mandibular characters). In the original analysis Lou *et al.* (2001a) did not provide statistical support measures for clades, or show trees including five key taxa that were included in their dataset (multituberculates, *Vincelestes*, tritylodontids, and the platypuses, *Obdurodon* and *Ornithorhynchus*). I have included parsimony analyses for both the 125 and 56 character datasets, with all 26 taxa, along with bootstrap support values.

Monotreme and marsupial specimens from the Australian Museum (AM) and the Queensland Museum (QM) were used for all anatomical examinations, including character state checking, and as reference material for the photographs and drawings. The specimen numbers were: platypus (*Ornithorhynchus anatinus*) JM6680 (QM), JM7014 (QM), JM9343 (QM), M8625 (AM), M26638 (AM); short-beaked echidna (*Tachyglossus aculeatus*) JM2276 (QM), JM7043 (QM); long-beaked echidna (*Zaglossus bruijnii bartoni*) M8263 (AM); Spotted-tailed quoll (*Dasyurus maculatus*)

JM9751 (QM); long-nosed bandicoot (*Perameles nasuta*) JM790 (QM); and northern hairy-nosed wombat (*Lasiorhinus krefftii*) JM7480 (QM). Echidna basicranial characters were also checked with Rougier *et al.* (1996).

### 3.2.2 Parsimony analysis

Maximum-parsimony (MP) analysis was conducted using PAUP 4.0b8 (Swofford 1998). All analyses were “standard parsimony”, with characters being unordered and equally weighted. Multistate characters were treated as polymorphic. Most-parsimonious trees were found via PAUP\* exhaustive searches. All bootstrap analyses were carried out using 1000 heuristic search replications. MacClade 3.05 (Maddison and Maddison 1995) was used to find tree length and indices of homoplasy for each of the 11 alternative monotreme placements among the background phylogeny. The Kishino-Hasegawa (1989) parametric, and Templeton (1983) non-parametric statistical tests were performed within PAUP\* 4.0b8, with two-tailed significance values given.

Congruence among the anatomical region (or meta-region) partitions was tested with the partition-homogeneity test (PHT; within PAUP\* 4.0b8) with 10000 branch-and-bound search replications. For the PHT, the shortest tree is calculated for each pre-defined data subset and the lengths summed. This value is compared with the summed length of trees calculated from randomly sampled partitions, each including the same number of characters as the pre-defined subsets. The more strongly the pre-defined subsets support different trees, the more homoplasy the overall dataset must contain and hence, the shorter the sum of the tree lengths will be, compared to those of the randomly sampled partitions.

Support for monotreme monophyly is high for the primary dataset with all characters included (95% MP bootstrap support). However, the level of bootstrap support differs for the anatomical regions, and paraphyly of the monotremes is favoured with the basicranial partition. These differences affect support for other relationships, including monotreme affinities with background taxa. To eliminate this source of error a sister group relationship between platypuses and echidnas was constrained (the unconstrained values are given in the figure captions where applicable). That echidnas and platypuses are sister taxa with respect to other taxa in this study is unchallenged by morphological studies and indeed analyses of DNA sequences (e.g. Retief *et al.* 1993; Gemmell and Westerman 1994; Messer *et al.* 1998; Janke *et al.* 2002) suggest the platypus and echidna lineages split during the early to mid Tertiary.

### 3.3 Results

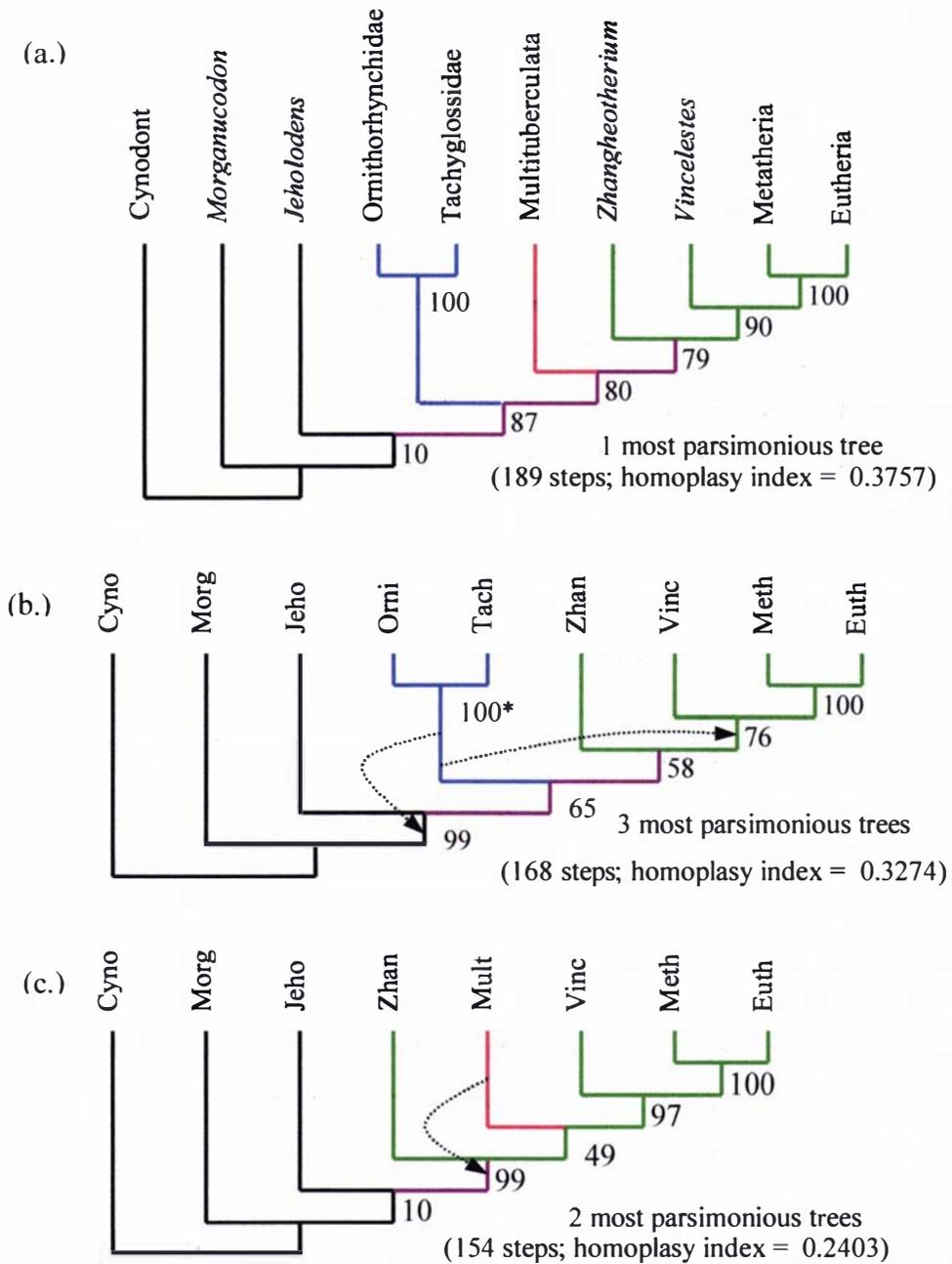
#### 3.3.1 *The background phylogeny of Mesozoic small terrestrial mammalian insectivores*

The null hypothesis for the background phylogeny (cynodonts, (morganucodontids, (triconodontids, (spalacotheriids, (eupantotheres, (metatherians, eutherians)))))) gains strong MP bootstrap support regardless of the inclusion, or exclusion of the dental and mandibular characters (Figure 3.1). With all characters included, alternative trees are rejected at  $p < 0.05$  by both the parametric (Kishino and Hasegawa 1989) and non-parametric (Templeton 1983) tests. The results are much the same with the dental and mandibular characters excluded, although *Zhangheotherium* as sister to Theria (as an alternative to Cladotheria) can only be rejected at  $p = 0.0832$  and  $p = 0.0833$  respectively by the parametric and non-parametric tests. However, it is worth noting that even with the dental and mandibular characters excluded, Cladotheria gains 95% bootstrap support and requires five fewer steps than a grouping of *Zhangheotherium* plus Theria.

With the dataset partitioned into the six anatomical regions, the partition homogeneity test indicates some incongruence for the background phylogeny ( $p = 0.0457$ ; see Table 3.1). This mild incongruence can largely be related to the mandibular characters, as with those excluded, congruence can only be rejected at  $p = 0.4718$ . This indicates a generally high level of congruence that is reflected by the background phylogeny null hypothesis (as in Figure 3.1) being the most (or equal most) parsimonious tree for each of the five non-mandibular anatomical region partitions. For the mandibular characters, the null hypothesis tree requires two more steps than the most-parsimonious solutions, which exclude Eutheria from other trechnotheres and/or require *Jeholodens* and *Zhangheotherium* to be sister taxa.

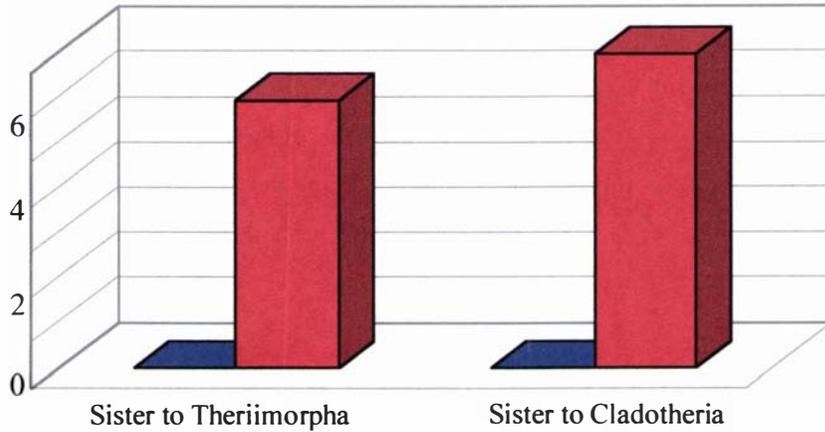
**Table 3.1** Partition homogeneity test (PHT) p-values for the primary dataset. These represent the probability of the observed (or greater) deviation from the null hypothesis (congruence among the partitions) being the result of sampling error for (a) the background phylogeny, which includes the taxa shown in Figure 3.1, (b) the background phylogeny plus monotremes, (c) the background phylogeny plus multituberculates and (d) the background phylogeny plus monotremes and multituberculates. The partitioning schemes are: 1. the six anatomical regions, 2. five anatomical regions (the mandibular region excluded), and 3. the three meta-regions.

partitions	PHT p-values			
	(a) Background phylogeny (BP)	(b) BP plus monotremes	(c) BP plus multituberculates	(d)BP plus monotremes and multituberculates
1. Anat. regions U, L, B, M, V, D	0.0457	<0.0001	0.0005	<0.0001
2. Mand. Excl. U, L, B, V, D	0.4718	<0.0001	0.0029	<0.0001
3. Meta-regions U, LB, MVD	>0.9999	<0.0001	0.0434	<0.0001

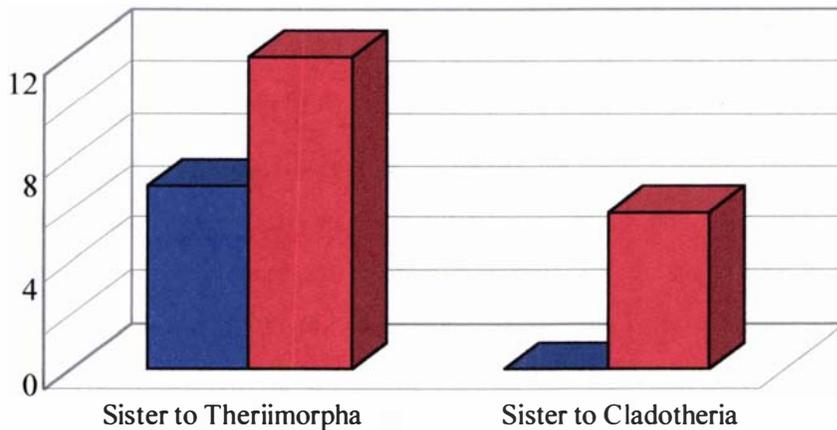


**Figure 3.2** Maximum parsimony trees (for the primary dataset) for the placement among the background phylogeny of (a.) monotremes and multituberculates together, (b.) just monotremes, and (c.) just multituberculates. Placements that are equally most-parsimonious with those that gain the highest bootstrap support are indicated by arrows. Bootstrap support values correspond to the clade above each of them. Branches are colour-coded as green for Trechnotheria, violet for the trechnothere stem lineage, red for multituberculates, and blue for monotremes. \*Note that monotreme monophyly is constrained (unconstrained it is supported in 95% and 71% of replicates in a. and b. respectively).

(a.) Steps saved by monotremes being placed on the trechnothere stem, compared with being placed as sister to either Theriimorpha, or Cladotheria, both without (blue) and with (red) multituberculates also being included with the background phylogeny



(b.) Steps saved by multituberculates being placed on the trechnothere stem, compared with being placed as sister to either Theriimorpha, or Cladotheria, both without (blue) and with (red) monotremes also being included with the background phylogeny



**Figure 3.3** The effect of multituberculates and monotremes being included with the background phylogeny (see figure 3.2) separately and together, on the number of steps saved by monotremes (a.) and multituberculates (b.) arising from the trechnothere stem, compared with being placed as sister to Theriimorpha, or as sister to Cladotheria.

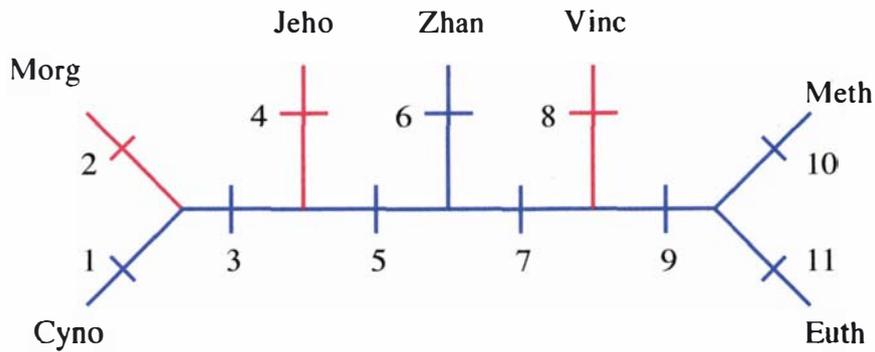
### 3.3.2 Apparent attraction between monotremes and multituberculates

Phylogenetic signal interaction between monotremes and multituberculates is a curious finding of this study. With both multituberculates and monotremes included for the MP analysis, they are strongly supported as consecutive sister groups of Trechnotheria (Figure 3.2a). However, with either monotremes or multituberculates included without the other, the most parsimonious placement is not exclusively with the trechnothere stem. As shown in Figure 3.2b, monotreme placement is equally most parsimonious as sister to Theriimorpha, or as sister to Cladotheria, or on the trechnothere stem. Likewise, multituberculates (Figure 3.2c) are most-parsimoniously placed either as sister to Cladotheria, or on the trechnothere stem, when monotremes are not included with them.

With the simultaneous inclusion of both monotremes and multituberculates, support for both arising from adjacent placements on the trechnothere stem is increased substantially relative to either being placed on neighbouring internodes. Figure 3.3a shows the effect of multituberculate inclusion on support for monotremes placement on the trechnothere stem, and Figure 3.3b shows the effect of monotreme inclusion on support for multituberculate placement on the trechnothere stem. With monotremes placed adjacent to multituberculates, support for monotremes arising from the trechnothere stem increases by 6 and 7 tree steps respectively, relative to placement as sister to Theriimorpha, and placement as sister to Cladotheria. Likewise, support for multituberculates arising from the trechnothere stem increases (upon monotreme inclusion) by 5 and 6 tree steps respectively, relative to placement as sister to Theriimorpha, and placement as sister to Cladotheria. Hence, the adjacent placement of monotremes and multituberculates effectively enhances signal supporting each others position on the trechnothere stem, relative to signal supporting positions on neighbouring internodes. This gives the impression of monotremes and multituberculates pulling each other together onto the trechnothere stem. Despite this apparent attraction, a monotreme plus multituberculate clade requires seven additional steps over their paraphyly with respect to Trechnotheria (as in Figure 3.2a).

### 3.3.3 The phylogenetic position of monotremes among the background phylogeny of generalized insectivores

The branch numbering on Figure 3.4 indicates eleven possible placements for monotremes on the background phylogeny of generalized insectivores. Parametric (Kishino-Hasegawa 1989) and non-parametric (Templeton 1983) tests indicate that with all 91 characters included, only monotreme placements as sister to either *Morganucodon*, *Jeholodens*, or *Vincelestes* can be rejected at  $p < 0.05$  (see the table associated with Figure 3.4). Monotremes associating with the outgroup is only



Monotreme position	All characters included			Specific anatomical regions					
	Tree steps	Statistical tests		most parsimonious tree (*) and extra steps required					
		K-H	Templeton	V	U	L	B	M	D
1	173	0.0584	0.0588	4	*	2	2	7	10
2	174	0.0135	0.0143	4	1	2	2	7	10
3	168	*	*	4	1	2	2	2	9
4	176	0.0106	0.0114	4	10	1	2	2	9
5	168	*	*	3	10	*	*	2	5
6	174	0.2757	0.2733	3	11	2	4	1	5
7	168	*	*	1	12	1	3	1	2
8	183	0.0104	0.0112	3	14	6	4	2	6
9	169	0.8825	0.8815	*	14	3	4	*	*
10	179	0.1316	0.1308	*	19	7	5	*	*
11	179	0.1316	0.1308	*	19	7	5	*	*

**Figure 3.4** Background phylogeny with red indicating at which placements a monotreme association (for the primary dataset with all characters included) is rejected ( $p \leq 0.05$ ) by both the Kishino-Hasegawa (K-H) and Templeton parsimony significance tests. Monotreme placement is not rejected (accepted at  $p > 0.05$ ) at internode placements that are coloured blue. The numbers of tree steps required for each of the eleven possible monotreme placements are noted in the table for analysis with all characters included. Additionally, the number of extra steps required beyond the most-parsimonious (\*) placement is also recorded in the table for each of the six anatomical region partitions: vertebral (V), upper appendicular (U), lower appendicular (L), mandibular (M), and Dental (D). See Figure 3.2 for taxonomic name abbreviations.

rejected at approximately  $p=0.06$ , while monotreme associations with marsupials or with placentals, are rejected at approximately  $p=0.13$ . Furthermore, Figure 3.4 shows that no monotreme placement along the backbone lineage, from outside the Theriimorpha (branch 3) up to being sister to Theria (branch 9) can be rejected at  $p < 0.88$ . These results illustrate that with the six anatomical regions combined, monotreme affinities are poorly resolved.

The inclusion of monotremes results in the homoplasy index increasing from 0.1270 (background phylogeny alone) to 0.3274. As indicated in Table 3.1, this increased conflict among characters coincides with extreme incongruence between the six anatomical regions ( $p < 0.0001$ ). This incongruence cannot be related to a specific anatomical region. In fact, with any one anatomical region left out, congruence is still rejected at  $p < 0.0001$  (for the generalized insectivore plus monotreme phylogeny).

The table associated with Figure 3.4 illustrates the topological nature of the extreme incongruence between anatomical regions, for monotreme affinities with the background phylogeny.

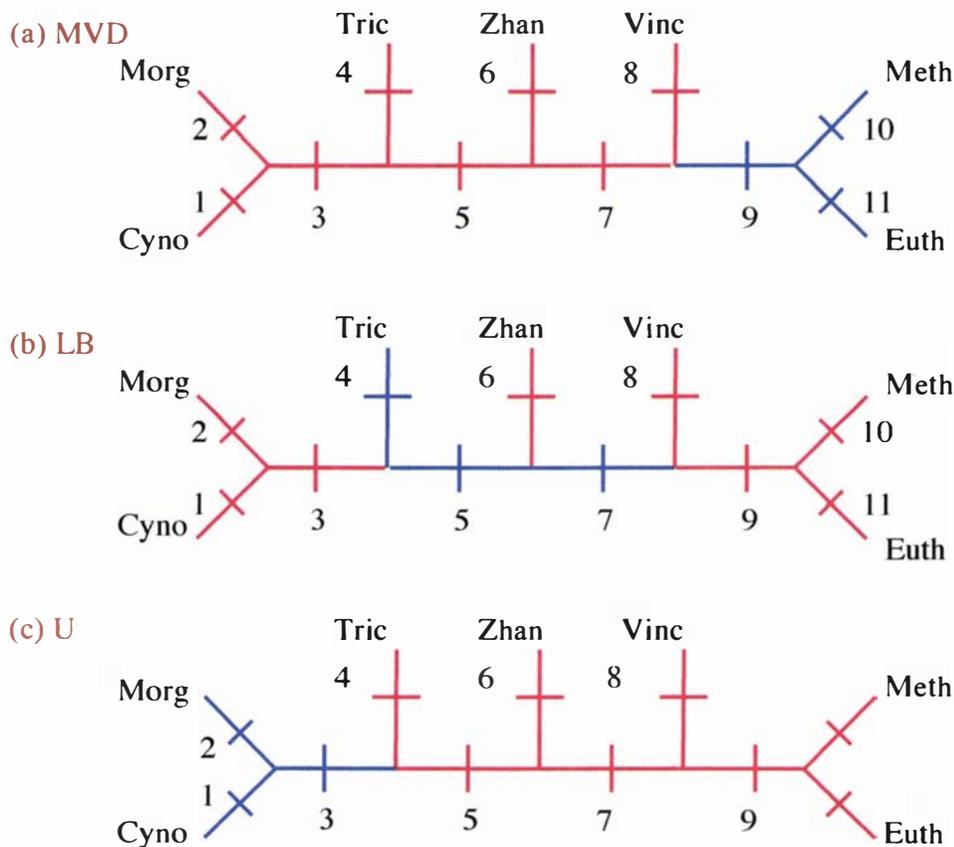
Monotremes can be most-parsimoniously associated with the same placement (with Theria) for the vertebral (V), mandibular (M) and dental (D) regions. Because these three anatomical regions are not in conflict they may be considered compatible. For both the lower appendicular (L) and basicranial (B) regions, the most parsimonious monotreme placement is an affinity with the trechnothere stem lineage, such that these regions are also compatible. For the upper appendicular region, monotremes are placed outside of Mammaliaformes (associating with the cynodont outgroup on the unrooted tree).

The phylogenetically compatible regions could be lumped together as meta-regions that represent the largest groupings of anatomical regions that could be a true reflection of monotreme affinities. In this regard the upper appendicular region (U) stands alone, while the vertebral, mandibular and dental regions may be lumped as one meta-region (MVD) and the lower appendicular and basicranial regions lumped as another meta-region (LB). This lumping together of anatomical regions into meta-regions might be expected to help isolate the source of incongruence, and so may also be useful for identifying the homoplastic mechanisms involved.

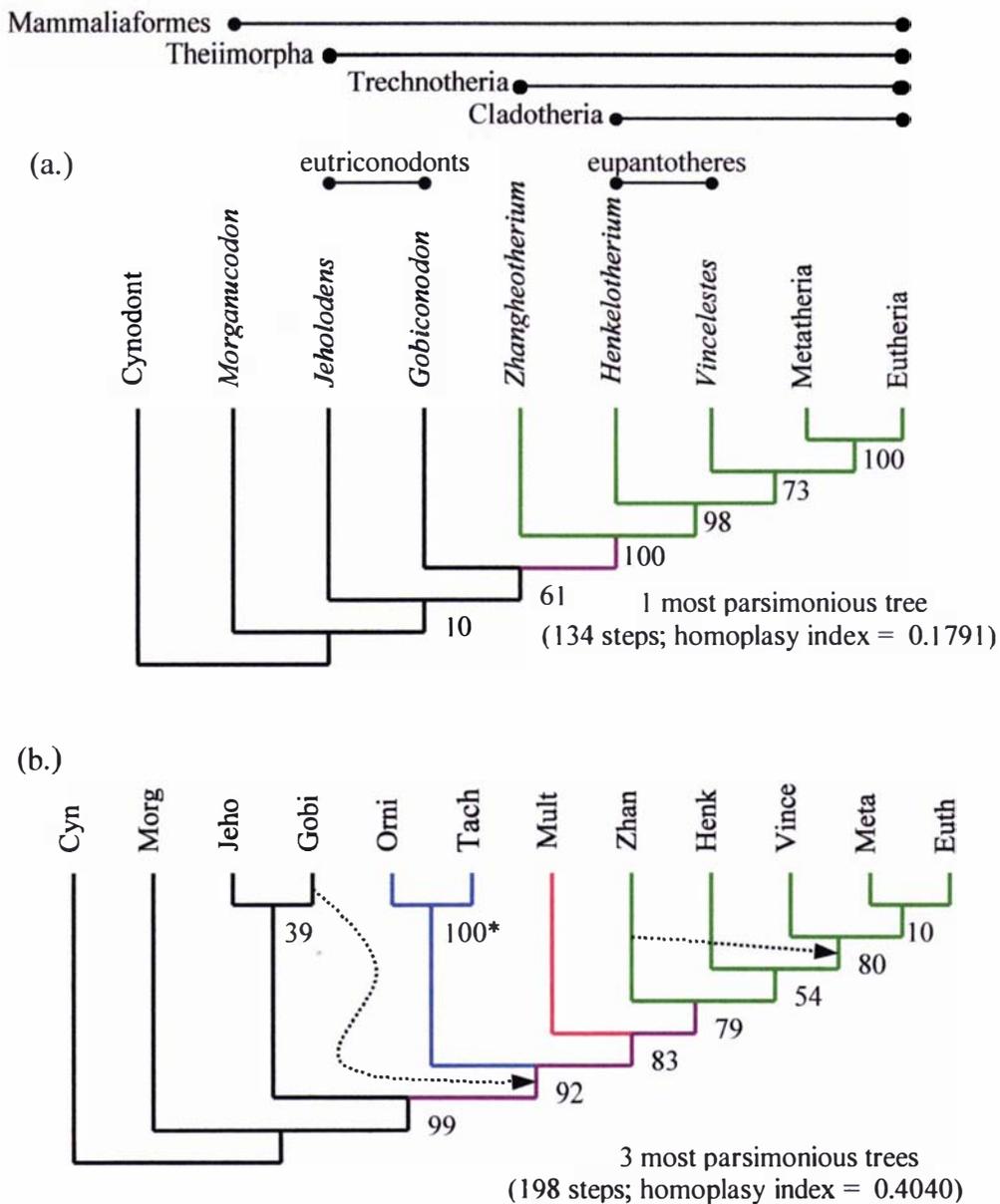
Congruence among the three meta-regions is rejected by the partition homogeneity test at  $p < 0.0001$  with monotremes included with the background phylogeny (Table 3.1). In contrast, for the generalized insectivores alone, the background phylogeny null hypothesis (Figure 3.1) is strongly favoured for each meta-region (MVD, LB, U), and congruence between the meta-regions cannot be rejected ( $p > 0.9999$ ).

The inability of the Kishino-Hasegawa and Templeton parsimony significance tests to reject (at  $p < 0.05$ ) eight of the eleven possible monotreme placements among the background phylogeny with all (91) characters included (Figure 3.4) contrasts with the situation for the meta-regions treated individually. Figure 3.5 shows that for MVD, LB and U respectively, only three, four and three of the 11 possible monotreme placements cannot be rejected (at  $p < 0.05$ ). The extent of the incongruence between the meta-regions is also indicated in Figure 3.5. No monotreme placement

Branch	Number of steps more than the MP tree (*) and statistical tests								
	(a.) MVD			(b.) LB			(c.) U		
	Tree steps	K-H	Templeton	Tree steps	K-H	Templeton	Tree steps	K-H	Templeton
1	+21	<0.0001	<0.0001	+4	0.0435	0.0455	32 (*)	*	*
2	+21	<0.0001	<0.0001	+4	0.0435	0.0455	+1	0.3287	0.3173
3	+15	<0.0001	0.0001	+4	0.0435	0.0455	+1	0.3287	0.3173
4	+15	<0.0001	0.0001	+3	0.1840	0.1797	+10	0.0004	0.0016
5	+10	0.0008	0.0016	53 (*)	*	*	+10	0.0004	0.0016
6	+9	0.0050	0.0067	+6	0.0314	0.0339	+11	<0.0008	0.0023
7	+4	0.0439	0.0455	+4	0.2111	0.2059	+12	0.0001	0.0005
8	+11	0.0004	0.0009	+10	0.0024	0.0039	+14	<0.0001	0.0002
9	63 (*)	*	*	+7	0.0505	0.0522	+14	<0.0001	0.0002
10	63 (*)	*	*	+12	0.0015	0.0027	+19	<0.0001	<0.0001
11	63 (*)	*	*	+12	0.0015	0.0027	+19	<0.0001	<0.0001



**Figure 5** Background phylogenies for MVD (a), LB (b) and U (c), with red indicating at which placements a monotreme association is rejected ( $p \leq 0.06$ ) by both the Kishino-Hasegawa (K-H) and Templeton parsimony significance tests. Monotreme placement is not rejected (accepted at  $p > 0.06$ ) at internode placements that are coloured blue. The number of extra steps required beyond the most-parsimonious (\*) placement is recorded in the table for each of the three meta-region partitions. See Figure 3.2 for taxonomic name abbreviations.



**Figure 3.6** Maximum parsimony trees (for the primary dataset) for the placement among the expanded background phylogeny for (a.) the generalized insectivores alone, and (b.) with monotremes and multituberculates also included. Placements that are equally most-parsimonious with those that gain the highest bootstrap support are indicated by arrows. Bootstrap support values correspond to the clade above each of them. Branches are colour-coded as green for Trechnotheria, violet for the trechnothere stem lineage, red for multituberculates, and blue for monotremes. \*Note that monotreme monophyly is constrained (unconstrained it is supported in 97% of replicates).

hypotheses that can be accepted (at  $p > 0.05$ ) for either MVD, or LB, can also be accepted ( $p > 0.05$ ) for U. Note that accepting the null hypothesis places the emphasis on the p-value being greater (rather than less) than the set value. Only one monotreme placement hypothesis (sister to Theria) can be accepted ( $p > 0.05$ ) for both MVD and LB. However, even this monotreme placement requires 7 additional tree steps for LB and is rejected by the Kishino-Hasegawa and Templeton tests at  $p = 0.0505$  and  $p = 0.0522$  respectively.

### 3.3.4 Expanded taxon sampling

The background phylogeny was expanded by including a second eutriconodont, *Gobiconodon* and a second eupantothere, *Henkelotherium*. Inclusion of these additional generalized insectivores provides a control treatment for examining the effect on homoplasy and congruence of including the ecologically-derived monotremes and multituberculates with the background phylogeny.

Maximum-parsimony bootstrap analysis was carried out on this expanded background phylogeny with all (91) characters included (Figure 3.6a). As before, the background phylogeny null hypothesis is well resolved, with Theria, Cladotheria, Trechnotheria and Theriimorpha each receiving 98% or greater bootstrap support. However the specific affinities of the two eutriconodonts and the two eupantotheres are less well resolved. Placing *Gobiconodon* (instead of *Jeholodens*) as the eutriconodont most closely related to Trechnotheria saves only one tree step. Similarly, placing *Vincelestes* (instead of *Henkelotherium*) as the eupantothere most closely related to Theria saves only two tree steps. Despite this uncertainty and the increase in the homoplasy index (from 0.1270 to 0.1791) that is induced by including the additional generalized insectivores, the significance level at which congruence among the six anatomical regions can be rejected increases. Upon expansion of the background phylogeny by inclusion of *Gobiconodon* and *Henkelotherium* the probability of the deviation from congruence resulting from sampling error (according to the PHT) increases from  $p = 0.0457$  to  $p = 0.1193$ .

High bootstrap support is maintained for the (adjacent) placement of monotremes and multituberculates on the trechnothere stem lineage when both are included together with the expanded background phylogeny (Figure 6b). However, the inclusion of these ecologically derived taxa reduces the resolution of the expanded background phylogeny. As depicted in Figure 3.6b (compare with Figure 3.6a), whether eutriconodonts are monophyletic, or paraphyletic becomes unresolved. More surprisingly, a clade that contains *Zhangheotherium* and *Henkelotherium* becomes an equally most-parsimonious alternative to cladothere monophyly.

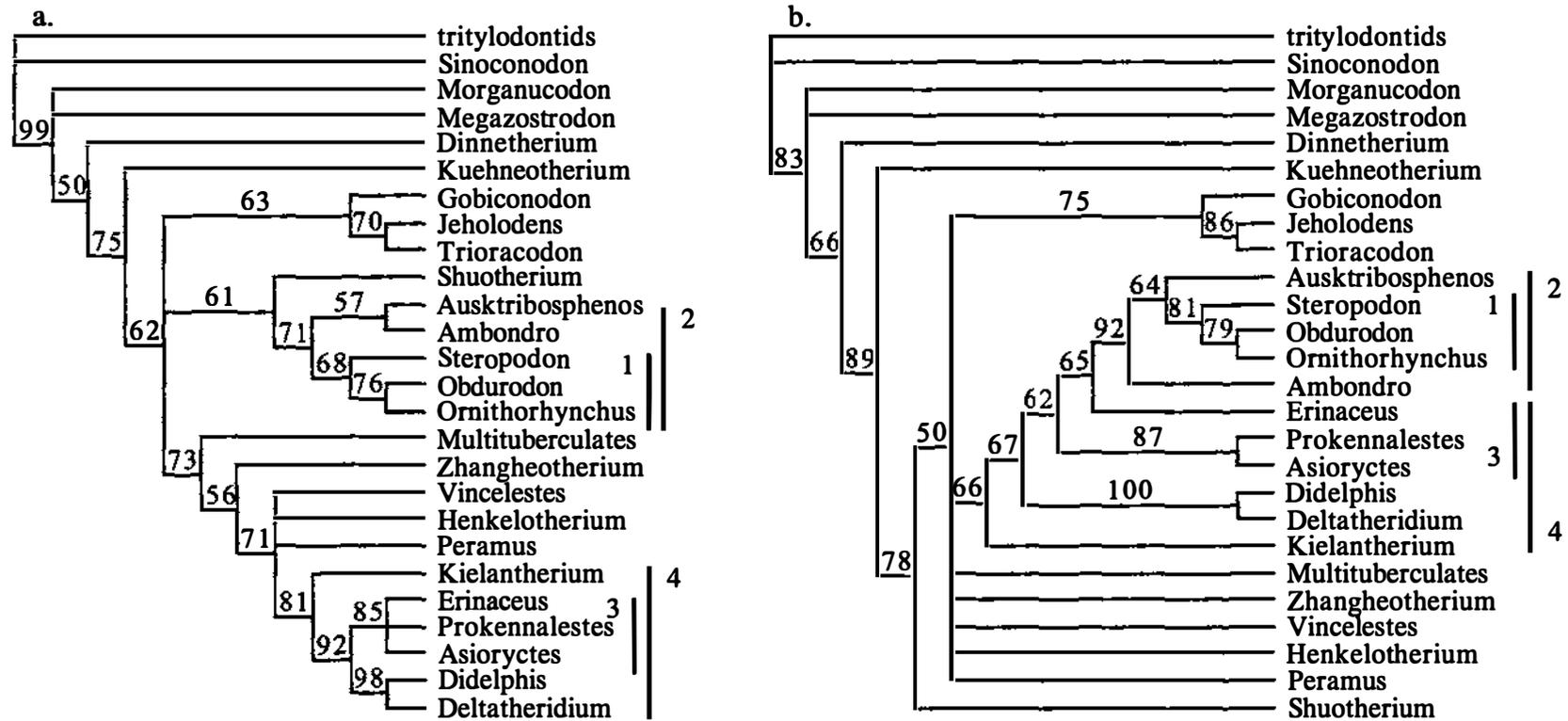
With just monotremes added to the generalized insectivores, incongruence according to the PHT ( $p < 0.0001$ ) and its topological nature (with respect to the anatomical regions) is essentially the same with or without *Gobiconodon* and *Henkelotherium*. In fact, with the expanded dataset, support is even stronger for monotremes arising from within Cladotheria for MVD and from the trechnothere stem for LB. For U, a monotreme association with cynodonts (unrooted tree) requires 8 steps fewer than for any position within or on the theriomorph stem. This is similar for the analogous situation without *Gobiconodon* and *Henkelotherium*, for which monotremes grouping with the cynodont outgroup saves 9 steps relative to any position with (or within) Theriimorpha.

The uncertainty of the positions of *Gobiconodon* and *Henkelotherium* in Figure 3.6b may be attributable to the monotremes and multituberculates disrupting character covariation among the generalized insectivores. Though as noted earlier, the positions of *Gobiconodon* and *Henkelotherium* relative to *Jeholodens* and *Vincelestes* (respectively) can be altered with only one or two tree step differences even with multituberculates and monotremes excluded. Given the possibility of errors in transformation estimation (by parsimony), *Gobiconodon* and *Henkelotherium* were not included for significance testing (Figures 3.4 and 3.5), which required an assumed background phylogeny (though their inclusion produces essentially the same results).

### 3.3.5 Analysis of australosphenidan relationships

If monotremes are closely related to ausktribosphenids, then using information from *Ausktribosphenos* (Rich *et al.* 1997, 1999) and the closely related *Bishops* (Rich *et al.* 2001b) may be the best way to estimate transformations that have occurred along the monotreme stem lineage. However, including these fragmentary taxa with the current dataset simply reduces the stability of the overall phylogeny. The alternative of constraining a monotreme plus ausktribosphenid clade would be too presumptuous until further confirmation of such a clade is available. Nevertheless, a brief analysis of the data provided by Lou *et al.* (2001a) is relevant for examining the stability of monotreme affinities with the inclusion of ausktribosphenids.

Lou *et al.* (2001a) found australosphenidan affinities to lie outside the Boreosphenida (including Theria) and even outside Theriimorpha. Unfortunately, no statistical support measure was employed so the potential effects of sampling cannot be inferred. Further, five key taxa for non-dental/mandibular data (multituberculates, *Vincelestes*, tritylodontids, and the platypuses, *Obdurodon* and *Ornithorhynchus*) were excluded from these analyses, despite their inclusion in the supplementary information. This is all the more curious considering that precedence above these for inclusion in the presented analyses, was given to particularly fragmentary taxa such as *Kuehneotherium* (Kermack *et al.* 1968) and *Shuotherium* (Chow and Rich 1982).



**Figure 3.7** Fifty percent consensus bootstrap (1000 replicates) parsimony cladograms based on (a.) the 125 character “whole skeletal”, and 56 character (dental and mandibular) extended taxon-sampling datasets of Lou, Cifelli *et al.* (2001). The following delineated taxonomic groups are delineated on each tree: Monotremata (1), Australosphenida (2), Eutheria (3) and Boreosphenida (4). Following Lou *et al.* (2001a), characters 6 and 21 were ordered, with the rest unordered for these PAUP\* analyses. Multiple states were treated as polymorphism.

To cover for the exclusion of key taxa, I have provided MP bootstrap analyses for both the 125 and 56 character datasets with all 26 taxa included (Figure 3.7). Two results are notable. Firstly, australosphenidan monophyly is favoured in both analyses, though with less bootstrap support when the non-dental/mandibular characters are included (71%, compared with 92% for the dental/mandibular characters alone). This drop in support mostly relates to replicates for which *Ornithorhynchus* is widely excluded from the remaining australosphenidans. Secondly, while Australosphenida is placed outside a grouping of Trechnotheria plus Multituberculata for the 125-character dataset, Australosphenida is nested within Boreosphenida for the dental/mandibular dataset. In fact, this latter result places the Gondwanan tribosphenic mammals as sister to the hedgehog (*Erinaceus*).

### 3.4 Discussion

#### 3.4.1 *The phylogeny of Mesozoic generalized mammalian insectivores*

It is encouraging that the background phylogeny of generalized insectivores is well resolved (Figures 3.1 and 3.6a). The finding that basicranial and post-cranial evidence support this phylogeny is important, as the dental and mandibular characters that have defined clades such as Theriimorpha, Trechnotheria and Cladotheria have been controversial (see McKenna 1975; Prothero 1981; Sigogneau-Russell and Ensom 1998; Pascual and Goin 1999; Sigogneau-Russell 1999). This result underlines the importance of the near-complete fossil finds of *Zhangheotherium* (Hu *et al.* 1997, 1998) and *Jeholodens* (Ji *et al.* 1999) and offers hope that taxa known from only dental/mandibular material, such as shuotheriids (Chow and Rich 1982; Sigogneau-Russell 1998), with their back-to-front molar structure, will be confidently placed on the mammalian phylogeny as more complete specimens are found.

The support for the background phylogeny that comes from bootstrap sampling and parsimony significance tests is complemented by very high congruence among most of the anatomical regions. It is impressive that five of the six (the mandibular partition being the exception) anatomical regions favour the background phylogeny null hypothesis, particularly considering the few characters in each partition and the existence of 945 topological permutations of the seven taxa. Homoplasy over the background phylogeny may be considered as more or less randomly distributed among characters of D, V, L, B, and U, given the high congruence under the PHT ( $p=0.4718$ , see Table 3.1) among these non-mandibular partitions.

Even the mild incongruence between partitions for the background phylogeny, which is induced upon inclusion of the mandibular partition, may be less the result of correlated homoplastic evolution, and more an effect of taxon sampling. Evidence for this comes from the reduced significance of the incongruence (from  $p=0.0457$  to  $p=0.1193$ ) with the addition of the eutriconodont (*Gobiconodon*) and eupantothere (*Henkelotherium*). Furthermore, inspection of the mandibular characters finds that only three conflict with the background phylogeny null hypothesis (Figure 3.1) and that these three characters do not support the same topological alternatives. Previous studies (character analysis and cladistic) are in agreement with the homoplastic status (and the taxonomic distribution of this homoplasy) indicated here for these three characters.

- The angular process (ch. 71) of cladotheres (which is posteriorly positioned) either evolved independently of the more anteriorly positioned angular process of non-theriimorphs (Jenkins *et al.* 1983; Rowe 1988), or alternatively, was lost (perhaps independently) among eutriconodonts, symmetrodonts and multituberculates (Sues 1986; Gambaryan and Kielan-Jaworowska 1995).
- Insertion of the pterygoid muscle on the mandible (ch. 72) is highly variable among Mesozoic mammals (see Cifelli and Madsen 1999; Wang *et al.* 2001).
- The absence of a scar or depression for the splenial bone on the dentary (ch. 76) of *Jeholodens*, but not *Zhangheotherium* is not surprising given that after the phylogenetic (rather than ontogenic) migration and cranial attachment of the postdentary (middle ear) bones, convergent loss of the paradentary bones (splenial and coronoid) is documented in numerous mammaliaform lineages during the Jurassic and Cretaceous (Krebs 1971; Kielan-Jaworowska and Dashzeveg 1989; Cifelli and Madsen 1999).

The limited incongruence that relates to homoplasy among a few mandibular characters is overshadowed by the strong support for the phylogeny of generalized insectivores, both with and without the dental and mandibular characters that originally defined most of the clades. Furthermore, recent studies that have described new cranial material (e.g. Lou *et al.* 2001b; Wang *et al.* 2001) also offer strong agreement with the phylogeny in Figure 3.1. These relationships can confidently be used to provide a background phylogeny for helping to infer the affinities of the enigmatic monotremes and multituberculates.

#### 3.4.2 Long-branch attraction between monotremes and multituberculates

As the dataset used here is modified from Ji *et al.* (1999), it is not surprising that with all taxa included, the most parsimonious tree (Figure 3.2a) is in agreement with their proposed phylogeny. Bootstrap support for multituberculates and monotremes being consecutive sister groups of

Trechnotheria is high and increases further with the inclusion of *Gobiconodon* and *Henkelotherium* (Figure 3.6b). If indeed multituberculates and monotremes are positioned adjacently with respect to the background phylogeny (but not as sister taxa), this would make them more susceptible to being erroneously favoured as sister taxa by any convergence or parallelism between them. Of course the converse is also true. If multituberculates and monotremes are sister taxa (with respect to the background phylogeny), that would make them more susceptible to being erroneously favoured as adjacent groups by any convergence or parallelism between one of them and the background phylogeny.

Support for a sister group relationship between multituberculates and monotremes is based almost entirely on cranial data (e.g. Hopson *et al.* 1993; Wible and Hopson 1993; Meng and Wyss 1995; Wang *et al.* 2001). For the present “whole skeletal” analysis, such a clade requires at least seven more steps than the relationship shown in Figure 3.2a. On this basis I tentatively consider the (few) cranial similarities between these two highly modified taxa to be homoplastic. Though as will be discussed later, the apparent stability of the placements of monotremes and multituberculates in Figures 3.2a and 3.6b is misleading.

Examining variation in apparent phylogenetic signal via bootstrap resampling relies on the assumptions that the characters evolve independently (Swofford *et al.* 1996), and that variation among the characters used is representative of the variation among the “universe” of characters (Goloboff 1991). The topological interaction effect between monotremes and multituberculates indicates that the data may not meet these criteria.

The relationships of both monotremes and multituberculates, with the background phylogeny are unresolved when each is included without the other (Figures 3.2a and 3.2b). Included together though (Figure 3.2c), they are drawn strongly to the trechnothere stem lineage, indeed towards each other. The adjacent (not monophyletic) placement of monotremes and multituberculates results in considerable tree step savings (Figure 3.3) over the neighbouring placements, which were equally favoured with these taxa included individually. The dataset offers only two potentially unequivocal synapomorphies for these two taxa (ch 49: presence of a distinct calcaneal peroneal process; ch 51: calcaneal contact with metatarsal V) forming a clade that arises from the trechnothere stem. As such, the strength of the apparent “long-branch attraction” between monotremes and multituberculates is somewhat surprising. Placing monotremes and multituberculates adjacently on the trechnothere stem saves at least six tree steps by comparison with moving one to a neighbouring internode that is an equally most-parsimonious placement without the other being included. Hence, it is of interest to identify the effect that placing

monotremes and multituberculates adjacently on the tree (on the trechnothere stem) has on the pattern of transformations across the phylogeny.

The “long-branch attraction” between monotremes and multituberculates primarily involves the adjacent placement of these taxa shutting down synapomorphies with, or among background phylogeny taxa that save tree steps when either is included alone. The inclusion of monotremes disrupts potential synapomorphies of a Cladotheria plus multituberculate clade (chs. 7, 36, 42, 69, 83) and the inclusion of multituberculates disrupts potential synapomorphies of a theriimorph clade excluding monotremes (chs. 24, 30). Adjacent placement of monotremes and multituberculates also saves steps by ensuring that a character state need only evolve once in the ancestors of these taxa and *Jeholodens* (ch.50), or in the ancestors of these taxa and *Zhangheotherium* (chs. 12, 54).

Because monotremes and multituberculates are highly derived relative to the generalized insectivores, the apparent attraction between the two ecologically derived taxa might be considered to be a morphological version of long-branch attraction (Hendy and Penny 1989). This phenomenon involves higher levels of (unobserved) transformations among highly derived taxa (long branches), relative to the rest of the tree, which tends to pull them together. As originally described (Felsenstein 1978), the bias resulted in a (false) sister group relationship between the long-branch taxa. However, long-branch attraction has also been shown to pull distantly related rodents and erinaceids into adjacent (non-sister) placements, with mitochondrial genome data (Lin *et al.* 2002).

Whether or not the adjacent position of monotremes and multituberculates found in this study (or the sister relationship in some other studies) is attributable to long-branch attraction deserves further examination. Regardless, exclusion of either monotremes or multituberculates demonstrates that placing confidence in the high bootstrap support for placing monotremes and multituberculates as consecutive sister taxa to Trechnotheria (Figure 3.2a) may be misleading.

### *3.4.3 Anatomical region dependence of phylogenetic signal and implications for monotreme affinities*

Although foreshadowed by Gregory (1910), it was not till much later (see Hopson and Crompton 1969) that monophyly of the mammalian crown group (monotremes and therians) with respect to cynodonts was fully accepted. No further resolution of monotreme affinities has met with universal support. Perhaps the most fundamental question to be answered is whether monotremes arose from within the Trechnotheria (branches 6,7,8,9,10,11 in Figure 3.4), arose from the trechnothere stem lineage (branch 5 in Figure 3.4), or are prototherian (considered here as branches 1,2,3,4 in Figure

3.4). Figure 3.4 shows that for monotremes, the vertebral (V), mandibular (M), and dental (D) partitions favour affinities within Trechnotheria, the upper appendicular (U) partition favours prototherian affinities, and for lower appendicular (L) and basicranial (B) partitions, a stem-trechnothere affinity is favoured. These results largely agree with previous studies of individual anatomical regions, as noted in the introduction.

Given that the primary (91 character) dataset strongly resolves the generalized insectivore phylogeny, it is perhaps surprising that the parsimony significance tests (Figure 3.4) cannot reject ( $p < 0.05$ ) 8 of the 11 possible monotreme placements. That prototherian, stem-trechnothere, and trechnothere placements are equally most-parsimonious is particularly surprising, because these placements are separated by many apomorphies along the mammalian backbone lineage (Figure 3.1, in red). It seems a remarkable failure that this “whole skeletal” analysis cannot reject ( $p < 0.05$ ) monotremes arising from the mammalian backbone lineage from before the node from which *Morganucodon* arises, right up to the last common ancestor of marsupials and placentals. Approximately 100 million years of mammalian evolution is represented between those nodes (see Chapter 2).

The individual meta-regions provide greater (though conflicting) resolution of monotreme affinities than does the overall dataset, with U, LB and MVD respectively favouring prototherian, stem-trechnothere and cladothere affinities. It is noteworthy that fewer monotreme placements among the background phylogeny could be rejected by the parsimony significance tests for the combined data (3: Figure 3.4), than could be for any of the three meta-region datasets alone (7-8: Figure 3.5). This is contrary to the expectation of “total evidence” approaches (e.g. Eernisse and Kluge 1993), that in spite of incongruence, relationships will become more robust to sampling effects as partitions are lumped, and will tend to converge upon the “true” phylogeny.

Rather than reducing the relative effect of stochastic error and converging upon the true phylogenetic position of monotremes, lumping the anatomical regions together simply results in an intermediate affinity being favoured as the most parsimonious compromise among the incongruent partitions. This would explain why Figure 3.2 and other “whole skeletal” cladistic analyses such as Rowe (1988), Hu *et al.* (1997, 1998), Ji *et al.* (1999) and Lou *et al.* (2001a), give the impression that monotreme (or Australosphenidan) affinities are at least locally stable, stemming from the mammalian backbone lineage either just before, or just after eutriconodonts. The phylogenetic instability of monotremes at the level of anatomical regions is illustrated by only two of the six anatomical regions (L and B) being compatible with this zone of local stability (placements 3, 4, 5 on Figure 3.4). In fact U favours monotremes being more primitive than *Morganucodon*, while V, M, and D favour monotremes being more advanced than is the eupantothere, *Vincelestes*. The

extent to which this anatomical region dependence overshadows any underlying phylogenetic signal for monotreme affinities is expressed by the partition homogeneity test (Table 3.1). With the monotremes included, incongruence is highly significant ( $p < 0.0001$ ) regardless of whether the data is partitioned by meta-region, or by anatomical region (with or without the mandibular region, which induced mild incongruence among the background phylogeny).

A close relationship between monotremes and tribosphenic cladotheres was suggested upon the discovery of near-tribosphenic teeth in *Steropodon* (Archer 1985; Kielan-Jaworowska 1987). Wible (1991) cited another prominent character, (partial) cochlea coiling, as further support for such a relationship. Outside of monotremes, cochlea coiling is only known from tribosphenic, or prototribosphenic mammals such as *Vincelestes* (Rougier *et al.* 1992). Perhaps more often though, the condition of the molars and cochlea coiling have perhaps more often been suggested to be convergent (e.g. Rougier *et al.* 1996; Fox and Meng 1997; Hu *et al.* 1997), in order to explain cladistic analyses finding monotremes to be excluded from Trechnotheria. Inspection of the tree steps table within Figure 3.4 shows that reconciling any monotreme phylogenetic affinity is considerably more complex than simply explaining away the coiled cochlea and reverse triangle molars with expanded talonids.

Not only are the most-parsimonious monotreme placements of U, MVD, and LB incompatible, but the ranges over which parsimony significance tests cannot reject monotreme affinities (Figure 3.5) for each of these meta-regions are also incompatible. This is consistent with the PHT results (Table 3.1), in indicating that the incongruence is unlikely to be a sampling artifact. In addition, the parsimony significance test results show that this incongruence cannot be the result of homoplasy among just one of the three meta-regions. Hence, homoplastic signal relating to monotreme affinities is not randomly distributed among characters, and occurs across at least two of the meta-regions, with the topological nature of the bias differing in each. The nature of this homoplasy is further explored in Chapter 4.

Little attention has been paid to the possibility of monotreme affinities being affected by homoplasy that occurs at the anatomical region level (but see Gregory 1947), aside from that concerning dental characters. However, it seems unreasonable to rule out correlated evolution of homoplasy across the characters of any of the individual anatomical regions, considering the functional and developmental linkage of their characters (see the section 3.4.5 and Chapter 4). Greater immunity to correlated evolution of homoplasy has however traditionally been perceived for cranial characters (e.g. MacPhee and Cartmill 1986; Parrish 1993). This seems to be largely due to these characters being considered as further separated from the selection pressures for dietary and locomotive adaptations that are obvious among dental, mandibular, and postcranial skeletal

characters. However, after examining homoplasy among 41 published mammalian datasets, Sánchez-Villagra and Williams (1998) argue that cranial characters are no less susceptible to homoplasy than are postcranial and dental characters.

High phylogenetic consistency for cranial (basicranial) characters is certainly a fair assessment for the background phylogeny. Even with *Gobiconodon* and *Henkelotherium* included, no homoplasy is observable among the 11 basicranial characters for the generalized insectivore taxa. This represents many hundreds of millions of years of evolution (combined) along the generalized insectivore lineages, without observed homoplasy for these characters. Unfortunately, this consistency does not hold for monotremes. Homoplastic evolution of at least six of these characters has occurred among monotremes since echidnas and platypuses split, which was apparently less than 55 million years ago (Retief *et al.* 1993; Gemmill and Westerman 1994; Janke *et al.* 2002, and see Chapter 2). How much more homoplasy might relate to the long monotreme stem lineage? Perhaps no characters should *a priori* be considered to be especially reliable for analyses where taxa with greatly varying ecological niches are included.

Whichever anatomical regions contain the homoplasy promoting the incongruence for the position of monotremes among a background phylogeny, that homoplasy could involve a transformation on the monotreme stem lineage and a transformation among the background phylogeny taxa (model A), or alternatively, two separate transformations among the background phylogeny taxa (model B). As an example, Ji *et al.* (1999) found monotremes to be more closely related to trechnotheres than is the eutriconodont, *Jeholodens*, despite the (early) trechnothere-like shoulder girdle of *Jeholodens* being substantially more advanced than the cynodont-like shoulder girdles of monotremes (See Jenkins and Parrington 1976; Sereno and McKenna 1995). Under model A, the therian-like shoulder girdle would have evolved once in the ancestor of theriomorphs and have been reversed during monotreme evolution. For the alternative (model B), therian-like shoulder girdles would have evolved independently (in parallel) in eutriconodonts and trechnotheres. Though non-committal, Ji *et al.* (1999) favoured model B.

Upon addition of monotremes to the background phylogeny, the homoplasy index swells by a factor of 2.58. Furthermore, the topological distribution of this additional homoplasy with respect to the three meta-regions results in an extreme swing in partition homogeneity test congruence p-values. The PHT p-value is  $>0.9999$  without monotremes and  $<0.0001$  with monotremes included (Table 3.1). These results point to model A being largely responsible for the homoplasy that results in incongruence between anatomical regions, for monotreme affinities.

It is possible for the addition of taxa to expose previously “hidden-homoplasy” (Archie 1989b). However, it is difficult to explain the extreme incongruence upon the inclusion of monotremes without much of the homoplasy being associated with the monotreme lineage. This (model B) would require a large-scale and complicated pattern of “hidden” homoplastic transformations occurring at many of the background phylogeny internodes and external branches. Complete congruence among the three meta-regions for the background phylogeny and low observed homoplasy gives no indication of this. As such, it must be expected that the majority of incongruence between the meta-regions for the placement of monotremes involves transformations that occurred in monotremes (model A). In fact, these homoplastic transformations can be further isolated to the monotreme stem lineage, as monophyly of the two monotreme families was constrained for the partition homogeneity tests.

The inclusion of *Gobiconodon* and *Henkelotherium* highlights a fundamental difference in the relationship between homoplasy and phylogenetic uncertainty that exists between the ecologically distinct taxa and the generalized insectivores. The specific affinities of *Gobiconodon*, *Henkelotherium*, monotremes and multituberculates are all uncertain among the background phylogeny that is shown in Figure 3.1. However, the distribution of homoplasy among the data matrix resulting from inclusion of the ecologically distinct taxa induces highly significant incongruence between the anatomical regions (Table 3.1). In contrast, the inclusion of *Gobiconodon* and *Henkelotherium* reduces the significance of incongruence between the anatomical regions despite (slightly) increasing the homoplasy index. A corollary of this is that uncertainty for the placement of *Gobiconodon* and *Henkelotherium* (Figure 3.6a) may simply be a stochastic effect related to sampling for shorter internodes than those separating the nodes of the background phylogeny. This offers hope that as character sampling increases across anatomical regions, the resolution of relationships among Mesozoic mammals that have strayed little from the ancestral mammalian niche will typically increase. Likewise, as character completeness increases for the generalized insectivores of uncertain affinities, such as the South American “symmetrodonts” (Bonaparte 1990), their position within Mammalia might be resolved.

That the affinities of monotremes (and multituberculates) depend on the anatomical regions examined, indicates that increased character sampling from currently described specimens may be of little benefit for inferring their placement on the background phylogeny. Fossils that break up the branches leading to monotremes and multituberculates may be required for this. Here, the ausktribosphenids may be helpful (see the section 3.4.4), at least for dental and mandibular characters. More generally though, the taxon-sampling problem for monotremes is further compounded by a recent tendency (e.g. Hu *et al.* 1997, 1998; Ji *et al.* 1999; Lou *et al.* 2001a, 2001b; Wang *et al.* 2001) to include only ornithorhynchids among cladistic analyses. In such cases,

estimation of the plesiomorphic monotreme condition relies mostly on the modern platypus (*Ornithorhynchus anatinus*). It is of course justifiable to exclude tachyglossids (echidnas) from dental, and perhaps mandibular studies on the basis of echidnas being edentulous and having a reduced dentary. However, there is little to suggest that the inclusion of both platypuses and echidnas together would not allow for a more reliable apomorphy distribution of monotreme non-dental/mandibular characters, with respect to the background phylogeny.

As with this study, cladistic analyses of morphological data that include both platypuses and echidnas have often provided only limited support for monotreme monophyly (e.g. Rougier *et al.* 1996). This is probably due to a combination of the highly modified nature of these monotremes, and the morphological characters being defined chiefly for determining relations among other Mesozoic mammal groups. This should not prohibit the inclusion of both monotreme families. Chromosomal (e.g. Wrigley and Marshall Graves 1988; Watson 1990) and DNA sequencing (e.g. Gemmell and Westerman 1994; Killian *et al.* 2001; Janke *et al.* 2002) studies provide overwhelming evidence of the monophyly of Monotremata. Because *a priori* knowledge of whether echidnas or platypuses best represent the plesiomorphic monotreme state does not exist for most of these characters, including both monotreme families as a constrained monophyletic group is the best null hypothesis for allowing parsimony analysis to distribute transformations.

As noted earlier, DNA sequence data provide compelling evidence for an early to mid Tertiary divergence between echidnas and platypuses. As such, the monotreme stem lineage, which stretches back into the Middle Jurassic (see Chapter 2), represents more than 100 million years of platypus/echidna common ancestry. Hence, the limited support for monotreme monophyly inspires little confidence in transformations along the much longer monotreme stem lineage being reliably accounted for by MP analysis of the same data. Considering this and the highly significant incongruence between anatomical regions that relates to monotreme affinities, little trust can be placed in proposals of monotreme placement that rely heavily on the monotreme crown group for provision of morphological characters.

#### 3.4.4 *Australosphenidan affinities of monotremes*

The proposed relationship (Australosphenida) between the Gondwanan tribosphenic mammals and monotremes (Lou *et al.* 2001a) threatens to unravel much of the uncertainty about the origins and evolutionary history of monotremes. The dataset of the above authors provides five unequivocal apomorphies (all dental) that support this clade (plus *Shuotherium* in some cases), that are present (at least ancestrally) in *Steropodon*, and/or *Obdurodon*, and are not shared by members of Boreosphenida. These are: (1) a buccal cingulid on the ultimate premolar; (2) a lingual cingulid on

the ultimate premolar; (3) a continuous mesial cingulid below the molar trigonid; (4) wrapping of the mesial cingulid around the anterolingual corner of molars; and (5) a very deep ectoflexid. However, Sigogneau-Russell *et al.* (2001) dispute some of these apomorphies and exclude *Ambondro* from Australosphenida.

Examining the evidence for monotremes being included within Australosphenida is beyond the scope of this study, and I have not examined material from ausktribosphenids or *Ambondro*. However, reasonable bootstrap support for Australosphenida is indicated in the current analysis (Figure 3.7) for both the 56 and 125 character datasets. Hence, at least for these data, this clade is robust to sampling effects and the addition of the five taxa (multituberculates, *Vincelestes*, Tritylodontids, and the platypuses, *Obdurodon* and *Ornithorhynchus*) that were excluded by Lou *et al.* (2001a). This provides further reason for optimism that “stem monotremes” that retain a generalized insectivore niche that is more or less ancestral for mammals, have at last been uncovered.

Inspection of Figure 3.7 shows that the inclusion of non-monotreme australosphenidans does not circumvent the anatomical region dependence of monotreme phylogenetic affinities. With the 125-character (“whole skeletal”) dataset, Australosphenida is placed outside the Boreosphenida (which includes modern tribosphenic mammals). However, for the 56 dental/mandible characters alone, Australosphenida is nested within Eutheria in each of the most-parsimonious trees, and more specifically as sister to the hedgehog in 65% of bootstrap replicates. This is not an expected relationship (though see Rich *et al.* 1997, 1999; Rich *et al.* 2001b). However, the dental and mandibular characters from the primary dataset of this study are also compatible with monotremes being derived from within or from close to modern tribosphenic cladotheres (Figure 3.5a). This testifies to the bulk of the evidence against a close relationship between Australosphenida and Boreosphenida being supplied by non-dental/mandibular characters, which among australosphenidans have only been published for Miocene to recent monotremes (see Griffiths *et al.* 1991; Musser and Archer 1998). As such, until more complete Mesozoic australosphenidan fossils are found, the affinities of this group according to “whole skeletal” parsimony analyses largely depend on extant, and Tertiary monotremes.

#### 3.4.5 Mechanisms generating homoplasy that can be related to the monotreme stem lineage

That incongruence relating to monotreme placement largely involves homoplastic evolution along the monotreme stem lineage, is consistent with the two factors hypothesized in the introduction as being important contributors to the uncertainty of monotreme affinities. These are (a) the extreme ecological niche-related modification of monotremes and (b) parallelism relating to trends that

were initiated before monotremes split from the mammalian backbone lineage. On the basis of relative expectations for these two factors to bias the placement of monotremes, the anatomical regions can be divided into partitions that are consistent with their compatibility (MVD; LB; U).

Whether monotreme traits are indicative of phylogenetic history, or instead are a reflection of fossorial/swimming/dietary adaptations is relevant to most aspects of the osteoanatomy of modern platypuses and echidnas (see Gregory 1947; Griffiths 1978; Augee and Gooden 1993; Grant 1995; Musser 1998). However, of the anatomical regions included in the current study, niche-related modification is particularly prominent among the characters of the upper appendicular, lower appendicular, and basicranial regions.

The locomotion of the generalized insectivores involves the limbs pushing off a solid substrate and swinging through air. In contrast, the pectoral girdle and forelimbs of monotremes are modified for digging/swimming through dense media (soil and water), while their hindlimbs play an important role in bracing against the powerful forelimb digging (echidnas and platypus), and possibly acting as a rudder (for the platypus). Jenkins (1970a, 1970b, 1973), Lewis (1983), Pridmore (1985), Szalay (1993b) and Gambaryan and Kielan-Jaworowska (1997) discuss many of the apparently niche-related modifications of the monotreme appendicular skeleton. Some of these appear to be unique, while some are shared with various groups of fossorial and aquatic therians.

Among generalized mammals, hearing has become specialized for transmittance of (often high frequency) airborne sound (Allin 1975; Fox and Meng 1997). However, the skull of foraging monotremes is often in contact with the substrate and many basicranial traits may be adaptations for (low frequency) bone-conducted hearing (Aitkin and Johnstone 1972; Griffiths 1978, Ladhams and Pickles 1996). Further modifications of monotreme basicranial architecture may be related to braincase expansion (Zeller 1993) and skull streamlining (Musser and Archer 1998). Zeller (1993) showed that braincase expansion proceeds differently (ontogenetically) between monotremes and modern therians, though he did not comment on the functional significance of these differences. Certainly the dorsoventral compression of platypus and echidna skulls has been hypothesized to be adaptive for swimming (Grant 1995) and soil leverage (see Augee and Gooden 1993) respectively.

Dental and mandibular material have been described from (late) Early Cretaceous monotremes such as *Steropodon* (Archer *et al.* 1985) and *Teinolophos* (Rich *et al.* 2001a), which were less derived than the modern monotremes, which lack teeth (at least as adults). Of the above taxa, the molar structure of *Steropodon* appears more plesiomorphic, while *Teinolophos* has a more completely preserved dentary that is perhaps closer to the primitive monotreme condition, at least in terms of its smaller size, and well developed angular process and ascending ramus. By

comparison with the extremely modified condition of monotreme appendicular and basicranial material that has been published, the dental (from *Steropodon*) and mandibular (from *Teinolophos*) character states appear to be relatively plesiomorphic (at least functionally).

Just as the fossorial/swimming habits have resulted in a modified functional relationship between the appendicular skeleton of monotremes and the environment, so too has the vertebral column been affected (Augee and Gooden 1993; Grant 1995). Fossorial/swimming niche-related vertebral adaptations typically relate to the shape of vertebral processes, muscle attachments, and interlocking mechanisms, and the way these alter along the vertebral series (Kielan-Jaworowska 1994; Jenkins 1974; Szalay 1994). Such characteristics are largely avoided in the current dataset (see Appendix D) and that of Ji *et al.* (1999). This does not mean that the monotreme conditions for the included vertebral characters are not at least in part a reflection of the habits of echidnas and the platypus. However, as for the mandibular and dental characters, the expectation is that the condition of the monotreme vertebral characters is less likely to be a reflection of niche-related adaptation than is the case for the appendicular and basicranial characters. This should not imply any belief that the vertebral, mandibular, and dental characters provide the best indication of monotreme affinities.

The second major factor proposed to bias monotreme affinities, parallelism with one or more groups of the background phylogeny, is exacerbated by the inadequacy of the transitional series provided by the monotreme fossil record. From among the dataset, the vertebral, mandibular, and dental characters appear to be most at risk for this source of homoplasy. These anatomical regions include character complexes that were subject to trends that were initiated before monotremes split from the mammalian backbone lineage, and may be expected to have continued in taxa that essentially retained that ancestral mammalian niche (including stem monotremes and the taxa of the background phylogeny). These trends include: fusion of atlas components and the fusion of various ribs to vertebrae (among the vertebral characters, see Jenkins 1970a; Jenkins and Parrington 1976); reduction and loss of postdentary, then parodontary bones, and loss of the groove for the meckelian cartilage (among the mandibular characters, see Krebs 1971; Allin 1986; Wang *et al.* 2001); and for dental characters, elaboration of basic inline “triconodont” molars, for oblique or transverse shearing and grinding functions, as occurred among numerous groups, including trechnotheres (Butler 1990; Setoguchi *et al.* 1999), docodonts (Lillegraven and Krusat 1991; Pascual *et al.* 2000), and shuotheriids (Chow and Rich 1982).

From the Permian pelycosaur synapsids, though to Triassic cynodonts, and along the backbone lineage up to the therians, the parasagittal (or near-parasagittal) gait gradually evolved. This transformation involves a number of interrelated trends (see Jenkins 1970a; Jenkins and Weijjs

1979; Kemp 1982), such as for the shoulder glenoid (and so the long axis of the humerus) to face more posteriorly and more ventrally, and for reduction of ossified shoulder girdle bracing. Ji *et al.* (1999) embrace the potential for these trends to have resulted in considerable parallel evolution of shoulder girdle and forelimb characters between trechnotheres and triconodontids.

Little attention has been paid to the possibility of monotreme affinities being affected by large scale parallelism that results from independent continuation of trends among character complexes within either of the lower appendicular or basicranial regions. Lower appendicular traits (which are absent among monotremes) such as a dorsally directed femoral greater trochanter, extension of the calcaneal tubercle, and superposition of the astragalus over the calcaneus may be related to a trend towards a more upright hindlimb posture and greater agility (Gambaryan 1974; Marshall and Sigogneau-Russell 1995; Muizon 1998). However, most of the other pelvic and hindlimb characters that are included in the dataset, such as the development of the patellar groove, and of the tarsal spur, do not appear to be related to long-term phylogenetic trends.

Trends affecting the structure of the petrosal are apparent during the late Triassic mammal-like reptile transition (Lou *et al.* 1995) and among early eutherians and metatherians (Wible *et al.* 2001), though these may respectively be too early, and too late to confuse monotreme placement. The basicranial characters among the dataset provided by Ji *et al.* (1999; see Appendix D) that are crucial for determining monotreme placement are mostly presence/absence architectural characters that do not appear to be following long-term phylogenetic trends. Though without eutriconodonts and early trechnotheres having been subjected to functional analyses of basicranial architecture, it is difficult to exclude the possibility of some influence by trends among Mesozoic mammals for improved acoustic insulation, and improved transmittance of (high frequency) airborne sound (Allin 1975; Lou *et al.* 1995; Fox and Meng 1997). However, it is noteworthy that as the functional relationships of the basicranial (and appendicular) characters of monotremes differ from those of the generalized insectivores, any parallelism for these is more likely between the background taxa, than between monotremes and one or more of the background taxa.

Both (a) ecological/functional modification and (b) trends among Mesozoic mammals (resulting in parallelism) may have influenced the apparent affinity of monotremes with the background phylogeny for any of the anatomical regions. However, upon consideration of the functional relations (for monotremes and the background taxa) of the included characters, the relative expectations for these two factors to bias monotreme placement differs according to anatomical regions. As a summary of the above discussion, these expectations follow.

1. The monotreme mandibular, vertebral, and dental (MVD) regions display the least niche-related modification and are the most susceptible to parallelism (with background taxa) that relates to trends that were initiated before monotremes split from the mammalian backbone lineage.

2. The monotreme lower appendicular and basicranial (LB) regions are ecologically (and functionally) highly modified among monotremes. However, they are less likely than MVD, to be susceptible to parallelism (with background taxa) that relates to trends that were initiated before monotremes split from the mammalian backbone lineage. LB is also expected to be less susceptible than MVD to parallelism between the background taxa themselves.

3. The upper appendicular (U) region is also ecologically (and functionally) highly derived among monotremes, and less likely than MVD, to be susceptible to parallelism (with background taxa) that relates to trends that were initiated before monotremes split from the mammalian backbone lineage. U is distinguished from LB by a higher expectation for the included characters to be influenced by trends (towards parasagittalism in this case) among Mesozoic mammals that could result in parallelism between the background taxa, and so bias the placement of monotremes.

Considered from the perspective of the functional and developmental dissimilarity between the anatomical regions, any correlated homoplastic evolution among them might reasonably be considered as coincidence. Cautioning against this is the fact that the anatomical regions can be divided into the same partitions (MVD; LB; U) by their phylogenetic compatibility and by relative expectations for apparent phylogenetic signal to be a reflection of niche-related modification, and for parallelism to bias the placement of monotremes.

The upshot of the above expectations is that even if little or no phylogenetic signal is preserved among the dataset for placing monotremes, apparent phylogenetic signals attributed to M, D and V might be expected to broadly group together, as might those for L and B. This is not to say that this dataset does not contain signal relating to the placement of monotremes. Rather, that although MVD and LB may not be natural partitions in terms of being functional or developmental units, for monotremes at least, they may be process partitions (Miyamoto and Fitch 1995) in terms of their susceptibility to phylogenetic biases. Furthermore, there seems to be no reason why any of the anatomical regions (or meta-regions) should carry higher weighting than the others.

### 3.5 Conclusions

Perusal of recent mammalian phylogenies that are based on “whole skeletal” datasets gives the impression that monotreme affinities are locally stable, in that they branch from the mammalian backbone lineage in the vicinity of eutriconodonts. Unfortunately, this local stability does not result from phylogenetic consistency (where a single tree is converged on as sampling increases). Instead, it can be inferred from partition homogeneity testing and homoplasy significance tests that monotreme placement in the vicinity of eutriconodonts is the most parsimonious compromise among the incongruent anatomical region partitions. Only a close association with Theria cannot be rejected ( $p < 0.05$ ; K-H and Templeton tests) for the mandibular, vertebral, and dental characters. In contrast, any monotreme placements within Theriimorpha are rejected ( $p < 0.003$ ) for the upper appendicular characters. Meanwhile, intermediate affinities are favoured for the lower appendicular and basicranial characters, with both therian and non-theriimorph affinities being rejected ( $p < 0.06$ ). A number of further suggestions and conclusions may be drawn from the current study.

- The null hypothesis for the “background phylogeny” of Mesozoic generalized mammalian insectivores (Figure 3.1) that was defined by dental/mandibular characters is consistent with and in fact reinforced by basicranial and postcranial data.
- The placements among the background phylogeny of two relatively non-specialized mammals (*Gobiconodon* and *Henkelotherium*) are only locally unstable (may move to a neighbouring branch). This uncertainty may be a stochastic effect of sampling for small internodes.
- The inclusion of multituberculates and particularly monotremes with the background phylogeny taxa induces extreme incongruence between the six anatomical region partitions.
- It is speculated that the tendency for monotremes and multituberculates to be pulled into adjacent positions on the trechnothere stem upon their being included together, is a long-branch attraction effect. It results from higher levels of (unobserved) transformations occurring among these ecologically-derived taxa, relative to the rest of the tree.
- A division of anatomical regions into three meta-regions: vertebral, mandibular, and dental (MVD), lower appendicular, and basicranial (LB) and upper appendicular (U) is consistent with grouping the anatomical regions by compatibility for monotreme affinities and by relative expectations for phylogenetic biases to effect monotreme affinities.

From the highly significant incongruence that is produced by conflicting signals for widely separated placements among the background phylogeny, it may be implied that resolution of monotreme affinities is at a stalemate. The discovery of further australosphenidans that are less ecologically derived than echidnas and platypuses may resolve this, though without such fossils (especially non-dental/mandibular elements), cladistic analysis alone cannot overcome the incongruence problem. Indeed the high level of homoplasy induced by the inclusion of

monotremes may itself present problems for reconstructing phylogenies with a maximum-parsimony optimality criterion. Huelsenbeck (1995, 1998) has shown that as levels of unobserved transformation increases (as occurs with homoplasy), parsimony becomes increasingly less consistent than methods in which evolutionary processes are modelled (especially where transformation probabilities differ among lineages). This should be taken as further warning for placing trust in the placement of monotremes with the current dataset, considering that the homoplasy index increases by a factor of 2.58 upon inclusion of the monotremes with the background phylogeny.

Contending with incongruence found under parsimony essentially requires favouring one apparent phylogenetic signal over another (or others). Maximum-likelihood (ML) offers one approach, whereby modelling the evolutionary process provides a basis for attempting to tease out phylogenetic signal from homoplastic bias. Maximum-likelihood models are in their infancy with morphological data, and erroneous parameter estimates can lead ML to perform less well than parsimony approaches that do not explicitly specify a model (Steel and Penny 2000; Swofford *et al.* 2001). Nevertheless, Lewis (2001) has shown that even simple ML morphological evolution models can be useful for overcoming biases such as long-branch attraction (which is inherently problematic for MP analyses).

Greater resolution of monotreme affinities might be better served by selecting placement(s) on the basis of consistency with what can be established on the nature of the incongruence between the anatomical regions (and is explored further in Chapter 4). This study however suggests that the incongruence relating to monotreme placement largely results from homoplasy that involves transformations along the monotreme stem lineage (as opposed to hidden-homoplasy among the background phylogeny taxa). If this is the case, then minimal requirements for anatomical region-level homoplasy along the monotreme stem lineage are: convergence/parallel evolution of MVD and LB with trechnotheres, for non-theriimorph affinities; reversal of U and LB, for affinities within Trechnotheria; and convergence/parallel evolution (with cladotheres) of MVD as well as reversal of U, for placement on the trechnothere stem.

### 3.6 Addendum

While this thesis was in the final stages of production, two papers relevant to this chapter (and Chapter 4) appeared. Lou *et al.* (2002) provide a large (275 characters) dataset in order to propose a phylogeny for the major groups of Mesozoic mammals, while Rauhut *et al.* (2002) describe a Middle-Late Jurassic mammal from South America that they assign to a basal position among Australosphenida. These papers further cement monotreme relations as being nested within Australosphenida. Unfortunately their conclusions on the placement of Australosphenida among other mammals suffer from the same problems as described in this chapter for Lou *et al.* (2001a). Tachyglossids are not included, incongruence between anatomical regions is not examined, and sampling error associated with monotreme affinities is not tested. Future examination of the nature of signal conflict between anatomical regions that relates to the affinities of Australosphenida should benefit from the additional characters used by Lou *et al.* (2002) and by increased confidence in the australosphenidan affinities of monotremes.

## **Chapter 4**

### **Character-map incompatibility and correlated homoplastic evolution of monotreme upper appendicular characters**



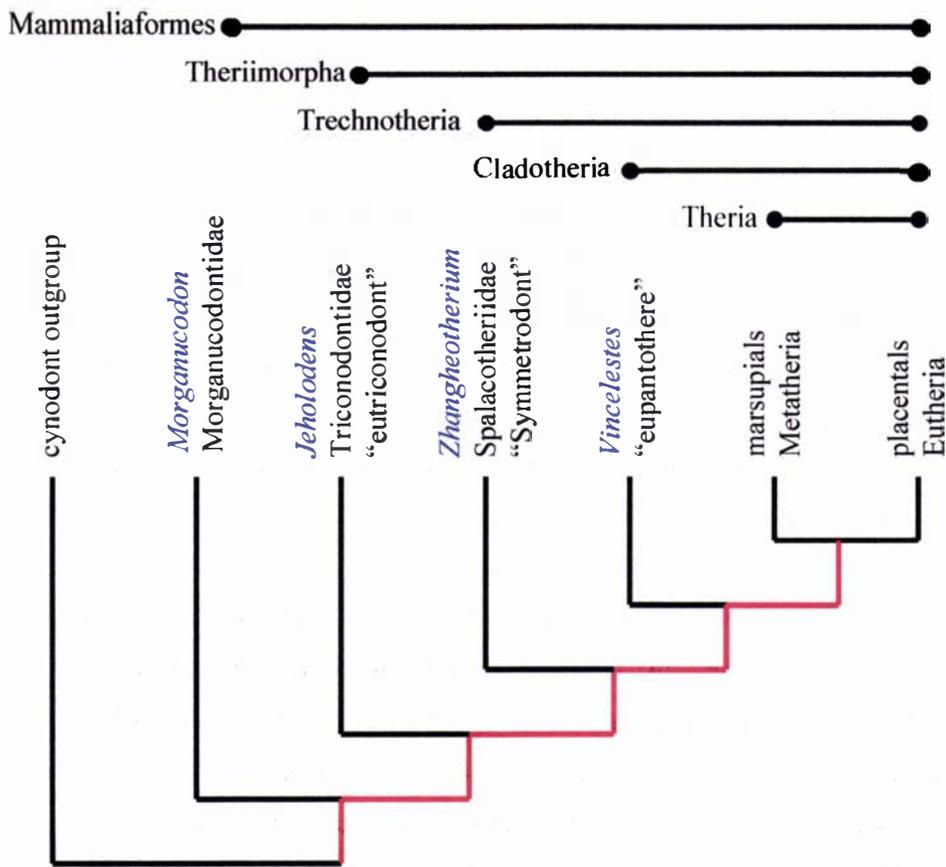
## 4.1 Introduction

### 4.1.1 *The phylogenetic affinity of monotremes*

At least since the Late Triassic, the ancestors of modern therians (marsupials and placentals) have been small terrestrial (or scansorial) forms, with niches probably ranging from those of shrews to those of small mustelids. For simplicity, I refer to these archetypal small mammalian insectivore/carnivores as generalized insectivores, on account of their having changed little from the ancestral mammalian niche. Fossils of offshoots from the mammalian backbone lineage are increasing our understanding of the patterns and processes of the morphological transformations that occurred over the ancestry of modern therians.

Reconstructions of *Morganucodon* (see Hopson and Crompton 1969; Mills 1971; Crompton and Lou 1993; Lou 1994), an insectivore from the Triassic/Jurassic boundary, have confirmed the presence of well developed squamoso-dentary articulation, an elongate and cylindrical cochlea housing, an unfused dentary symphysis allowing jaw rotation, and postcanine teeth differentiated into premolars and molars, with positional correspondence of upper and lower cusps. As such, many prerequisites for modern therian hearing and mastication had evolved before the Jurassic. Major transformations further along the mammalian backbone lineage define membership of increasingly exclusive clades that include therians (Theria: Metatheria and Eutheria). For example, numerous shoulder girdle traits that trechnotheres share with *Jeholodens* (Ji *et al.* 1999) and *Gobiconodon* (Jenkins and Schaff 1988) imply that much of the shoulder flexibility and upright (parasagittal) gait of non-specialized modern therians probably existed in the last common ancestor of trechnotheres and these eutriconodonts. Within the Trechnotheria, examination of the spalacotheriid “symmetrodont”, *Zhangheotherium* (Hu *et al.* 1997, 1998) indicates the basic patterns of modern therian basicranial and hind limb architecture evolved before this early trechnothere split from therians.

From Late Jurassic eupantotheres such as *Henkelotherium* (Krebs 1991) through to the earliest metatherians (including marsupials) and eutherians (including placentals) of the Early Cretaceous, many transformations occurred that have been suggested to be of key importance for the later diversification of therians. These include further adaptations for upright locomotion, such as reduced torsion between the proximal and distal ends of the humerus (Kielan-Jaworowska and Gambaryan 1994), cochlea coiling for improved high frequency hearing (Wible *et al.* 2001), and tribosphenic cheek teeth (for which the protocone of the upper molars occludes with the talonid of the lower molars, such that a grinding (*tribein*) function combines with the shearing (*sphen*) and puncturing function of the more anterior reverse triangle cusps: see Crompton 1971; Butler 1990).



**Figure 4.1** The phylogeny of major groups of Mesozoic generalized insectivores that is used in the CMI analysis as the background phylogeny for determining the pairwise character compatibility with respect to monotreme placement. The names of genera used are in blue, while their higher order affinities (as used in the text) are in black, including informal higher order groupings that may not be monophyletic (“ ”). The mammalian backbone lineage is denoted red and the taxonomic ranges of the nested clades are indicated by the spans above the phylogeny.

Outlining some of the steps involved in the evolution of modern therians illustrates that as whole organisms, this process was gradual, and implies that much morphological evolution occurred along the mammalian backbone lineage (denoted red in Figure 4.1) between each of the nodes of the background phylogeny.

There is considerable confidence in the relationships (Figure 4.1) of the major groups of Mesozoic generalized insectivores. This is underpinned by general phylogenetic agreement among data from different anatomical regions (see Ji *et al.* 1999; Lou *et al.* 2001a; Wang *et al.* 2001). A corollary of this is that homoplasy among the generalized insectivores can be mapped onto trees. Unfortunately, the apomorphies used to define the branching order of the generalized insectivores are not applicable to all Mesozoic mammals.

Groups that have diverged substantially from the ancestral mammalian niche appear instead as a mix of (more or less) primitive and advanced characters. Such mosaic evolution has been shown to be prominent for multituberculates (Miao 1993; Kielan-Jaworowska 1996), which were apparently largely herbivorous (Krause 1982), and for the (poorly known) docodonts (Lillegraven and Krusat 1991; Pascual *et al.* 2000), which Krusat (1991) considered to be semi-fossorial. Perhaps the most highly derived and phylogenetically controversial of all Mesozoic mammals are the aquatic and/or fossorial monotremes (Kemp 1983; Archer *et al.* 1993; Musser 1998). Kielan-Jaworowska (1996) considered that many skeletal comparisons between monotremes and other mammals (multituberculates and therians in that case) are rendered fruitless by the fossorial/swimming adaptations of echidnas and platypuses.

With molecular data, the statistical effects resulting from “long-branch” taxa with high rates of evolution, or composition heterogeneity, has drawn considerable attention (e.g. Hendy and Penny 1989; Waddell *et al.* 1999a; Phillips *et al.* 2001). Similar distortion of morphology-based trees is not as intuitive as is the case for molecular-based trees, where the presumed stochastic nature of changes among the data allows for simple statistical proofs of phylogenetic bias (Felsenstein 1978). Biases affecting the placement among conservative taxa, of highly derived (long-branch) taxa is a recurrent theme of this chapter.

It may be important to distinguish between an ecological long-branch, such as monotremes, and taxa that are often considered highly derived, but may have retained an essentially ancestral ecological niche, such as *Shuotherium* (Chow and Rich 1982; Sigogneau-Russell 1998; Wang *et al.* 1998). *Shuotherium* has a highly unusual molar design, with a talonid (or pseudotalonid) anterior, rather than posterior to the trigonid. While this has led to the dentition of *Shuotherium* being considered highly derived, it appears to be just an alternative way to “solve” the same ecological dilemma as did tribosphenic mammals, that of achieving puncturing, slicing and crushing with the same molars. There is no *a priori* reason to expect other aspects of *Shuotherium* morphology to be highly derived. In contrast, evolution is “solving” very different ecological problems for the generalized insectivores and for the fossorial/semiaquatic monotremes. Hence, any long-branch related phylogenetic biases for the placement of monotremes among the generalized insectivores might be expected to apply across many anatomical regions.

As well as the scapular and clavicle, which have been retained by most marsupials and placentals, the platypus and the echidna shoulder girdles include the procoracoid, metacoracoid and interclavicle (Klima 1973). No other mammals more closely related to therians than *Morganucodon* are known to have retained the full complement of “reptilian” shoulder elements into adulthood. Other notable upper appendicular traits of monotremes that have not advanced

beyond those of mammal-like reptiles (but have among eutriconodonts and trechnotheres), are condylar (rather than trochlear) articulation of the humerus with the ulna (Jenkins 1973), a scapular spine that forms the scapular anterior border (rather than dividing the scapular into anterior (supraspinous) and posterior (infraspinous) halves: see Jenkins and Weijs 1979; Ji *et al.* 1999). In contrast, the condition of numerous characters from other anatomical regions tend to indicate that monotremes share a closer affinity with trechnotheres than do eutriconodonts. These include the lack of a rib attached to the atlas vertebrae (Rowe 1988), a well developed patellar facet (or groove) on the femur (Szalay and Trofimov 1996; Ji *et al.* 1999), a partly coiled cochlea (Allin and Hopson 1992), and upper and lower molar “wear facets” that match upon eruption from the jaws (at least for monotremes that had retained teeth into adulthood: Archer *et al.* 1992).

Monotremes were first excluded from Theria (and indeed Trechnotheria) because of the cusp topology of the molars that briefly appear in *Ornithorhynchus* juveniles. Hopson and Crompton (1969) reasoned that it was easier to derive these unusual vestigial teeth from molars with linearly arranged cusps (such as those of *Morganucodon*), than from therian (Holotherian) teeth, which ancestrally had the three primary cusps arranged such that lines connecting them would form a triangle. The inclusion within Holotheria (crown and stem trechnotheres) of *Kuehneotherium* from the Triassic/Jurassic boundary of Wales (Kermack *et al.* 1968) shows the extent to which upper and lower molars occluding as the reverse of each others triangular cusp formation has held sway over Mesozoic mammal phylogeny reconstruction.

Like its contemporary *Morganucodon*, *Kuehneotherium* retained a jaw articulation consisting of the quadrate and articular (plus prearticular), which were later transformed into the incus and malleus middle ear bones (Goodrich 1930; Allin and Hopson 1992). Many authors (e.g. Prothero 1981; McKenna and Bell 1997; Wang *et al.* 1998; Sigogneau-Russell 2000) continue to recognize *Kuehneotherium* as a holotherian, but not eutriconodonts and multituberculates, despite these latter taxa (which lack reverse triangle occlusion) sharing the triossicular ear with trechnotheres.

A second major factor in the exclusion of monotremes from the Theria was the formation of the sidewall of the braincase. In modern therians an ascending process of the alisphenoid and a large portion from the squamosal make up the sidewall. In contrast, Watson (1916) showed that an anterior lamina of the periotic is the major contributor to the platypus braincase sidewall and that the squamosal contributes very little. Hopson (1970) and Kermack *et al.* (1981) argued that the structure of the braincase sidewall unites monotremes with multituberculates and triconodonts (such as *Morganucodon*) as the mammalian subclass Prototheria. However, Presley and Steel (1976) and Presley (1981) showed that the braincase sidewalls of modern therians and the platypus develop from a homologous ossification, with the differences being as superficial as which other

ossifications this fuses with later. Further, Griffiths (1978) showed that the development of the echidna braincase sidewall did not fit easily with the simple pattern of evolution argued for the Prototheria hypothesis. In his thoughtful discussion of monotreme affinities, Kemp (1983) argued against both the molar cusp topology and the braincase sidewall cases for Prototheria, while predicting that further discovery of Mesozoic therian and monotreme fossils would see monotremes considered as more closely related to therians than is *Kuehneotherium*.

Rather than resolve the phylogenetic position of monotremes, the discovery in Australia of an Early Cretaceous monotreme lower jaw fragment with three molars (*Steropodon galmani*; Archer *et al.* 1985), reinforced the mosaic nature of monotreme evolution. The lower molars of *Steropodon* exhibited not only the trechnothere trademark of reverse triangle main cusps, but also an expanded posterior heel (talonid) with two cusps. The expanded talonid and the morphology of the talonid cusps and crests (crisids) led to monotremes being considered as tribosphenic (Archer *et al.* 1985), or near-tribosphenic (Kielan-Jaworowska *et al.* 1987) mammals. Thus they were nested well within Trechnotheria, sharing a close relationship with marsupials and placentals. Two further Early Cretaceous monotremes have since been described from lower jaws, *Kollikodon ritchiei* (Flannery *et al.* 1995) and *Teinolophos trusleri*. The latter was initially considered to be a eupantothere (Rich *et al.* 1999), though removal of further matrix from its only *in situ* molar revealed it to be a monotreme (Rich *et al.* 2001a). Unfortunately the molars of *Teinolophos* and particularly *Kollikodon* are highly derived and lend little further information for determining the relationships of monotremes.

In Chapter 3 it was shown that the placement of monotremes on a background phylogeny of generalized insectivores (Figure 4.1) depended on which anatomical regions were examined. Of the six partitions, the upper appendicular region (U) favoured monotremes arising from the backbone lineage even earlier than *Morganucodon*, the lower appendicular (L) and basicranial (B) regions both favoured monotremes arising from the trechnothere stem-lineage (as sister to trechnotheres), and the mandibular (M), vertebral (V) and dental (D) regions each favored monotremes being more closely related to marsupials and placentals than are non-tribosphenic cladotheres such as *Henkelotherium* and *Vinceletes*. Hence, the anatomical regions could be divided into meta-regions on the basis of congruence: mandibular, vertebral and dental (MVD); lower appendicular and basicranial (LB); and upper appendicular (U).

Clearly the phylogenetic placement of monotremes that is attributable to at least two of the meta-regions must reflect a dominance of homoplasy over phylogenetic signal. Considered from the perspective of the functional and developmental dissimilarity between M, V, and D, or between L, and B, any correlated homoplastic evolution among them might reasonably be considered as

coincidence. However, in Chapter 3 it was suggested that the same meta-region partitions (MVD; LB; U) are consistent with expectations of the relative potential for apparent monotreme affinities to in fact be a reflection of (1) niche-related modification, and (2) parallelism, following trends among Mesozoic mammals.

From consideration of the functional relations (among monotremes and the background taxa) of only the characters included in the dataset the following was suggested in Chapter 3:

- The monotreme mandibular, vertebral, and dental (MVD) regions display the least niche-related modification and are the most susceptible to parallelism (with the background taxa) that relates to trends initiated before monotremes split from the mammalian backbone lineage.
- The monotreme lower appendicular and basicranial (LB) regions are ecologically (and functionally) highly modified among monotremes. However, they are less likely than MVD to be susceptible to parallelism (with the background taxa) that relates to trends that were initiated before monotremes split from the mammalian backbone lineage. LB is also expected to be less susceptible than MVD to parallelism between the background taxa themselves.
- Monotreme upper appendicular characters are also ecologically highly modified. However, U is distinguished from LB by a higher expectation for the included upper appendicular characters to be influenced by trends (towards parasagittalism in this case) among Mesozoic mammals that could result in parallelism between the background taxa, and so bias the placement of monotremes.

A consequence of the above points is that even if little or no phylogenetic signal is preserved among the dataset for the placement of monotremes, apparent phylogenetic signals attributed to M, D and V might be expected to broadly group together, as might those for L and B. This is not to say that this dataset does not contain signal (apparent phylogenetic signal, as character covariance may occur by ways other than shared genetic history) relating to the placement of monotremes. Rather, that although MVD and LB may not be natural partitions in terms of being functional or developmental units, for monotremes at least, they may be natural partitions in terms of their susceptibility to phylogenetic biases. In this sense, the meta-regions may be considered to be process partitions (Miyamoto and Fitch 1995) as groups of characters that are apparently evolving under similar evolutionary rules. Indeed, the three meta-regions also fulfill the criteria of Bull *et al.* (1993) for being considered as process partitions, simply in that they are incongruent.

The three meta-region partitions are congruent for the generalized insectivores. Incongruence was rejected ( $p > 0.9999$ ) by the partition homogeneity test (Incongruence length difference test: Farris *et al.* 1994). In contrast, extreme incongruence is indicated ( $p < 0.0001$ ) with the inclusion of

monotremes with the generalized insectivores. This level of significance is maintained for rejecting congruence when the data is partitioned into the six anatomical regions, though lumping the characters into the three meta-regions more efficiently isolates the source of the incongruence, which may help in identifying (or confirming) the mechanisms involved. Either way, the widely differing opinions in the literature on the placement of monotremes along the mammalian backbone lineage is not limited to a few isolated characters that are randomly distributed across anatomical regions. Rather, it derives from sets of characters.

Departing for a moment from the question of monotreme placement on the background phylogeny, a close relationship with multituberculates has often been suggested for monotremes (e.g. Broom 1914; Kermack and Kielan-Jaworowska 1971; Hopson and Rougier 1993; Meng and Wyss 1995; Wang *et al.* 2001). This relationship has largely been based on the ossification of the braincase sidewall (as discussed earlier) and the structure of the hearing apparatus. However, the similarities between the two taxa have been argued as plesiomorphic or convergently adapted for fossorial habits (see Griffiths 1978; Miao 1993). Furthermore, postcranial anatomy does not support a close relationship between monotremes and multituberculates (Rowe 1988; Sereno and McKenna 1995).

A new hypothesis (Lou *et al.* 2001a) proposes that monotremes are highly derived members of a Gondwanan radiation of mammals with tribosphenic molars, that also includes *Ausktribosphenos* (Rich *et al.* 1997) from the Early Cretaceous of Australia, and the Middle Jurassic aged *Ambondro* (Flynn *et al.* 1999) from Madagascar. Lou *et al.* (2001a) named this clade Australosphenida (defined by a number of dental synapomorphies), which their cladistic analysis found to be widely excluded from the Laurasian tribosphenic mammals (Boreosphenida), which include therians and close relatives such as *Kielantherium*. Chapter 3 suggests the conclusion that these two groups of tribosphenic mammals are diphyletic is premature. Regardless of whether or not monotremes are closely related to *Ausktribosphenos* and *Ambondro*, neither is known from non-mandibular/dental material and so they are not included in this examination of conflict among anatomical regions.

The focus for this chapter is the placement of monotremes with respect to the background phylogeny of generalized insectivores (Figure 4.1). Multituberculates were not included with the background phylogeny because of the added phylogenetic uncertainty and possible character state transformation errors their inclusion induces. The placement of multituberculates the background phylogeny is highly controversial (Rowe 1988; Miao 1993; Kielan-Jaworowska 1996; Ji *et al.* 1999). Further, in Chapter 3 it was shown that monotremes and multituberculates distort each others phylogenetic signal with respect to the generalized insectivores, possibly as a form of long-branch attraction. An additional reason to include only generalized insectivores in the background phylogeny, is that homoplasy among that tree cannot be attributed to major ecological-niche shifts.

Hence for examining the effect of niche shifts on homoplasy, the background phylogeny might be viewed as a control for the subsequent inclusion of monotremes. Further, if multituberculates were included, the uncertainty of their position would leave the background phylogeny unresolved, reducing the resolution of the character-map incompatibility analysis (see section 4.1.2).

Placing limits on the possible affinities of monotremes with the background phylogeny would be useful for understanding both Mesozoic mammal biogeography and the evolution of the many highly autapomorphic characters of monotremes. However, little confidence can be placed in any hypothesis of monotreme affinities, which does not also explain the incongruence (between anatomical regions) induced by their inclusion with the background phylogeny. The partition homogeneity test (PHT) provides little insight into the nature of incongruence. It simply indicates that conflict among signals attributable to different partitions is greater than expected from stochastic variation alone. The PHT does not directly examine the relationship between signal and “noise” among characters within the individual partitions. Further, the PHT does not indicate the topological nature of the incongruence induced upon including monotremes with the generalized insectivores. In order to remedy these shortfalls of the PHT, I develop in this chapter an analysis to test for and quantify signal (and conflict) that relates to the placement of a “problem” taxon, on a background phylogeny, both within and between partitions.

#### 4.1.2 *Pairwise character incompatibility analysis*

A number of methods have been developed to measure, or infer the presence of signal above noise. Such methods for inferring signal include permutation tail probability (PTP) tests (Archie 1989a; Faith and Cranston 1991), tree-length distribution (TLD) central moments (Hillis 1991; Huelsenbeck 1991) and more recently, relative apparent synapomorphy analysis (RASA: Lyons-Weiler *et al.* 1996). These methods determine apparent phylogenetic signal over entire data matrices. As such, examining the signal associated with the relationship of a specific taxon (such as monotremes) to a background phylogeny is problematic. Fu and Murphy (1999) used taxon pruning with PTP tests to locate character covariance on trees. However, such tests only associate apparent phylogenetic signal to a specific clade by its exclusion rendering the signal among the remnant taxa as insignificant. If, for example, signal was shown to be highly significant with monotremes included and excluded, then little useful information is yielded on the apparent phylogenetic signal attributable to monotreme placement.

As with the tests for signal (apparent phylogenetic signal), tests for congruence among data partitions are useful over entire datasets, but limited in their applicability for relating conflicting signals to a specific taxon or clade. The partition homogeneity test (PHT), first defined by Farris *et*

*al.* (1994) has become standard for testing congruence between data subsets. The shortest tree is calculated for each pre-defined data subset and the lengths summed. This value is compared with the summed length of trees calculated from randomly sampled partitions, each including the same number of characters as the pre-defined subsets. The more strongly the pre-defined subsets support different trees, the more homoplasy the overall dataset must contain and hence, the shorter the sum of the tree lengths will be, compared to those of the randomly sampled partitions.

Like PTP tests and TLD central moments, the PHT sums across all taxon/character state interactions. This problem may be avoided if all relationships are known but for that of the taxon of interest, by constraining the phylogeny for all other relationships. However, if congruence cannot be rejected, the PHT is unable to distinguish whether the congruence is attributable to noise or signal homogeneity. Further, the PHT cannot establish whether the individual partitions themselves contain signal (above noise). Because determining compatibility among character pairs allows for variation in signal to be examined within datasets or partitions (e.g. Gower and Sennikov 1996; O'Keefe and Wagner 2001), it follows that utilizing character compatibility allows congruence between partitions and signal within partitions to be examined simultaneously.

Le Quesne (1969; 1972) showed that among random data, the probability of a character being incompatible with another depends on the numerical distribution of character states among taxa. For example, with 10 taxa, a character (A) with two states split 5 versus 5 among taxa, is more likely to be incompatible with a random character, than is a character (B) with two states split 8 versus 2 among taxa. For character B, another character need only be compatible with monophyly of the 2 taxa, while for character A, another character must not conflict with the monophyly of at least one of the groups of 5 taxa. This observation that the numerical distribution of character states effects the probability of character compatibility led Salisbury (1999) to suggest that "a character that is compatible with a phylogenetic hypothesis supports the tree only to the degree at which this compatibility would be improbable under a model of cladistic dissociation between character state distribution and the tree". Similar logic has provided the basis for numerous phylogenetic applications of character compatibility. These have included providing optimality criteria for selecting from equally parsimonious trees (Rodrigo 1992), character weighting (Gauld and Underwood 1986, Wilkinson 1994) and character exclusion (Meacham 1994; Sharkey 1994).

Determining expected levels of incompatibility given the distribution of character states among taxa has allowed for testing the null hypothesis that characters or data matrices are random, or uninformative with respect to phylogeny (Le Quesne 1989; Alroy 1994; Meacham 1994; Gower and Sennikov 1996; Day *et al.* 1998). These tests essentially compare counts of pairwise incompatibility (or compatibility) with an expected value, generated using knowledge only of the

numerical distribution of character states among taxa. As an example, assume characters A and B each have two taxa with state “0” and five taxa with state “1” and that for each character, these states are randomly distributed across the seven taxa. The combinatorial probability of characters A and B being incompatible is 0.4762. Meacham (1981) provides formulae for determining the probability of incompatibility for data randomized across taxa, as above. However, analogous to PTP, RASA, TLD central moments and PHT, these methods examine (in)compatibility across entire data matrices. In this study I describe an analysis of pairwise character incompatibility that uses character mapping. This method, which extends existing techniques allows the relative strength and significance of signal within and between (congruence) data partitions to be examined with respect to a specific taxon on a background phylogeny.

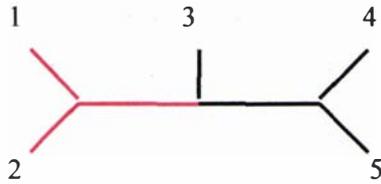
In the character-map incompatibility (CMI) analysis of this study, for each character, the equally most-parsimonious positions of the taxon of interest are mapped onto the background phylogeny to determine pairwise compatibility among characters. The background phylogeny need not be fully resolved, though similar to increasing taxon sampling, the fewer unresolved nodes, the more precise will be the count of pairwise character incompatibility. The use of a background phylogeny allows CMI analysis to measure incompatibility with respect to specific taxa. Further, character-mapping allows CMI analysis to incorporate information about the relative topological association of most-parsimonious placements of the taxon of interest on the background phylogeny.

For characters with little homoplasy, states tend to be clumped together on the background phylogeny. Clumped character states provide connected character-maps (Figure 4.2a), as opposed to non-connected (or compound) character-maps (Figure 4.2b). Characters with states that are clumped will tend to have a greater probability of being incompatible than characters with states that are dispersed over the background phylogeny (as results from homoplasy).

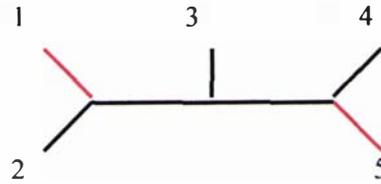
The model used in this study for generating expected incompatibility tests the relative topological association on a specified tree of the most-parsimonious positions of the taxon of interest for each character. In this study the specified tree is the background phylogeny and Monotremata is the taxon of interest. The sub-tree made up of the branches that are equally most-parsimonious positions for monotremes on the background phylogeny is said to be the monotreme character-map.

Character-mapping allows the integration of information from both the numerical distribution of character states across taxa, and the clumping of those states on the background phylogeny. For example, consider the 5-taxon unrooted phylogenies below, onto which hypothetical monotreme character-maps are overlaid (in red). The character-map includes the branches of the background phylogeny on which monotremes could most parsimoniously have arisen for two characters (A, B).

(A.) connected character-map



(B.) compound character-map



**Figure 4.2** Hypothetical character-maps (in red) for Monotremata, superimposed onto a 5-taxon background phylogeny. The equally most-parsimonious placements are adjacent for character (A), conferring a connected character-map, but are non-connected for character (B), conferring a compound character map

If the monotreme character state is shared by two background taxa and the background phylogeny contains no homoplasy for that character, the monotreme character-map must be connected and monophyletic (at least with respect to the unrooted tree). On a 5-taxon tree, two such two-taxon, monophyletic character-maps are possible, and character-map A represents one of these. In contrast, character B is represented by a two-taxon, non-connected (compound) character-map. There are four possible character-maps of this type on a five-taxon phylogeny. Note that for a five-taxon phylogeny, a two-taxon, compound character-map cannot include the external branch leading to taxon 3, because if taxon 3 and any other taxon share the monotreme character state, the internode connecting them and the other external branch connected to this, would also be equally most-parsimonious monotreme placements, so a three-taxon connected character-map would result.

The probability of two randomly selected two-taxon, monophyletic character-maps being incompatible is  $1/2$ , while the probability of two randomly selected two-taxon, non-connected character-maps being incompatible is  $1/4$ . Hence, incorporating the relative topological association of most-parsimonious positions can make a considerable difference for determining expected CMI values even for a phylogeny with few taxa. This is likely to be important, because unless homoplasy among the background phylogeny is very high, character states will not be randomly distributed across taxa as has been assumed for previous incompatibility analyses (e.g. Meacham 1994; Wilkinson and Nussbaum 1996).

The character-map incompatibility (CMI) analysis of this study examines character incompatibility within and between anatomical region partitions (and meta-region “process partitions”) for the placement of monotremes on the background phylogeny of generalized insectivores. These analyses provide quantitative (relative) measures of the strength of apparent phylogenetic signal and conflict, both within and between data partitions, allowing assessment of the evolutionary independence of characters and the significance of anatomical region incongruence. The CMI

results are discussed with respect to monotreme evolutionary ecology and possible phylogenetic biases (particularly long-branch outgroup-attraction) that result from being ecologically (and functionally) highly derived. Examining the evolution of monotreme upper appendicular characters receives particular attention and a framework for assessing monotreme affinities in terms of homoplasy options is presented.

## 4.2 Methods

### 4.2.1 Data

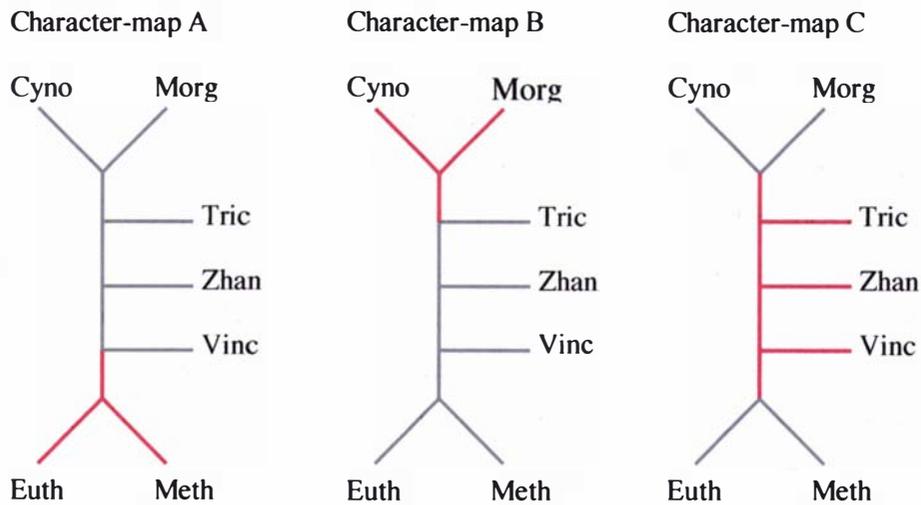
Both the cladistic and CMI analyses used the 91-character dataset from Chapter 3 (see Appendix D). The seven generalized insectivore taxa are used as a background phylogeny that has 11 branches (external and internal) from which monotremes potentially arise (see Figure 4.4). Numerous recent studies (Rowe 1993; Hu *et al.* 1997; Ji *et al.* 1999) have found the relationships among the groups represented in the background phylogeny to be well resolved. This background phylogeny includes: the Early Jurassic “Triconodont”, *Morganucodon*, the Early Cretaceous eutriconodont, *Jeholodens*, the Early Cretaceous “symmetrodont”, *Zhangheotherium*, the Early Cretaceous eupantothere, *Vincelestes*, marsupials, and placentals, together with an outgroup comprising cynodont (mammal-like reptiles) characters. Each of the clades that define the background phylogeny (Figure 4.1) was shown in Chapter 3 to retain high bootstrap support, with or without, the dental and mandibular characters that they were initially defined by.

*Zhangheotherium* is the sister taxon of the spalacotheriid symmetrodonts (Hu *et al.* 1998; Cifelli and Madsen 1999) and likewise, *Jeholodens* is sister to the triconodontid eutriconodonts (Ji *et al.* 1999; Lou *et al.* 2001a; Wang *et al.* 2001). For both of these near-complete specimens, the authors are rather non-committal on inclusion or exclusion from the above-noted families. For simplicity I will refer to *Zhangheotherium* as a spalacotheriid and to *Jeholodens* as a triconodontid. In fact, the 4 unknown basicranial characters of *Jeholodens* were replaced by the states that Rougier *et al.* (1996) allotted to (and are conserved across) the triconodontid eutriconodonts: *Trioracodon*, *Triconodon* and *Priacodon*. Allotting the 4 triconodontid characters to *Jeholodens* means this OTU (operational taxonomic unit) is in fact a composite that potentially offers a better estimate of triconodontid affinities than does *Jeholodens* alone. Nevertheless, referring to this OTU as *Jeholodens* is perhaps the most informative option, as the vast majority of characters describe this taxon.

As with Chapter 3, but unlike Ji *et al.* (1999), which included only the extant ornithorhynchid (*Ornithorhynchus anatinus*: platypus), I have included the second extant monotreme family (Tachyglossidae: echidnas). As tachyglossids lack teeth and only vestigial juvenile teeth are retained by the platypus, monotreme dental characters are represented by those discernible from the extinct Early Cretaceous monotreme, *Steropodon galmani* (Archer *et al.* 1985), or if not discernible in that, then the Oligocene/Miocene platypus genus, *Obdurodon* (Archer *et al.* 1992, 1993; Musser and Archer 1998). This increases the number of dental characters applicable to monotremes from 5 to 16 (of 21). These characters are attributed to an OTU I refer to as Ornithorhynchidae. Molecular dating studies (Retief *et al.* 1993; Gemmell and Westerman 1994; Messer *et al.* 1998; Janke *et al.* 2002) clearly show that the Early Cretaceous *Steropodon* diverged from *Ornithorhynchus* before ornithorhynchids and tachyglossids split. However, allowing ornithorhynchid dental characters to be represented by *Steropodon* simply defines the best current estimate of the plesiomorphic monotreme dental condition within the context of the current study, as all the corresponding tachyglossid characters are defined as unknown.

Monotreme and marsupial specimens from the Australian Museum (AM) and the Queensland Museum (QM) were used for all anatomical examinations, including checking character states, and as reference material for the photographs and drawings. These specimen numbers were: platypus (*Ornithorhynchus anatinus*) JM6680 (QM), JM7014 (QM), JM9343 (QM), M8625 (AM), M26638 (AM); short-beaked echidna (*Tachyglossus aculeatus*) JM2276 (QM), JM7043 (QM); long-beaked echidna (*Zaglossus bruijni bartoni*) M8263 (AM); Spotted-tailed quoll (*Dasyurus maculatus*) JM9751 (QM); long-nosed bandicoot (*Perameles nasuta*) JM790 (QM); and northern hairy-nosed wombat (*Lasiorhinus krefftii*) JM7480 (QM). Echidna basicranial characters were also checked with Rougier *et al.* (1996).

The 91 characters are assigned to 6 anatomical region subsets: vertebral (8); mandibular (9); upper appendicular (22); lower appendicular (20); dental (21); and basicranial (11). For most analyses data is analyzed as the three meta-region (process) partitions, MVD, LB and U, where only the upper appendicular (U) region is treated in isolation. The small number of characters, particularly for the vertebral, mandibular and basicranial regions, results in a lack of statistical power for the CMI analyses. Lumping the six regions into the three meta-regions largely overcomes this, and also is useful for further isolating the source of incongruence relating to the placement of monotremes. Additionally, analysis utilizing the meta-regions allows further examination of the suggestion (Chapter 3) that MVD and LB (although not natural partitions in terms of being functional or developmental units) may for monotreme affinities be natural partitions in terms of their susceptibility to phylogenetic biases.



**Figure 4.4** Character-map (in)compatibility across the background phylogeny. Monotreme most-parsimonious placements are indicated in red for character maps A, B, and C. Note that character maps A and B are incompatible as they share none of the background phylogeny branches. However, character map C is compatible with both A and B. Taxon abbreviations are: Cyno (Cynodont); Morg (*Morganucodon*); Tric (Triciconodontidae); Zhan (*Zhangheotherium*); Vinc (*Vincelestes*); Meth (Metatheria); and Euth (Eutheria).

#### 4.2.2 Cladistic analysis

Maximum-parsimony analysis was conducted using PAUP 4.0b8 (Swofford 1998). All analyses were “standard parsimony”, with characters being unordered and equally weighted. Multistate characters were treated as polymorphic. Most-parsimonious trees were found via PAUP\* exhaustive searches. All bootstrap analyses were carried out using 1000 heuristic search replications. MacClade 3.05 (Maddison and Maddison 1995) was used to find tree length and indices of homoplasy for each of the 11 alternative monotreme placements among the background phylogeny. The partition-homogeneity test (within PAUP\* 4.0b8), with 10000 branch-and-bound search replications was used to test congruence among the anatomical region (or meta-region) partitions.

Monotreme monophyly is supported for the dataset as a whole in 71 % of bootstrap replicates, though echidnas being more closely related to trechnotheres than platypuses saves 1 tree step by comparison with monotreme monophyly. Of the six anatomical regions, only the basicranial partition is not compatible with monotreme monophyly, though the level of bootstrap support for this differs for each anatomical region. These differences affect support for other relationships, including monotreme affinities with background taxa. As such, a sister group relationship between

platypuses and echidnas was constrained for individual anatomical region (and meta-region) analyses in order to eliminate this source of error (The unconstrained values are given in the figure captions where applicable). That echidnas and platypuses are sister taxa with respect to other taxa in this study is unchallenged by morphological studies. Furthermore, analyses of DNA sequences (e.g. Retief *et al.* 1993; Gemmell and Westerman 1994; Messer *et al.* 1998; Janke *et al.* 2002; and see Chapter 2) suggest that the platypus and echidna lineages diverged during the early to mid Tertiary.

#### 4.2.3 Pairwise character-map incompatibility

For a specific taxon of interest (monotremes in this case), a character-map (the set of most-parsimonious positions on a background phylogeny) is determined for each character. Figure 4.4 shows character-maps for three monotreme characters on the background. With seven background taxa there are eleven positions (internal and external branches) with which monotremes may associate on the unrooted tree. If the set of most-parsimonious positions do not intersect for two characters, these characters are incompatible for monotreme placement with respect to the background phylogeny. As such, from Figure 4.4, characters A and B are incompatible, while characters A and B are both compatible with character C.

Observed character-map incompatibility ( $CMI_O$ ) is simply the proportion of character pairs for which character-maps are incompatible.  $CMI_O$  was determined for all pairs of characters, both within and between anatomical region (and meta-region) partitions. The total number of pairwise character-map comparisons within regions is  $n(n-1)/2$ , where  $n$  is the number of characters the region includes. For example, with 21 characters, 210 pairwise character-map comparisons may be made among dental (D) characters (all possible pair combinations among the 21 dental characters).  $CMI_O$  for pairwise comparison within regions is denoted,  $CMI_O[\text{region}]$ , such as  $CMI_O[D]$ . The total number of pairwise comparisons between regions is  $n_1*n_2$ , where  $n_1$  and  $n_2$  are the number of characters included in two different anatomical regions. For example, between the dental region (21 characters) and upper appendicular region (22 characters) there are 462 pairwise character-map comparisons (all pair combinations where one of the 21 dental characters is one member of a pair, and one of the 22 upper appendicular characters is the other member of the pair).  $CMI_O$  for pairwise comparison between regions is denoted,  $CMI_O[\text{region1}*\text{region2}]$ , such as  $CMI_O[U*D]$ .

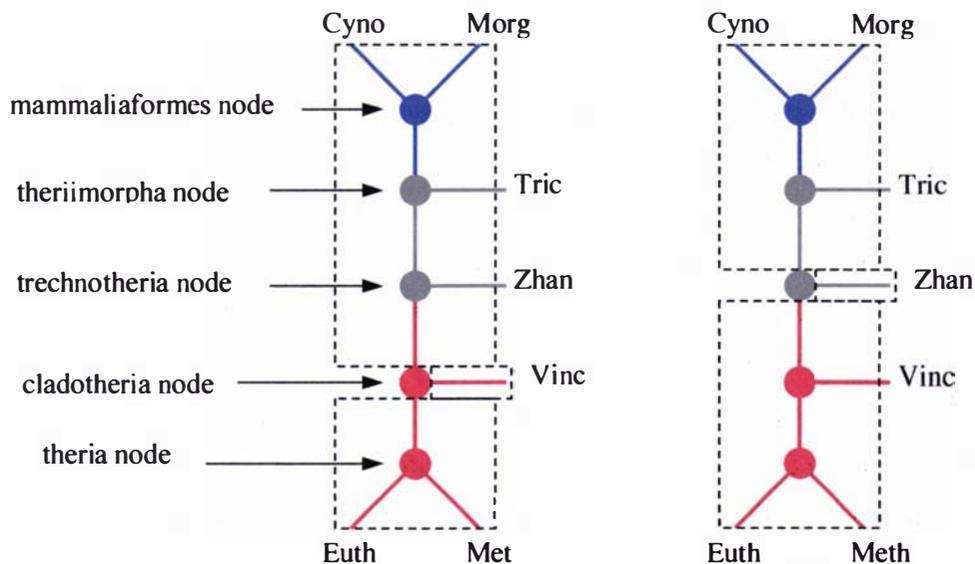
The need to examine the distribution of incompatibility across the tree is addressed by measuring character-map incompatibility across internal nodes. Character-maps may be incompatible with respect to only a subset of the background phylogeny nodes. For a bifurcating tree, any node is the intersection of three branches. As such, each node represents the last common ancestor of three

(potential) clades on an unrooted tree. In Figure 4.5, character-maps for hypothetical characters A (blue) and B (red) are shown on the same unrooted trees. At least one position from both character-maps is included within one of the three clades defined by the cladotheria node. Hence, although character-maps A and B are incompatible with respect to the background phylogeny overall, they are not incompatible across the cladotheria node. In contrast, character-maps A and B are incompatible across the trechnotheria node as character-maps A and B are wholly contained within different clades of the three clades defined by the trechnotheria node. Similarly, characters A and B are also incompatible across the theriiomorpha node, but are not incompatible across the mammaliaformes and trechnotheria nodes.

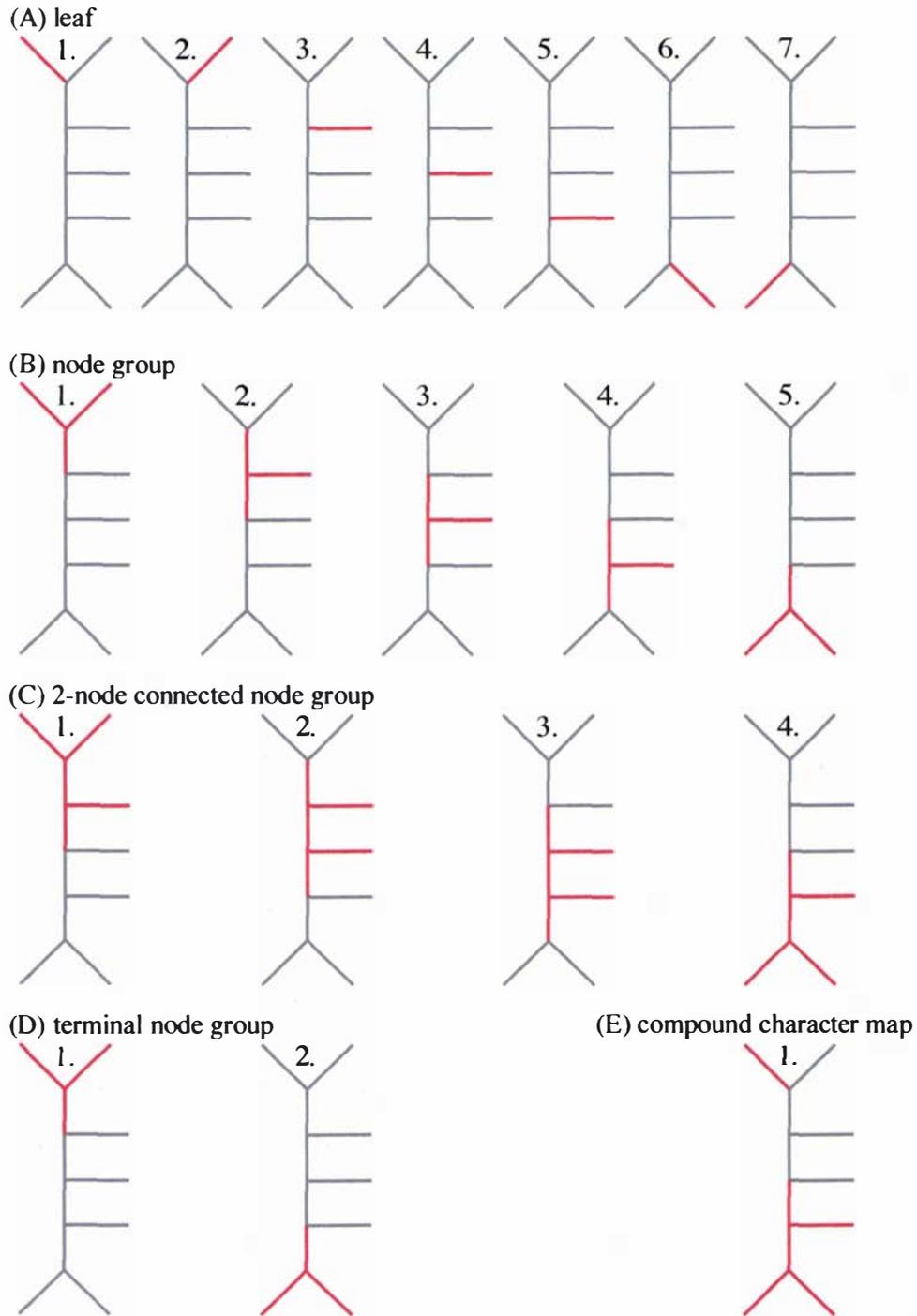
Note that where a clade name is used to define a node (as the last common ancestor of its members), the first letter is treated as lower case (e.g. trechnotheria node), as opposed to upper case, which is reserved for the taxon name (e.g. Trechnotheria). Additionally, incompatibility (or related statistics) over the background phylogeny refers to the whole topology, unless otherwise stated, in which case, nodes will be referred to (e.g. incompatibility across the cladotheria node).

(1) The cladotheria node defines three potential clades on the unrooted tree

(2) The trechnotheria node defines three potential clades on the unrooted tree



**Figure 4.5** Character-map (in)compatibility across specific nodes of the background phylogeny for pairwise comparison of two hypothetical monotreme characters (A: blue, and B: red). Rectangles represent three potential clades defined by the cladotheria node (1), and the trechnotheria node (2). The red and blue character maps are incompatible (as they share no most-parsimonious placements). However, both character maps (red and blue) have at least one most-parsimonious position contained within one of the potential clades defined by the cladotheria node. As such the characters are not incompatible across cladotheria. In contrast, the red and blue character maps are wholly contained by different potential clades that are defined by the trechnotheria node, so are incompatible across trechnotheria. Taxon abbreviations are: Cyno (Cynodont); Morg (*Morganucodon*); Tric (Triciconodontidae); Zhan (*Zhangheotherium*); Vinc (*Vincelestes*); Meth (Metatheria); and Euth (Eutheria).



**Figure 4.3** Character-map categories as most-parsimonious monotreme placements (in red) on the unrooted background phylogeny. Taxon labels are missing but are as for Figure 4.4. See section 4.2.4 for explanation of each of the categories (A-E). Note that the compound character map (E.) combines a leaf and a 2-node connected node group. The numbers correspond to the different topological permutations of each category. As the unrooted background phylogeny is symmetrical, permutations such as A.3 and A.5 have the same symmetry relation, but must be differentiated as they correspond to terminal branches that lead to different taxa (and also in this case are not compatible).

#### 4.2.4 *Expected pairwise character-map incompatibility*

The number (a) and relative topological association (b) of the equally most-parsimonious positions of the taxon of interest on the background phylogeny, as well the number of tree steps required (c) are used to parameterize models for determining expected character-map incompatibility (CMI<sub>E</sub>).

The latter two parameters determine five basic character-map categories for monotreme characters: leaf, node-group, connected node-groups, terminal node-groups and compound character-maps.

- **Leaf:** The most parsimonious monotreme position on the background phylogeny being with just one taxon (or leaf) of the background phylogeny. With seven background taxa, there are seven possible monotreme leaf character-maps, as shown in Figure 4.3A.
- **Node-group:** For a single unordered character, it is not possible for equally most-parsimonious monotreme positions to occur on two branches attached to a node, without the third branch attached to that node also being an equally most-parsimonious monotreme position on the background phylogeny. So equally most-parsimonious character-map positions will be arranged into node-groups (Figure 4.3B). For any unrooted background phylogeny, the number of potential node-group positions for a character is two less than the number of taxa. As such there are five monotreme character-map possibilities for single node-groups.
- **Connected node-groups:** A character-map is considered a connected node-group if it includes equally most-parsimonious monotreme positions distributed over adjacent (connected) node-groups on the background phylogeny. These positions will include each branch attached to each node within the connected node-group. The number of potential character-map possibilities for connected node-groups on the background phylogeny depends on the number of connected nodes. As the background phylogeny includes five nodes, connected node-group character-maps may include 2 – 5 nodes. With two-node groups connected, for example, there are four monotreme character-map possibilities (Figure 4.3C).
- **Terminal node-groups:** If the most-parsimonious tree(s) including monotremes and the background phylogeny require only one tree step for a character, either the monotreme character-map, or the remaining taxa of the background phylogeny must be monophyletic. As such, if the monotreme character-map is either a single node-group, or a connected node-group, it must include a terminal node (see Figure 4.3D). If more than one tree step is required for any topology containing the background phylogeny and monotremes, monophyly of the character-map (for the unrooted topology) is not required, and so node-groups need not include a terminal node.

- **Compound character-maps:** If equally most-parsimonious monotreme positions on the background phylogeny are not connected, the character is defined as a compound of the minimum number of connected character groups present. Figure 4.3E shows an example of a character-map that is a compound of a two-node connected node-group and a leaf. Compound characters do not occur in this dataset, indicating that convergences/reversals/parallelisms among the background taxa are not widely separated, at least for states shared with monotremes.

Table 4.1 shows the combinatorial probability of monotreme character-maps being incompatible, given random shuffling of each character-map category over the background phylogeny, for each character in pairwise comparisons. The workings (and equations) for these expected incompatibility data are provided in Appendix E. Figure 4.3 can be used to provide a simple example. Note that the taxon labels are missing from the unrooted trees of Figure 4.3, but that these would be the same as for Figure 4.4. The character-maps, (most-parsimonious monotreme placements) are shown in red in Figure 4.3. The monotreme character-map for the condition of the crista interfenestralis (ch. 61) corresponds to tree C.1 in Figure 4.3. This is a 2-node connected node-group (for which there are four permutations: C.1-4). The monotreme character-map for procoracoid presence versus absence (ch. 18) corresponds to tree D.1. This is a single-node, terminal node-group (for which there are two permutations: D.1-2). The character-maps C.1 and D.1 are compatible. As these two character-maps are randomly shuffled over the background phylogeny, the proportion of incompatible pairings will converge on the combinatorial probability of pairwise incompatibility for the respective character-map categories of these two characters (0.5). This is the proportion of incompatible pairs (4) from among the eight pairwise combinations of the four permutations of the 2-node connected node-group, with the two permutations of the single node, terminal node-group. The four incompatible pairs are (C.1, D.2), (C.2, D.2), (C.3, D.1), and (C.4, D.1).

As for the above example that used the character-maps in Figure 4.3, expected pairwise character-map incompatibility ( $CMI_E$ ) is determined under the assumption that monotreme character-maps are randomly distributed over the background phylogeny. Hence,  $CMI_E$  is the sum of the combinatorial probabilities of incompatibility for each pairwise comparison of characters, given the character-map category of each member of the character pairs. In this way  $CMI_E$  values were determined among the characters, both within and between each of the anatomical region partitions. Monotreme phylogenetic signal will result in monotreme character-maps being non-randomly distributed over the background phylogeny and hence lower observed than expected incompatibility. The z-ratio binomial probability two-tailed test (see Steel and Torrie 1980) was used to determine the significance of differences between observed character-map incompatibility

(CMI<sub>O</sub>) and the character-map incompatibility that is expected (CMI<sub>E</sub>) under random shuffling of character-maps (RSCM) over the background phylogeny.

**Table 4.1** Combinatorial probability of pairwise incompatibility for character map category combinations (see section 4.2.4 for category definitions). Terminal node-group character maps [indicated by (term)] are differentiated from standard node-group character maps by the requirement to include a terminal node. For each probability entry, the denominator represents the number of topographic combinations on the background phylogeny possible for the given character map category pair. The numerator is the number of these that are incompatible. As the unrooted background phylogeny is symmetrical, the theriimorpha and cladotheria nodes have the same combinatorial probability of pairwise incompatibility, as do the mammaliaformes and theria nodes. All character map category pairwise combinations not included (e.g. 3 node \* 4 node) have a pairwise incompatibility of zero with respect to the generalized mammal background phylogeny.

Character map category pairwise comparison	Combinatorial probability of pairwise incompatibility			
	Entire background phylogeny	trechnotheria node	theriimorpha and cladotheria nodes	mammaliaformes and theria nodes
leaf * leaf	42/49	30/49	28/49	22/49
leaf * node (term)	10/14	8/14	8/14	2/14
leaf * 2 node (term)	8/14	8/14	3/14	2/14
leaf * 3 node (term)	6/14	0/14	3/14	2/14
leaf * 4 node (term)	4/14	0/14	0/14	2/14
leaf * node	28/35	16/35	14/35	8/35
leaf * 2 node	18/28	8/28	6/28	6/28
leaf * 3 node	10/21	0/21	3/21	4/21
leaf * 4 node	4/14	0/14	0/14	2/14
node (term)* node (term)	2/4	2/4	2/4	0/4
node (term)* 2node (term)	2/4	2/4	1/4	0/4
node (term)* 3node (term)	2/4	0/4	1/4	0/4
node (term)* node	6/10	4/10	4/10	0/10
node (term)* 2node	4/8	2/8	2/8	0/8
node (term)* 3node	2/6	0/6	1/6	0/6
2 node (term)* 2 node (term)	2/4	2/4	0/4	0/4
2 node (term)* node	4/10	4/10	1/10	0/10
2 node (term)* 2 node	2/8	2/8	0/8	0/8
3 node (term)* node	2/10	0/10	1/10	0/10
node * node	12/25	8/25	6/25	0/25
node * 2 node	6/20	4/20	2/20	0/20
node * 3 node	2/15	0/15	1/15	0/15
2 node * 2 node	2/16	2/16	0/16	0/16

Relative character-map incompatibility (CMI<sub>R</sub>) for monotremes, with respect to the background phylogeny, is the relative deviation of the observed character-map incompatibility from the expected character-map incompatibility.

$$CMI_R = (CMI_O - CMI_E) / CMI_E$$

For the following results and discussion, significant (or insignificant) differences between  $CMI_O$  and  $CMI_E$  are reported as significant (or insignificant)  $CMI_R$  deviations (from zero), where a positive value indicates conflict, and a negative value indicates signal homogeneity.

### 4.3 Results and Discussion

#### 4.3.1 *Phylogenetic signal and conflict within and between anatomical meta-region partitions*

Observed ( $CMI_O$ ), expected ( $CMI_E$ ), and relative ( $CMI_R = \{CMI_O - CMI_E\} / CMI_E$ ) incompatibility values are shown in Table 4.2 for the three meta-region partitions and provided in Appendix F for the six individual anatomical region partitions. Of the 4095 pairwise comparisons among all 91 characters in the dataset, 494 are incompatible over the background phylogeny. This compares with an expected value of 425.826, such that  $CMI_R$  is +0.160. This difference between observed and expected incompatibility is significant at  $p=0.0005$  (two-tailed test), given a normal approximation of a binomial error distribution. Further  $p$ -values associated with CMI are similarly defined.

Incompatibility studies typically expect that comparison of observed and expected incompatibility will either indicate an absence of phylogenetic signal ( $CMI_O \approx CMI_E$ ), or a significant apparent phylogenetic signal ( $CMI_O < CMI_E$ ). In contrast, the significant phylogenetic conflict ( $CMI_O > CMI_E$ ) over all pairwise character comparisons indicates that incongruence dominates any phylogenetic signal for monotreme placement. Day *et al.* (1998) considered greater than expected incompatibility to be consistent with genetic recombination, such as occurs with hybridization, or meiosis. Such mechanisms are of limited explanatory value for the current study, with the background taxa being distantly related (above species, and indeed family level). Hence, explaining the significant phylogenetic conflict among characters for monotreme placement requires other mechanisms, such as reversals, parallelisms, or convergences that are correlated between many characters. It is important to test whether correlation of homoplastic evolution among characters can be isolated with respect to the anatomical region (or meta-region) partitions.

Pairwise comparisons may be divided into those within partitions, where both members of character pairs are from the same partition, and those between partitions, where the members of character pairs are from different partitions. For the six regions, the sum of the within-region pairwise comparisons is 750, and with these regions lumped into the three meta-regions this number increases to 1399. This increase is due to within meta-region comparisons including the 750 within anatomical region comparisons, as well as the comparisons between the anatomical

regions that are lumped into the same meta-region. For example  $CMI_O[LB] = CMI_O[L] + CMI_O[B] + CMI_O[L*B]$ .

**Table 4.2** Relative CMI ( $CMI_R = (CMI_O - CMI_E) / CMI_E$ ) within and between the three anatomical meta-regions (U; LB; MVD) over the entire background phylogeny and across the following nodes: mammaliaformes; theriimorpha; trechnotheria; and cladotheria. Note that n = the number of pairwise character comparisons. p-values indicate the level of significance for the difference between  $CMI_O$  and  $CMI_E$  according to the z-ratio binomial probability test (for n pairwise comparisons).

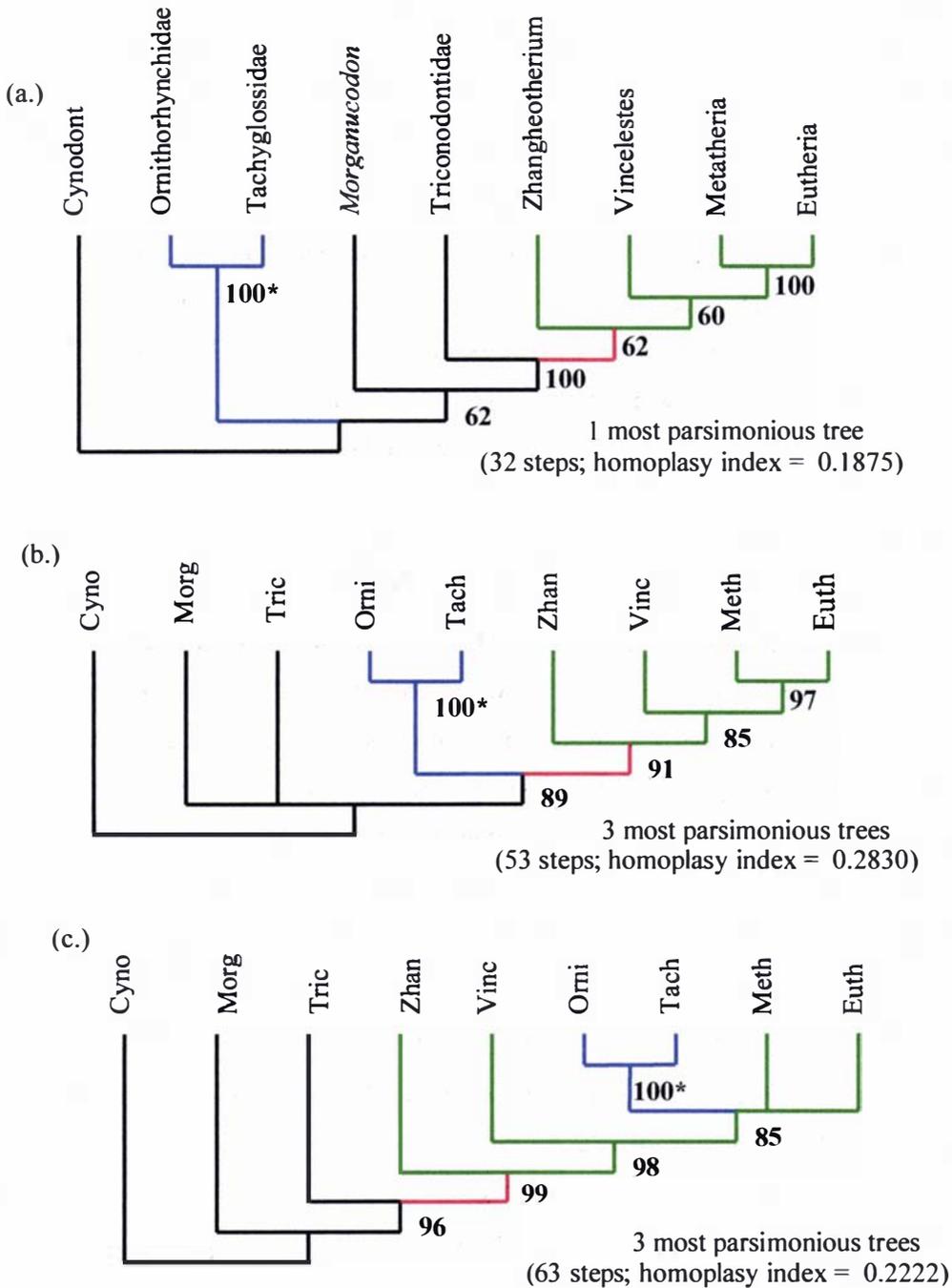
	n	$CMI_O$	$CMI_E$	Resolvability ( $CMI_E/n$ )	$CMI_R$	p
<b>Over the entire background phylogeny</b>						
within U	231	12	55.99	0.242	-0.7857	<0.0001
within LB	465	39	42.242	0.091	-0.0767	0.6599
within VMD	703	10	30.718	0.044	-0.6745	0.0002
U*LB	682	109	117.26	0.172	-0.0704	0.4295
U*VMD	836	206	103.462	0.124	0.9911	<0.0001
LB*VMD	1178	118	76.154	0.065	0.5495	<0.0001
<b>across the mammaliaformes node</b>						
within U	231	1	6.030	0.026	-0.8342	0.0615
within LB	465	0	7.335	0.016	-1.0000	0.0111
within VMD	703	0	3.686	0.005	-1.0000	0.0251
U*LB	682	9	14.263	0.021	-0.3690	0.2041
U*VMD	836	22	11.060	0.013	0.9892	0.0016
LB*VMD	1178	0	11.712	0.010	-1.0000	0.001
<b>across the theriimorpha node</b>						
within U	231	10	35.233	0.153	-0.7162	<0.0001
within LB	465	8	8.771	0.019	-0.0879	0.9283
within VMD	703	0	11.334	0.016	-1.0000	0.0012
U*LB	682	91	51.204	0.075	0.7772	<0.0001
U*VMD	836	160	50.214	0.060	2.1864	<0.0001
LB*VMD	1178	16	22.192	0.019	-0.2790	0.2225
<b>across the trechnotheria node</b>						
within U	231	12	38.247	0.166	-0.6862	<0.0001
within LB	465	35	30.641	0.066	0.1423	0.4715
within VMD	703	10	16.439	0.023	-0.3917	0.1389
U*LB	682	59	74.482	0.109	-0.2079	0.0658
U*VMD	836	142	54.958	0.066	1.5838	<0.0001
LB*VMD	1178	101	49.405	0.042	1.0443	<0.0001
<b>across the cladotheria node</b>						
within U	231	0	35.233	0.153	-1.0000	<0.0001
within LB	465	0	8.771	0.019	-1.0000	0.0048
within VMD	703	4	11.334	0.016	-0.6471	0.0414
U*LB	682	0	51.204	0.075	-1.0000	<0.0001
U*VMD	836	60	50.214	0.060	0.1949	0.177
LB*VMD	1178	48	22.192	0.019	1.1629	<0.0001

Comparison of the incompatibility that occurs within partitions for monotreme placement over the background phylogeny, with that occurring between partitions, illuminates a fundamental pattern for this dataset. A  $CMI_R$  of  $-0.527$  for the sum of all 1399 within meta-region pairwise comparisons indicates strong apparent phylogenetic signal within the meta-region partitions, while a positive  $CMI_R$  of  $+0.459$  for the remaining 2696 (between meta-region) pairwise comparisons indicates strong conflict between the partitions. Both these values deviate significantly ( $p < 0.0001$ ) from the values expected under RSCM (random shuffling of character maps). If instead the data is partitioned as the six anatomical regions, the corresponding within partition  $CMI_R$  value is  $-0.609$ . The  $CMI_R$  deviation for pairwise comparisons between the six anatomical regions, of  $+0.357$ , is 22% less than the corresponding value with the regions lumped as the three meta-regions. Hence, this CMI analysis provides agreement with the cladistic analysis (Chapter 3), by showing that anatomical region lumping further isolates the incongruence for monotreme placement and in addition, quantifies this in terms of relative character incompatibility.

Examining monotreme placement incompatibility over the background phylogeny may highlight general trends, though for testing signal homogeneity and conflict, incompatibility needs to be examined across individual nodes. On an unrooted bifurcating tree each node represents a three-way split. The theria node for example, represents the split between 1. the marsupial lineage, 2. the placental lineage, and 3. the lineage representing the common ancestors of therians. These lineages provide for three alternative hypotheses for monotreme placement with respect to the theria node. None of the 91 characters in this study are incompatible with monotreme placement on the therian stem lineage (3; from above), whether that is as sister to Theria, or as distantly as with the cynodont outgroup. Hence, despite the significantly higher-than-expected incompatibility between meta-regions, over the background phylogeny overall, none of this incompatibility occurs across the trechnotheria node (for which the  $CMI_R$  deviation is  $-1.000$ ). As such, the 38 character-maps for which monotremes are excluded from the therian (marsupial/eutherian) clade, are without conflict. Assuming RSCM for all pairwise comparisons among the 91 characters, the (homogenous) signal these 38 characters confer is highly significant ( $p < 0.0001$ ). This conveys the extent to which morphological data that includes information from fossil taxa, is at odds with grouping monotremes with marsupials (Marsupionta: Gregory 1947), as has recently been favoured by a number of molecular studies (Janke *et al.* 1996, 1997, 2002; Kirsch and Mayer 1998; Toyosawa *et al.* 1998).

Progressing towards the outgroup from the theria node, the  $CMI_R$  deviations (and two-tailed binomial probability for the significance of the deviation of  $CMI_O$  from  $CMI_E$ ) for all pairwise comparisons among the 91 characters are:  $-0.374$  ( $p < 0.0001$ ) across the cladotheria node,  $+0.359$

( $p < 0.0001$ ) across the trechnotheria node, +0.593 ( $p < 0.0001$ ) across the theriiomorpha node, and -0.408 ( $p = 0.0032$ ) across the mammaliaformes node.



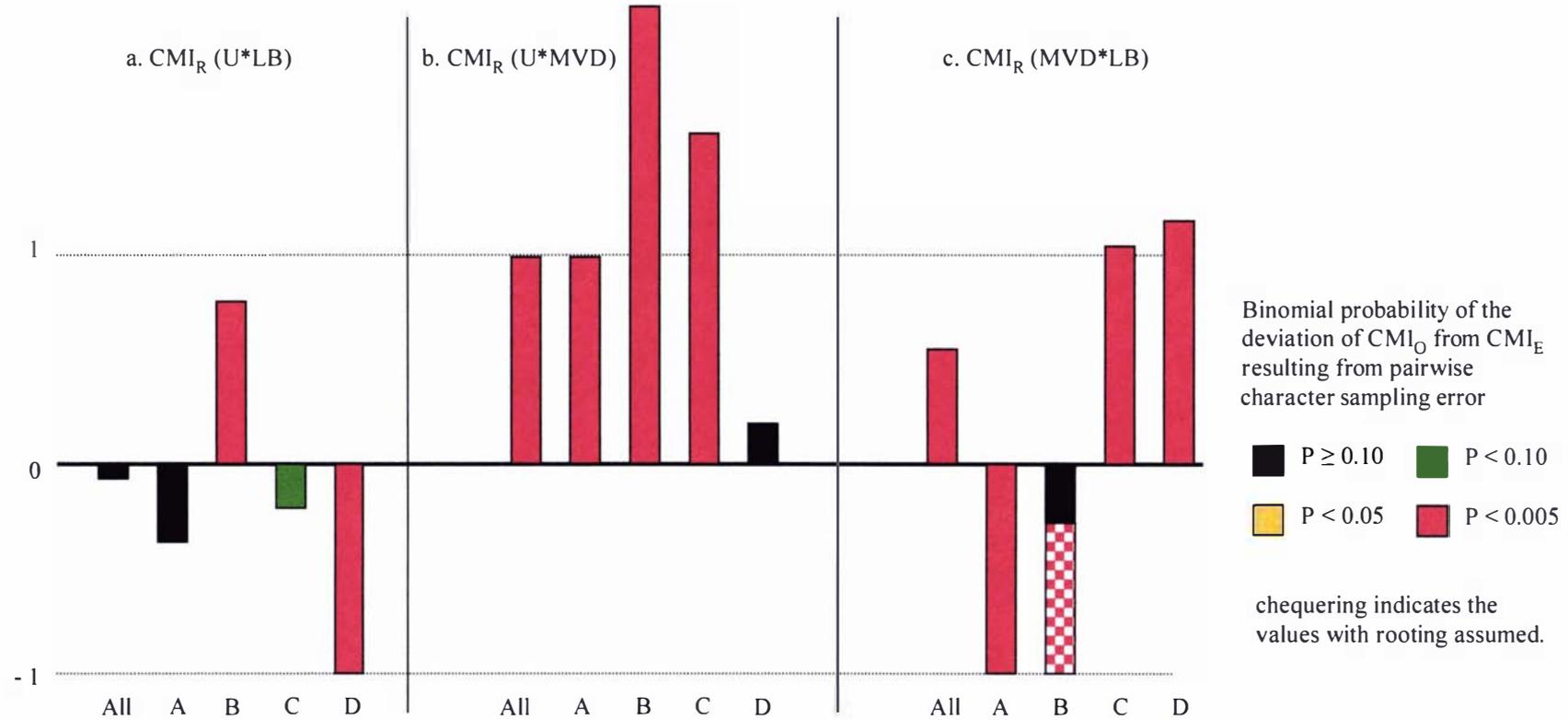
**Figure 4.6** MP 50% bootstrap consensus trees for the placement of monotremes among the background phylogeny of generalized mammal for U (a.), LB (b.) and MVD (c.). Bootstrap support values correspond to the nodes above them. Branches are colour-coded as green for Trechnotheria, red for the trechnothere stem, and blue for monotremes. Note that monotreme monophyly is constrained (unconstrained it is supported in 40%, 12%, and 38% of replicates in a., b., and c. respectively).

Characters that more precisely pinpoint the monotreme position on the background phylogeny will result in higher phylogenetic resolvability (the average probability under RSCM of the given pairwise comparisons being incompatible given the distribution of monotreme most-parsimonious positions: see Table 4.1). Phylogenetic resolvability over the background phylogeny varies considerably among partitions. Phylogenetic resolvability of within-meta-region pairwise character-map comparisons are respectively 0.242, 0.091, and 0.044 for U, LB, and MVD. Inspection of Table 4.2 shows that this basic rank order of  $U > LB > MVD$  for phylogenetic resolvability holds across each of the nodes.

The topological nature of the incongruence among meta-regions that was found in Chapter 3 is illustrated in Figure 4.6. Monotremes are excluded from Mammaliaformes for U, are sister to Trechnotheria for LB, and share a close relationship with Theria for MVD. Inferring (equally) most-parsimonious monotreme placements on the background phylogeny of generalized insectivores for each character, as a character-map, provides the opportunity to investigate how well the bootstrap results (Figure 4.6) are reflected by between-meta-region pairwise incompatibility.

Over the background phylogeny, the excess of observed incompatibilities is most prominent for pairwise character comparisons between U and MVD, with  $CMI_O[U * MVD]$  being 206, compared with the RSCM expected value of 103.462 (Table 4.2). Cladistic analyses (Figure 4.6) place monotremes outside of Mammaliaformes for U, but place monotremes in a trichotomy with marsupials and placentals for MVD. Accordingly, Figure 4.7b shows positive  $CMI_R[U * MVD]$  deviations (indicating  $CMI_O > CMI_E$ ) across mammaliaformes (column A), cladotheria (column D) and for the two nodes internal to these, theriimorpha (column B) and trechnotheria (column C). Among these, the excess of observed incompatibilities across mammaliaformes, theriimorpha and trechnotheria is highly significant ( $p < 0.005$ ).

By favouring monotremes arising from the trechnothere stem (Figure 4.6b), LB provides a phylogenetic hypothesis that is intermediate to those favoured by U and MVD. Two nodes, mammaliaformes and theriimorpha separate this LB monotreme placement from that indicated for U. Figure 4.7a shows that  $CMI_R[U * LB]$  across mammaliaformes is negative, though is not significant (at  $p \leq 0.10$ ). In contrast,  $CMI_R[U * LB]$  across theriimorpha is positive (+0.7772) and represents highly significant ( $p < 0.0001$ ) signal conflict between U and LB. These results correspond well with the cladistic analyses, considering that exclusion of monotremes from mammaliaformes for U (Figure 4.6a) is only weakly supported (62% of bootstrap replicates), while for U, monotremes are excluded from theriimorpha in 100% of bootstrap replicates. Significantly less than expected incompatibility across the trechnotheria and cladotheria nodes (Figure 4.7a,



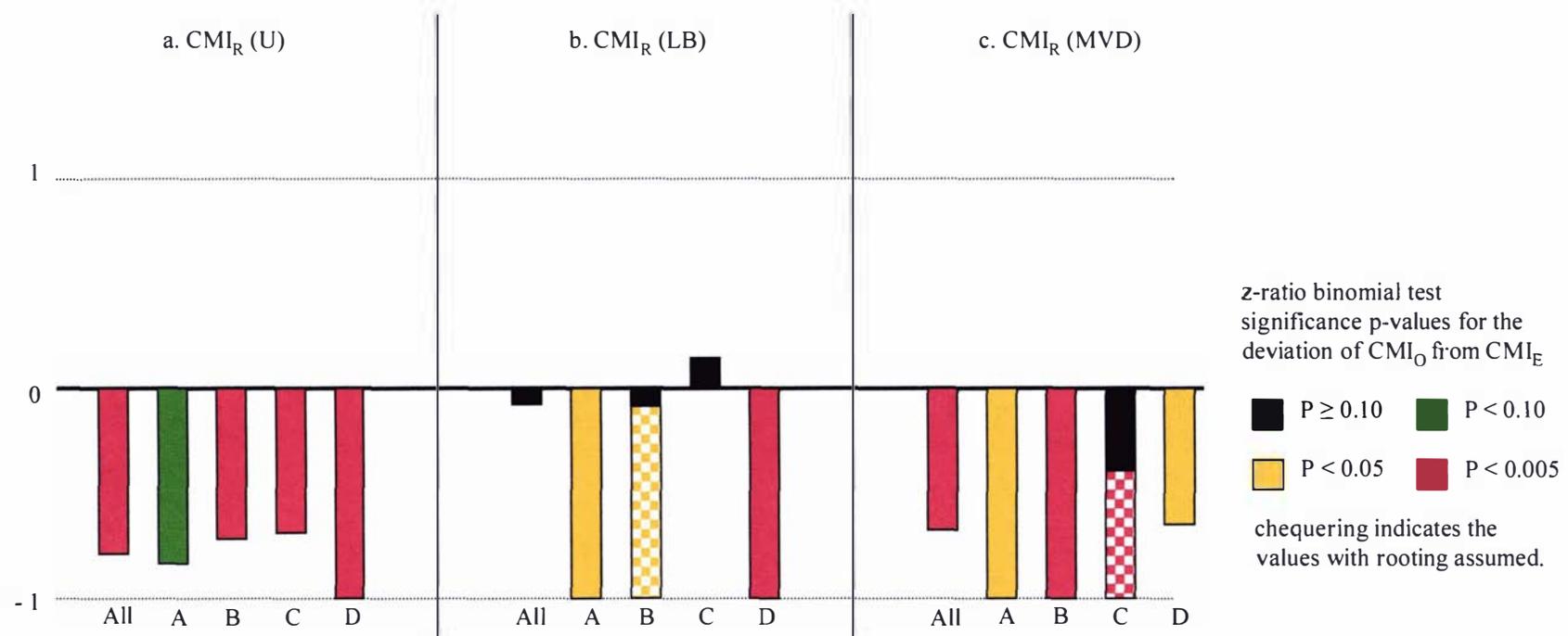
**Figure 4.7** Between-meta-region relative character-map incompatibility ( $CMI_R$ ) for: a. U\*LB; b. U\*MVD; and c. MVD\*LB.  $CMI_R$  and two-tailed p-values are for character-map incompatibility over the entire tree (All), across the mammaliaforms node (A), across the theriiomorpha node (B), across the trechnotheria node (C) and across the cladotheria node (D). Further details are shown in Table 4.2. Note that a compound graph is drawn for  $CMI_R$ (MVD\*LB) across theriiomorpha. The black indicates the  $CMI_R$  value with the theriiomorpha node designating three potential clades on an unrooted tree (as per the methods section, and each of the other data points). The (chequered) extension shows the  $CMI_R$ -value with the theriiomorpha node designating clade inclusion/exclusion (so assuming rooting: see Section 4.3.1)

columns C and D) represents further agreement with the parsimony results, which show that both U and LB place monotremes more primitively than these nodes.

Figure 4.6 shows that the most parsimonious monotreme placement for MVD (with Theria) is separated from their most parsimonious placement for LB (on the trechnothere stem) by two nodes, trechnotheria and cladotheria. Again  $CMI_R$  values correspond well with the incongruence indicated by the cladistic analyses. Observed incompatibility across both of these nodes (trechnotheria and cladotheria) is more than double the expected values, as indicated by  $CMI_R[LB*MVD]$  deviations being greater than +1.000 in each case (Figure 4.7c, columns C and D). Furthermore, agreement between MVD and LB for monotremes arising from within the Theriimorpha (Figure 4.6) is consistent with lower-than-expected incompatibility across the non-trechnothere nodes (mammaliaformes and theriimorpha). Although the  $CMI_R[MVD*LB]$  deviation across theriimorpha is  $-0.279$  (Table 4.2), this is not a significant ( $p \leq 0.10$ ). However, all 16 observed incompatibilities result from conflict between characters supporting theriimorph affinities for monotremes, and characters supporting monotreme affinities with the eutriconodont, *Jeholodens*. No characters among either LB, or MVD support monotreme placement outside the Theriimorpha. Hence, the  $CMI_R[MVD*LB]$  deviation is  $-1.000$  across the theriimorpha node, if treated as a two-branch intersection, for which support for monotreme placement with *Jeholodens* and with trechnotheres is pooled (so simply a Theriimorpha inclusion/exclusion split). This negative  $CMI_R$  represents significant ( $p < 0.005$ ) signal homogeneity between MVD and LB for the placement of monotremes within Theriimorpha.

The benefit of examining incompatibility across nodes is revealed by inspection of Figure 4.7a. For incompatibility over the (entire) background phylogeny the  $CMI_R[U*LB]$  deviation is only slight ( $-0.0704$ ) and is not significant at  $p \leq 0.10$ . In contrast, between these meta-regions (U and LB) there is conflict across theriimorpha, and agreement (significant signal homogeneity) across the trechnotheria and (particularly) cladotheria nodes. Similarly, the significant ( $p < 0.0001$ ) conflict between LB and MVD (Figure 4.7c) over the background phylogeny as a whole, provides no indication of the less-than-expected incompatibility across the mammaliaformes and theriimorpha nodes for pairwise comparisons between these two meta-regions.

It is important that signal homogeneity and conflict across nodes, between meta-region partitions (Figure 4.7) are so closely reflected by differences and similarities between the phylogenetic reconstructions for U, LB, and MVD (Figure 4.6). This indicates that random shuffling of character-maps (RSCM) has successfully dissociated signal for monotreme placement, from signal among the background taxa, as well as from the inherent phylogenetic resolvability bestowed by



**Figure 4.8** Within-meta-region relative character map incompatibility ( $CMI_R$ ) for: a. (U); b. (LB); c. (MVD).  $CMI_R$  and two-tailed p-values are for character-map incompatibility over the entire tree (All), across the mammaliaforms node (A), across the theriimorpha node (B), across the trechnotheria node (C) and across the cladotheria node (D). Further details are shown in table 4.2. Note that compound graphs are drawn for  $CMI_R$ (LB) across theriimorpha, and for  $CMI_R$ (MVD) across trechnotheria. For these, the black indicates the  $CMI_R$  value with nodes designating three potential clades on an unrooted tree (as per the methods section, and each of the other data points). The (chequered) extensions show  $CMI_R$  values with the nodes designating clade inclusion/exclusion (so assuming rooting: see Section 4.3.1)

the distribution of character states. The confidence this provides in the effectiveness of RSCM is particularly encouraging, because incongruence testing does not directly test for the presence of signal within partitions, but CMI analysis does.

In sharp contrast with the between-meta-region  $CMI_R$  deviations (Figure 4.7), a prominent feature of the within-meta-region  $CMI_R$  deviations (Figure 4.8) is the absence of significantly greater-than-expected incompatibility (at  $p \leq 0.10$ ) across any node, among characters within any of the three meta-regions. Observed character-map incompatibilities across each node are less than one third of the values expected under RSCM for comparisons within U, as indicated by each  $CMI_{R[U]}$  deviation being less than  $-0.67$ . Over the (entire) background phylogeny and across each node (except mammaliaformes), the  $CMI_{R[U]}$  deviation is significantly negative at  $p < 0.0001$  (Table 4.2). That the deviation of  $CMI_{R[U]}$  across mammaliaformes is a sampling effect can only be rejected at  $p = 0.0615$ . However, the relatively small sample of U characters (22) and low phylogenetic resolvability across the mammaliaformes node (Table 4.2) conspire against the possibility of finding high statistical significance for the  $CMI_{R[U]}$  deviation, which is very low at  $-0.8342$ .

$CMI_{R[MVD]}$  deviations (Figure 4.8c) display a similar pattern to those for  $CMI_{R[U]}$  and indicate that incompatibility among characters within MVD is considerably less than expected, over the background phylogeny overall and across each of the nodes. In each case the observed values deviate significantly from the expected values at  $p < 0.05$ , except across the trechnotheria node, for which the deviation of  $CMI_{R[MVD]}$  being a sampling effect can only be rejected at  $p = 0.1389$  (Table 4.2). All ten  $CMI_{O[MVD]}$  incompatibilities across the trechnotheria node result from conflict between characters supporting cladothere affinities for monotremes, and characters supporting monotreme affinities with *Zhangheotherium*. As such, no characters among MVD support monotreme placement outside Trechnotheria. The  $CMI_{R[MVD]}$  deviation across the trechnotheria node treated as a three-branch intersection is  $-0.3917$ . However, treated as a two-branch intersection, for which support for monotreme placement with *Zhangheotherium* and with cladotheres is pooled (so simply a Trechnotheria inclusion/exclusion split), the  $CMI_{R[MVD]}$  deviation is  $-1.000$ . This negative  $CMI_{R[MVD]}$  deviation represents significant ( $p < 0.005$ ) signal homogeneity among the characters within MVD, for the placement of monotremes within Trechnotheria.

In contrast to pairwise comparisons within U and within MVD, incompatibility among characters within LB over the background phylogeny does not deviate significantly from that expected with RSCM, even at  $p \leq 0.10$  (Figure 4.8b, column "All"). However, none of the 465 LB character-map pairwise comparisons are incompatible across either the mammaliaformes or the cladotheria nodes.

Furthermore, a caveat similar to that for the  $CMI_R[MVD]$  deviation across the trechnotheria node exists for incompatibility within LB, across theriiomorpha. The  $CMI_R[LB]$  deviation across theriiomorpha is not significant (at  $p \leq 0.10$ ), but the observed incompatibility relates to conflict between one character supporting monotreme affinities with *Jeholodens*, and five characters supporting closer monotreme ties to trechnotheres. Hence, treated as a two-branch split with support for monotreme placement with *Jeholodens* and with trechnotheres pooled (so simply a Theriiomorpha inclusion/exclusion split), the relative CMI is reduced to  $-1.000$ . This negative  $CMI_R$  deviation represents significant ( $p < 0.05$ ) signal homogeneity among the characters within MVD, for the placement of monotremes within Theriiomorpha.

While the significance of a negative deviation of observed from expected incompatibility is a test for the existence of signal above “noise”, the actual  $CMI_R$  deviation is essentially a measure of the homogeneity of apparent phylogenetic signal among the given characters. With the theriiomorpha and trechnotheria nodes considered as clade inclusion/exclusion splits for LB and MVD respectively,  $CMI_R$  values are less than  $-0.64$  across each of the nodes for pairwise comparisons among characters within U and within MVD. The only exception to this for pairwise comparisons within LB, is across the trechnotheria node.

The CMI analysis goes further than the cladistic analysis of Chapter 3, which showed that in terms of monotreme placements, L and B were congruent, as were M, V, and D.

There is an important consequence for the  $CMI_R[LB*MVD]$  deviation across cladotheria being significantly positive (indicating signal conflict between LB and MVD: Figure 4.7c), while most  $CMI_R[LB]$  and  $CMI_R[MVD]$  values are significantly negative (Figure 4.8), including those across cladotheria. This is that, whichever of LB and MVD place monotremes erroneously, the homoplasy is correlated across the anatomical region members of that meta-region, rather than being randomly distributed among characters. This conclusion is further supported by investigation of incompatibility over the background phylogeny within and between individual anatomical regions (Appendix F). All between-anatomical-region  $CMI_R$  deviations are negative, when character pairs are from the same meta-region ( $[L*B][M*V][M*D][V*D]$ ). In contrast, all between-anatomical-region  $CMI_R$  deviations are positive when character pairs are from different meta-regions ( $[L*M][L*V][L*D][B*M][B*V][B*D]$ ). These results are consistent with the suggestion from Chapter 3 that although MVD and LB may not be natural partitions in terms of being functional or developmental units, for monotremes at least, they may be natural partitions in terms their susceptibility to phylogenetic biases.

The lack of uniform signal for monotreme placement across the trechnotheria node for LB is also consistent with previous examination of these anatomical regions. A partially coiled cochlea and

fused sutures between the pelvic bone of adults (Ji *et al.* 1999) support monotreme placement within Trechnotheria, while the lack of a caudal tympanic process of the inner ear containing petrosal bone (Wible 1990), and the lack of a prominent heel (calcaneal tubercle: see Szalay 1993b; Hu *et al.* 1997) indicate monotreme affinities lie outside Trechnotheria. The lack of a uniform signal for monotreme placement with respect to the trechnotheria node for comparisons within LB cannot simply be related to the lumping together of the lower appendicular and basicranial regions. Inspection of Appendix F shows that over the background phylogeny,  $CMI_R$  deviations are close to zero for pairwise character comparisons even within these regions separately.

Being a measure of signal homogeneity, rather than of support for phylogenetic hypotheses,  $CMI_R$  values among characters within partitions are not directly comparable with statistical support for trees inferred from the same characters. Nevertheless, signal homogeneity within U and within MVD, but extreme conflict between U and MVD is consistent with monotreme upper appendicular characters typically being primitive (Sereno and McKenna 1995; Hu *et al.* 1998), and monotreme vertebral (Jenkins 1970a; Hu *et al.* 1997 supp. info.) and mandibular/dental (Archer *et al.* 1985; Kielan-Jaworowska *et al.* 1987; Rich *et al.* 2001a) characters typically being at an advanced eupantothere level. That CMI indicates significant phylogenetic conflict also occurs across at least one node for pairwise comparisons between LB and U as well as between LB and MVD demonstrates the complex nature of the incongruence among anatomical regions for the placement of monotremes among the generalized insectivores.

Partition homogeneity testing (Chapter 3) was able to show highly significant incongruence among the three meta-regions. Just how much more informative CMI analysis is with respect to this incongruence is showcased by comparison of Figure 4.8 with Figure 4.7. Not only does CMI provide a relative measure of the strength of phylogenetic conflict between U, LB, and MVD, the nodes across which the conflict occurs are identified. Furthermore, the CMI analysis shows that across each of the nodes for which there is significant ( $p < 0.005$ ) conflict between meta-regions, there is greater than expected signal homogeneity within each of those meta-regions. The importance of this is discussed in the following section, for which this CMI analysis helps provide a framework within which character analysis may be used to explore monotreme affinities in terms of homoplasy options.

#### 4.3.2 A framework for assessing monotreme affinities in terms of homoplasy options

There is a considerable anatomical region bias for signal relating to the placement of monotremes among the background phylogeny (Figure 4.1) of generalized insectivores. This is clearly shown by the parsimony bootstrap analyses (Figure 4.6) and between-meta-region CMI results (Figure 4.7) and is in agreement with the parsimony significance tests and partition homogeneity testing of Chapter 3. Although there are eleven possible positions (branches) on the background phylogeny from which an additional taxon (monotremes) may have arisen, for the purposes of the following discussion I will condense these into three placements: (1.) exclusion from Theriomorpha; (2.) placement on the trechnothere stem; and (3.) placement within Trechnotheria. These cover all eleven possible placements on the background phylogeny and as indicated in Figure 4.6, are respectively the favoured monotreme placements for U, LB and MVD.

In the absence of incongruence, homoplastic transformations can be assumed to be randomly distributed among branches, with respect to data partitions. For such ideal cases, increased character sampling will tend to increase phylogenetic resolution. Indeed this desire to further tease phylogenetic signal from “noise” is typically the major aim of lumping, or concatenating datasets (Bledsoe and Raikow 1990; Kluge and Wolfe 1993). Some authors, espousing a “total evidence” approach (e.g. Eernisse and Kluge 1993) expect that even in spite of significant incongruence, data lumping will tend to converge upon the “true” phylogeny. In Chapter 3 it was shown that the opposite in fact occurs for defining the placement of monotremes. Fewer monotreme placements among the generalized insectivores could be rejected for the combined data, than could be for any of the three meta-region datasets alone. In light of this, embracing the most parsimonious compromise among the incongruent meta-regions would be unfounded on the basis of teasing phylogenetic signal from “noise”, and in fact would amount to a loss of information on the nature of apparent phylogenetic signal within and between the data partitions. Hence, in the face of extreme and topologically complex incongruence, I contest that selecting one of the alternative monotreme placements requires some biological basis for explaining one or more of the conflicting meta-region signals for monotreme affinities, as having resulted from homoplasy.

Ecological and functional examination of character state covariance, implying convergence of hind-limb characters among diving ducks (McCracken *et al.* 1999) and of life history strategies among wasps (Quicke and Belshaw 1999) have been successful in explaining incongruence, and led to phylogenetic resolution. Similarly, monotreme affinities may be viewed as competing homoplasy options.

Monotreme placement outside Theriimorpha requires considerable homoplasy among LB and MVD (see Figure 4.6). Similarly, trechnothere stem, or trechnothere affinities for monotremes require considerable homoplasy among U and MVD, or U and LB respectively. The parsimony and CMI results provide important information on the nature of this homoplasy. Firstly, this homoplasy is not randomly distributed among the phylogeny with respect to the anatomical regions. Although the incongruence (PHT) and parsimony significance testing (Chapter 3) showed this, the CMI results allow further interpretation of the nature of the homoplasy.

There is highly significant ( $p < 0.005$  see Figure 4.7) phylogenetic conflict for U\*MVD across the theriimorpha and trechnotheria nodes, while incompatibility within U and within MVD across these nodes (at least when treated as clade inclusion/exclusion splits) is significantly ( $p < 0.005$ ) less than expected (Figure 4.8). Similarly, significant ( $p < 0.005$ ) conflict between meta-regions, within each of which, a homogenous signal is indicated, is also seen for U\*LB (across theriimorpha) and LB\*MVD (across trechnotheria and cladotheria).

There is an important consequence of incompatibility among characters within each of two meta-regions being significantly less than expected (Figure 4.8) across nodes for which there is phylogenetic conflict between those two meta-regions (Figure 4.7). That is, that homoplastic evolution relating to monotreme placement among the generalized insectivores has evolved non-independently among characters within one or both of the meta-regions. Hence, signal relating to monotreme placement varies at the level of meta-regions, rather than being randomly distributed among characters. Any proposal of monotreme affinities must explain the non-independence of homoplastic character evolution within at least two of the three meta-regions (U, LB, MVD).

In Chapter 3 it was suggested that the anatomical region dependence of monotreme affinities was likely to result mostly from homoplasy that could be related to transformations along the monotreme stem lineage. This was supported by the inclusion of monotremes with the generalized insectivores resulting in the homoplasy index inflating by a factor of 2.58 and the induction of highly significant incongruence (PHT:  $p < 0.0001$ ). Alternatively, anatomical region dependence of monotreme affinities could result from monotreme inclusion exposing apparent synapomorphies of sister taxa among the background phylogeny to in fact be independently derived. For example, among a phylogeny of various fish and amphibian groups, birds and mammals would be included as sister taxa and homeothermy would be an apparent synapomorphy of this group. Inclusion of other reptile groups would indicate that homeothermy was in fact derived independently in birds and mammals (e.g. Gauthier *et al.* 1988; Janke and Arnason 1997). Hence, the homoplastic nature of the evolution of “warm bloodedness” in birds and mammals is effectively “hidden” without the inclusion of other reptile groups.

Explaining the extreme incongruence upon the inclusion of monotremes, as hidden-homoplasy among the generalized insectivores, would require a large-scale and complicated pattern of unobserved homoplastic transformations occurring at many of the background phylogeny internal and external branches. Complete congruence among the three meta-regions for relationships among the background phylogeny gives no indication of this. Indeed observed homoplasy is low across the whole topology for the taxa that have essentially retained the ancestral mammalian niche. As such, it must be expected that the majority of incongruence among the meta-regions for the position of monotremes involves transformations that occurred during monotreme evolution. If this is the case, then these correlated homoplastic transformations can be further isolated to the monotreme stem lineage, as monophyly of the two monotreme families was constrained for the partition homogeneity tests.

Although the extreme nature of the incongruence and inflation of homoplasy induced upon monotreme inclusion appear as a “smoking gun” for the meta-region incongruence relating to monotreme placement, it would be premature to dismiss the possibility of hidden-homoplasy among background taxa being at least partly responsible. Even assuming saltational or punctuated models of evolution (see Gould and Eldredge 1977; Mayr 1988; Hoffman 1992), the apparent synapomorphies of the generalized insectivore clades probably did not occur as transformations over the duration of a single generation (or even a single species). In consideration of this, Gow (1985) suggested that trends that were initiated prior to speciation events were frequently responsible for promoting parallel evolution among mammal-like reptiles and early mammals. Of course it is more likely that trends will continue to be followed, after divergence, by taxa that have maintained the ancestral niche (and so a similar functional relationship with the environment, for characters involved with the trends). Hence, expectations for parallelism among the background taxa should be higher than for parallelism between monotremes and the background taxa, at least for evolution that has occurred since the fossorial/semiaquatic monotreme niche transition.

Evolutionary trends among small terrestrial mammal-like reptiles and early mammals have been documented for a more upright gait (Kemp 1982; Blob 2001), improved acoustic insulation and transmittance of (high frequency) airborne sound (Lou *et al.* 1995; Allin 1975; Fox and Meng 1997;), and the loss and reduction of postdentary (middle ear) and parodontary (coronoid and splenial) bones (Allin 1986; Heinrich 1998; Wang *et al.* 2001). However there is no evidence at least among the taxa of this study to indicate parallel evolution of correlated characters among the background phylogeny, except perhaps for multiple separate losses in adults (among Theriimorpha) of the meckelian groove and one or more parodontary bones. Among trechnotheres alone, independent losses of these characters have been suggested for spalacotheriid symmetrodonts (Cifelli and Madsen 1999), dryolestid eupantotheres (Krebs 1971), eutherians (Kielan-Jaworowska

and Dashzeveg 1989), and hence by inference, also along the separate lineages leading to marsupials and *Vincelestes*. These characters may be particularly susceptible to homoplasy due to their slow loss (both phylogenetically and ontogenetically) and possible functional reincorporation, such as for muscle attachment as Wang *et al.* (2001) suggest for the ossified meckelian cartilage of gobiconodontids. In fact, even the postdentary bones are still attached to the dentary and forming the primary jaw joint in early pouch young of marsupials (Maier 1993; Sánchez-Villagra *et al.* 2002).

Evolutionary trend related promotion of parallelism of characters among taxa for which those characters have a similar functional relationship to the environment has a corollary. This is, that reversal of the trends would not be expected unless there is a change in the functional relationship between those characters and the environment. This principle fits well with published examples for which correlated reversal among characters have occurred. Pectoral reduction, pelvic enlargement and changes in various aspects of skull morphology of flightless birds are a reversal of evolutionary trends along the lineage from theropod dinosaurs to modern birds (Cubo and Arthur 2001). The evolution of tree kangaroos has involved pectoral enlargement, relative length reduction of distal (relative to proximal) hind limb elements and freedom from the locomotory constraints of bipedal hopping (Grand 1990). Each of these apomorphies represent a reversal of evolutionary trends along the lineage from primitive macropodoids to modern macropodid kangaroos. Trend reversals that are coincident with major ecological shifts have also been shown among various groups of lizards (Arnold 1990), and chaenopsid fishes (Emerson and Hastings 1998).

The apparent importance of ecological changes for reversing evolutionary trends among character complexes is of interest for this study. If the correlated homoplastic evolution inducing the incongruence upon monotreme inclusion is hidden-homoplasy among the background phylogeny (taxa with a similar niche, relative to monotremes), it is likely to be parallelism/convergence rather than reversal. This is significant as it means that correlated hidden-homoplasy among the background phylogeny may be erroneously pushing monotremes toward the outgroup, but is unlikely to be erroneously pushing monotremes closer to therians. For example, given the cladothere trend toward increased coiling of the cochlea that continued right through to early marsupials and placentals (Wible *et al.* 2001), the uncoiled cochlea of *Zhangheotherium* (Hu *et al.* 1997) would not be expected to be a reversal, and the 270° cochlea coiling of *Vincelestes* (Rougier *et al.* 1992) is more likely to be greater (rather than less) than that of its LCA with therians. It follows that if the extent of monotreme cochlea coiling (180°-270°, depending on the authority and differing slightly between echidnas and the platypus: see Griffiths 1978; Ladhams and Pickles 1996; Fox and Meng 1997) has changed little along their stem lineage, a close affinity of

monotremes with therians or with *Vincelestes* could be reconciled by parallelism among trechnotheres. However, an affinity with, or more primitive than *Zhangheotherium* could not. Hence, where characters are following trends among the generalized insectivores, hidden-homoplasy among the background phylogeny is not expected to bias monotreme placement towards a position that is closer to therians, though parallel or convergent evolution along the monotreme stem lineage (with one or more background taxa) may.

Arnold (1990) considered niche shifts to be the major factor confounding phylogeny reconstruction. Szalay (1994) similarly observes the importance of the relationship between form and function, when interpreting mammalian postcranial characters for phylogenetic analysis. In his examination of early mammal relationships, Lou (1994) wrote that, "To understand what kinds of characters have made up the apomorphies is as meaningful as the number of apomorphies, if not more so". These views however are often ignored in an age where relationships tend to stand or fall purely on the basis of tree length, or the absolute number of shared apomorphies. Nevertheless, a number of authors (e.g. Maynard Smith and Savage 1956; Jenkins 1970a; Griffiths 1978; Archer *et al.* 1992, 1993; Gambaryan and Kielan-Jaworowska 1997) have stressed the potential importance of understanding monotreme ecology for determining monotreme affinities. The large increase in homoplasy and extreme incongruence induced by adding the monotremes to the background phylogeny should serve to reinforce the importance of considering the nature of the niche changes that have occurred along the monotreme lineage since monotremes split from the mammalian backbone lineage.

It has long been considered that the backbone lineage of mammalian evolution (Figure 4.1, in red), from transitional mammal-like reptiles/mammals through to the LCA of marsupials and placentals, comprised small, terrestrial forms that fed on insects and other small prey (e.g. Hopson and Crompton 1969; Kermack and Kermack 1984). As the early mammalian family tree has continued to be filled out by discoveries such as the early trechnother, *Zhangheotherium*, the eutriconodont, *Jeholodens* and the Early Jurassic triconodont, *Hadrocodium* (Lou *et al.* 2001b), this conclusion has been cemented. Hence, it is clear that the LCA of monotremes and therians was in fact a small terrestrial (or perhaps scansorial) insectivore/carnivore, regardless of what point that pertains to along the backbone lineage of mammalian evolution. The oldest known monotreme fossil, *Teinolophos trusleri* (Rich *et al.* 1999; Rich *et al.* 2001a) from the early Aptian (approx. 120 mybp) of southern Victoria (Australia) appears to have retained a small insectivore niche, though with aberrant diagonally aligned molar lophs, its specific relations among the monotremes are unclear.

Characteristics that echidnas and platypuses share might be expected to have existed in their LCA. Shared characters that are relevant for determining the nature of this LCA are a diet of invertebrates (Faragher *et al* 1979; Griffiths 1968), considerable fossorial (burrowing) adaptation (Augee and Gooden 1993; Grant 1995) and by phylogenetic inference from the background phylogeny, a marked increase in size over their LCA with the mammalian backbone lineage (*Ornithorhynchus anatinus* is the smallest of the known crown group monotremes, and these are known to reach up to 2.5kg: Grant 1995). Whether the platypus and echidna LCA was aquatic or terrestrial is uncertain, though as discussed by Musser and Archer (1998), a combination of molecular and morphological suggests this ancestor was more similar to platypuses than echidnas.

Aside from the adult retention of teeth, the mid Miocene (approx. 15 mybp) *Obdurodon dicksoni*, which is known from an extraordinarily well preserved skull, dentary and isolated teeth (Archer *et al.* 1992, 1993; Musser and Archer 1998), differs only in minor details from the living platypus. The hypertrophied bill, dorsoventral flattening of the skull, and numerous other details suggest that *Obdurodon*, like *Ornithorhynchus* foraged in an aquatic environment. Details of the dentary suggest that the Late Oligocene (approx. 25 mybp) *Ob. insignis* may have been more closely related to the modern platypus than the Miocene *Ob. dicksoni*.

An *Obdurodon*-like upper molar is known from the early Paleocene (approx. 63 mybp) of Patagonia (Pascual *et al.* 1992a; 1992b). This *Monotrematum americanum* tooth is so similar to those of *Obdurodon*, that it should perhaps be reassigned to *Obdurodon* (Pers. comm. Anne Musser). Given the similarity of the highly derived crown pattern shared by these taxa, it would seem probable that *Monotrematum* had a similar diet to *Obdurodon*, feeding on invertebrates and perhaps small vertebrates in an aquatic environment. If correct, this deduction implies that the LCA of the monotreme crown group also was an aquatic, platypus-like creature. This is because recent dating estimates from DNA sequence data (Retief *et al.* 1993; Gemmell and Westerman 1994; Messer *et al.* 1998; Janke *et al.* 2002) indicate this LCA of platypuses and echidnas existed less than 55Mya (early Eocene), and probably less than 45Mya (late Eocene or early Oligocene: see Chapter 2), well after *Monotrematum* and *Obdurodon* diverged. The absence of any echidna-like mammals, or putative tachyglossids from fossil faunas prior to the mid-Miocene (approx. 14 mybp) is consistent with echidnas being derived from a platypus-like ancestor.

Flannery *et al.* (1995) evoke the possibility that the monotreme transition to a semi-aquatic lifestyle occurred during the Early Cretaceous, by attributing a function of crushing hard crustacean exoskeletons or shellfish, to the bunodont molars of the Aptian (approx 110 mybp) monotreme, *Kollikodon ritchiei*. An early transition among stem monotremes to a semi-aquatic niche requires a subsequent reversal for echidnas. Though beyond the scope of this chapter, such a history for

echidnas may in fact be helpful for explaining a number of their traits. For example, the skull and thorax of echidnas are unusually flattened for a synapsid. More extreme dorsoventral flattening is a characteristic of the platypus that is considered to be associated with reduction of aquadynamic drag (Griffiths 1978), and helps reduce the energetic cost of swimming (Fish *et al.* 1997). Another unusual feature of both extant monotremes is a standing posture with an out-turned pes (hind foot and ankle). Szalay (1993b) proposed this to be an adaptation related to fossorial activity. Counting against this being the primary selective factor for an out-turned pes is that similar adaptation is not known to occur among any of the many groups of extant or extinct fossorial, or semi-fossorial mammals. Alternatively, this modification (due largely to the relative positions of the tibia and fibula: Lewis 1983) may allow the platypus pes to be held fast to the body for drag reduction (Fish 2000), in an orientation that allows it to work as a rudder (Grant 1995). Among echidnas the ankle and claws are also rotated, such that the pes appears to face posteriorly (Pridmore 1985). Perhaps this further (more distal) rotation was selected for improved terrestrial mobility, as compensation for a stance inherited from a platypus-like, semiaquatic ancestor.

*Steropodon galmani* and its dentally unusual Aptian contemporary *Kollikodon ritchiei* both share the hypertrophied mandibular canal that contains the nerves and blood vessels that supply the extensive rhinarium of the platypus. The presence of large mandibular canals in the Early Cretaceous monotremes is consistent with habits that involve fossorial or aquatic foraging. The rhinarium of both platypuses and echidnas is specialized for short-range physical and electrical detection of (often) visually obscured prey (Andres *et al.* 1991; Pettigrew *et al.* 1998). These functions are integrated with large and complex brains (Manger and Pettigrew 1995). A further characteristic common to *Steropodon* and *Kollikodon* is large size (relative to almost all other known Early Cretaceous mammals). The tooth and jaw measurements given for these taxa respectively by Archer *et al.* (1985) and Flannery *et al.* (1995) indicate they were about the size of a small river otter.

During the Cretaceous, southern Australia was within, or close to the Antarctic Circle and had mean annual temperatures probably less than 5 °C (Rich *et al.* 1988). For a small homeothermic animal entering a cold aquatic environment, selection is expected for larger body size. Wolff and Guthrie (1985) showed that semiaquatic mammals are generally larger than close terrestrial relatives, with increased thermal inertia and relative increase of propulsion over drag being associated with larger size. It would not seem inappropriate to speculate that the evolution of increased body size in monotremes coincided with an ecological transition to a semi-aquatic niche, and occurred before the divergence of *Steropodon* and *Kollikodon*.

Although molecular biology techniques are not directly applicable for taxa known only from Mesozoic fossils, they may still be used to place limits on the affinities of monotremes among the background phylogeny. In contrast to the original phylogenetic analyses involving complete mitochondrial (mt) genomes of monotremes (Janke *et al.* 1996, 2002) it was shown in Chapter 2 that by nullifying nucleotide composition bias, mtDNA supports monotremes being excluded from a therian clade. This conclusion is further cemented by the results of Killian *et al.* (2001) for the nuclear gene, mannose 6-phosphate/insulin-like growth factor II receptor (MP6/IGF2R). However, MP6/IGF2R may be unreliable for molecular dating estimates, as the study of Killian *et al.* (2000) reveals that this gene is evolving under quite different functional constraints in monotremes, in comparison to placentals and marsupials. Similar criticism cannot be leveled at mt genes, as their respiratory (protein-coding genes) and RNA-processing (ribosomal RNA and transfer RNA genes) functions are highly conserved across all vertebrates and most other animals (Slonimski 1982; Boore 1999).

The mtDNA analysis of Chapter 2 confirms the conclusion of a smaller (partial 12S RNA only) study of Gemmell and Westerman (1994) that monotremes could have split from therians only a few million years before marsupials and placentals diverged. For the complete protein-coding and RNA-coding mt sequences, the small amount of evolution that occurs from the LCA of monotremes and therians, to the LCA of marsupials and placentals, could hardly account for even 20 mybp. This would certainly rule out any hypotheses of monotremes arising from the backbone lineage of mammalian evolution prior to the beginning of the Jurassic (50-65 million years before the LCA of marsupials and placentals; see Chapter 2). Hence, monotremes must share a closer relationship with therians than does *Morganucodon*. I am not aware of any recent (since and including Kemp 1983) morphological studies that contradict this.

Upon consideration of the current CMI analysis, the incongruence and cladistic analyses, as well as aspects of monotreme palaeoecology, I suggest six points that may be considered as a framework for assessing the placement of monotremes among the background phylogeny.

1. Homoplastic “phylogenetic” signal relating to monotreme placement varies at the level of meta-regions, rather than being randomly distributed among characters. Any proposal of monotreme affinities must explain the non-independence of homoplastic character evolution within at least two of the three meta-regions (U, LB, and MVD).
2. Much of the incongruence is expected to involve transformations along the monotreme stem lineage, as opposed to homoplasy that is “hidden” among the background phylogeny.
3. Reversal of an evolutionary trend within a lineage is unlikely unless there is a change in the functional relationships of the characters involved.

4. As a consequence of point 3, any correlated homoplasy that is “hidden” among the background phylogeny (of taxa retaining plesiomorphic mammalian niches) is likely to be parallelism or convergence, so would tend to pull (bias) the placement of monotremes toward the outgroup.
5. Monotremes stem from small terrestrial (or perhaps scansorial) insectivore/carnivores and by the last common ancestor of platypuses and echidnas, fed mostly on invertebrates, were mid-sized, fossorial and probably semiaquatic.
6. Study of molecular sequence data requires that monotremes arose from the mammalian backbone lineage prior to the placental/marsupial split, but more recently than morganucodontids. Any character in conflict with this must be considered as homoplastic.

It is hoped that examining monotreme affinities within the framework set out above will provide more insight than does simply combining incongruent data partitions for parsimony analysis. The former task involves comparing (between Monotremata and the generalized insectivores) phylogenetic trends for the functional relations of character complexes, and treating potential monotreme positions among the background phylogeny as competing options for homoplasy. Such a method should converge on the placement of monotremes among the background phylogeny as the relationship between form and function of Mesozoic mammal characters (especially those of monotremes) becomes better understood.

Without access to the relevant fossil material, the anatomical meta-region-level homoplasy that is effecting monotreme affinities cannot be thoroughly assessed against the framework set out above. However, as a case study, I have made a preliminary comparison of phylogenetic trends (among the generalized insectivores) for the functional relations of shoulder and forelimb characters, and compared these with the conditions in monotremes.

#### *4.3.3 Parasagittalism as a phylogenetic trend among generalized Mesozoic mammals*

A “reptilian” grade shoulder girdle and oviparity (egg-laying) are the classic features cited (e.g. Simpson 1945; Romer and Parsons 1977) in establishing monotremes as a group that separated from the mammalian backbone lineage well before marsupials and placentals diverged. Recent improvements in the fossil record have had little bearing on understanding reproduction in Mesozoic mammals (though see Kielan-Jaworowska 1979), but have further highlighted the primitive nature of monotreme shoulder girdles and forelimbs (e.g. Jenkins and Schaff 1988; Sereno and McKenna 1995; Ji *et al.* 1999).

In the present analysis, the upper appendicular characters place monotremes outside the Mammaliaformes. However, Figure 4.6a shows this is only supported in 62% of bootstrap replicates, and in fact only one character, the presence of an incipient (scapular) supraspinous fossa supports monotreme exclusion from Mammaliaformes. This placement conflicts with 32 apparent synapomorphies among the dataset that support monotreme affinities with, or within the Theriimorpha, as well as the mitochondrial genome data (Chapter 2), which requires that monotremes be placed more closely to therians than is *Morganucodon*. In fact, Sereno and McKenna (1995) recognize three shoulder girdle synapomorphies that place monotremes closer to therians than is *Morganucodon*, though I suggest that each of these characters (which are not included in the current dataset) is unreliable for determining monotreme placement.

The three putative shoulder girdle synapomorphies for a Monotremata-Theriimorpha clade (absence of the procoracoid foramen, the absence of the coracoid groove, and the fusion of the scapulocoracoid suture) may simply be autapomorphic for monotremes. Among trechnotheres, eutriconodonts and multituberculates (but not monotremes), these characters should perhaps be considered as inapplicable, as the above states cannot be distinguished from the loss or reduction, and exclusion from the glenoid of the coracoids. Furthermore, my inspection of monotremes indicates that the scapulocoracoid suture remains unfused in subadult platypuses and echidnas, in agreement with McKenna (1961).

The important test for the reliability of the upper appendicular characters is across the theriimorpha node, which defines the intersection of three branches (or alternate monotreme placement hypotheses), exclusion from Theriimorpha, placement with triconodontids, or placement with trechnotheres. As shown in Table 4.3, among the 91 characters there are 10 apparent synapomorphies for monotreme placement outside Theriimorpha, and all of these are upper appendicular characters. Only one character, contact of the cuboid on the anteromedial aspect of the calcaneum (an ankle bone) could be considered an apparent synapomorphy of monotremes and triconodontids (or at least *Jeholodens*). However, multituberculates also share this character. Further, comparison of the drawings of Szalay (1993b) and Ji *et al.* (1999) reveals that the relationships between the cuboid, calcaneum, navicular and astragalus of monotreme feet are so different from the relationships of those bones in the feet of *Jeholodens* and multituberculates, that similarity in the angle of contact between the calcaneum and cuboid appears rather superficial. Meanwhile, 25 apparent synapomorphies of monotremes with, or within Trechnotheria are spread out among U (1), LB (8) and MVD (16). It is of course this anatomical region dependence of apparent synapomorphies for monotreme placement that provides the highly significant ( $p < 0.0001$ ) excess of observed incompatibility above that expected under RSCM, across the theriimorpha node for both U\*LB and U\*MVD (Table 4.2; Figure 4.7).

**Table 4.3** Anatomical region distribution of apparent synapomorphies for competing monotreme placements with either of the three potential clades (or associations) defined by the theriomorpha node on the unrooted background phylogeny: outside Theriomorpha; with triconodontids; or with trechnotheres (including the trechnothere stem). Superscript denotes the supporting characters, for which numbering follows Ji *et al.* 1999: <sup>a</sup>(13, 15, 16, 18, 19, 22, 23, 24, 30, 32); <sup>b</sup>(50); <sup>c</sup>(12); <sup>d</sup>(34, 36, 41, 42, 54); <sup>e</sup>(57, 58, 60); <sup>f</sup>(69, 73, 75); <sup>g</sup>(2, 3, 6, 7); <sup>h</sup>(83, 90, 91, 92, 93, 94, 97, 98, 101).

Anatomical region	Number of characters supporting monotreme placement		
	Outside Theriomorpha	With triconodontids	With trechnotheres
Upper appendicular	10 <sup>a</sup>	-	1 <sup>c</sup>
Lower appendicular	-	1 <sup>b</sup>	5 <sup>d</sup>
Basicranial	-	-	3 <sup>e</sup>
Mandibular	-	-	3 <sup>f</sup>
Vertebral	-	-	4 <sup>g</sup>
Dental	-	-	9 <sup>h</sup>
Total	10	1	25

Explaining the conflicting apparent phylogenetic signals with respect to the theriomorpha node (U\*LB, U\*MVD: see Figure 4.7) requires correlated homoplastic evolution of characters across U, or both LB and MVD. Upon consideration of the factors listed in the framework for assessing monotreme affinities, the signal associated with the upper appendicular region is shown to be a strong candidate for reflecting correlated homoplasy (rather than monotreme affinities). This could in turn explain the incongruence across the theriomorpha node for the placement of monotremes among the background phylogeny.

Before examining the monotreme upper appendicular characters I will briefly overview the (upper appendicular) trend from sprawling to parasagittal posture along the synapsid lineage leading up to modern therians. Firstly, Gambaryan and Kielan-Jaworowska (1997) point out that tetrapod postures cannot be dichotomously lumped (sprawling versus parasagittal) as simply as many discussions of locomotion imply. Lizards typically sprawl (see Jenkins and Goslow 1983; Pridmore 1985), with the manus (front foot and wrist) placed well lateral to the shoulder joint. Cursorial (running specialist) therians such as dogs and most ungulates have an essentially parasagittal gait, with the elbow and manus being held almost in line (sagittally) with the shoulder glenoid throughout the swing. Matters are somewhat confused by the fact that for most generalized therians, while the manus may be placed under the glenoid, or even closer to the midline, the humerus is abducted 10°-30° from the sagittal plane (Jenkins 1971a) so that the elbow is placed lateral to the shoulder joint. These generalized therians (such as *Didelphis* and *Rattus*) have what I call an abducted-parasagittal gait, because the proximal limb elements (humeri, femora) are

somewhat abducted, but unlike their reptilian ancestors, they possess a trochlea articulation between the humerus and ulna that restricts movement at that joint to one plane (Jenkins 1973). For simplicity, I will use the term, near-parasagittal to refer to both the slightly abducted parasagittal gait of generalized therians, and the more fully parasagittal gait of many ungulates and carnivores.

Of the monotremes, the platypus has a sprawling posture, with the manus typically planted just lateral to the elbow (Pridmore 1985). The echidna however appears to be unique, in that the manus is typically planted under the glenoid, as for most therians (Jenkins 1970b), but the humerus is abducted near horizontal and like the platypus and other sprawlers, the elbow joint does not include a humeroulnar trochlea (see Figure 4.11).

Permian synapsid pelycosaurs such as the carnivore, *Dimetrodon*, and the herbivore *Edaphosaurus* had a purely sprawling posture (Jenkins 1970a, 1971b), with the humerus held approximately horizontal and the manus placed well lateral to the shoulder glenoid. Forward propulsion was due mostly to a combination of horizontal retraction and long-axis rotation of the humerus (Jenkins 1970a, 1973). Such locomotion places considerable compressional forces on the thorax (Jenkins 1970a), which have to be accommodated by a massively robust shoulder girdle. Among mammal-like reptiles (and monotremes) this shoulder girdle includes an interclavicle, a thick clavicle, and a scapulocoracoid plate that consists of the scapula, posterior coracoid (metacoracoid), and anterior coracoid (procoracoid).

From pelycosaurs, though Triassic cynodonts, and along the backbone lineage up to therians, the near-parasagittal gait gradually evolved. This transformation involves a number of interrelated trends. The trend for the glenoid (and so also the long axis of the humerus) was to face more posteriorly and more ventrally (Jenkins and Weijs 1979; Kemp 1982). With this reorientation, medial rotation about the long-axis of the humerus translates less efficiently into forward momentum, as a greater lateral component of momentum is produced. Elevation of the distal end of the humerus (relative to the proximal, or shoulder joint end), adduction of the humerus (bringing the elbow closer to the body), and extension/flexion at the elbow contributed more to forward movement (Jenkins 1973).

The directional change in glenoid facing results in the manus being brought closer to the midline of the body. As a result, the medial vector of the ground force in reaction to locomotion (or posture) is reduced (see Blob 2001) so that the compressional force on the thorax is reduced. This in turn is thought to have allowed for the trend toward less massive shoulder girdle bracing, culminating in the loss or reduction of the metacoracoid, procoracoid and paired desmal interclavicle elements

(see Klima 1973). Indeed, Jenkins (1974) demonstrated that the clavicle of abducted-parasagittal mammals was under compression and suggested that it was the reduced medial forces imparted by “fully” parasagittal mammals that permitted further reduction of the shoulder girdle with the loss of this element in many taxa.

Upon a more parasagittal gait (than cynodonts and *Morganucodon*) being evolved (possibly independently) among trechnotheres, triconodontids, and multituberculates, a lessened requirement for shoulder bracing allowed for freedom, both at the clavicle/interclavicle (or sternum) joint and for the clavicle with respect to the acromion of the scapula (see Sereno and McKenna 1995; Hu *et al.* 1997; Ji *et al.* 1999). Along with the reduced size of the coracoids, this permitted movement of the scapula, with the clavicle acting both as a spoke and a strut (Jenkins 1974). Gambaryan and Kielan-Jaworowska (1997) dispute the claim of Sereno and McKenna (1995) that taeniolabidoid multituberculates had near-parasagittal forelimb posture, instead explaining multituberculate upper appendicular characters as being adapted for jumping with abducted forelimbs.

A number of factors have been suggested to be important in selection for parasagittalism among mammals. Parasagittalism, particularly with the incorporation of scapular rotation (through a vertical plane), can increase stride length (Jenkins and Weijs 1979), while placement of the manus closer to the midline and centre of gravity may increase agility (Rewcastle 1981), perhaps for direction changing in particular. Less abducted proximal limb elements also enhance the potential for conserving energy by using elastic mechanisms and pendular movements (Cavagna *et al.* 1977; Alexander 1991). Reduced emphasis on humeral long-axis rotation allows for smaller tubercles on either side of the humeral head (Gambaryan and Kielan-Jaworowska 1997), which in turn allows greater forelimb mobility. Both Muizon (1998) and Argot (2001) applied a similar causal link to explain the presence of lower humeral tubercles in arboreal (as compared with terrestrial) marsupials. Furthermore, Heath (1968) and Carrier (1987) have convincingly argued that the evolution of locomotory endurance among mammals was tied to the evolution of a more parasagittal gait, with lung expansion able to be synchronized with dorsoventral vertebral flexion. In fact Carrier (1987) suggested upright posture, endurance and endothermy to be correlated in both mammals and the ancestors of birds. Certainly the increased food supply that must have been necessary to support a higher level of resting metabolism would likely require a greater foraging range, and hence locomotory endurance.

Given the variety of potential selective advantages associated with the evolution of parasagittalism among non-specialized mammals, it is not surprising that (as discussed earlier) phylogenetic inference indicates a more or less continuous postural trend from the sprawling pelycosaurs through to the near-parasagittal therians. Extensive parallelism between spalacotheriids and

triconodontids, that is associated with these taxa independently following the trend towards increased parallelism, is one hypothesis for explaining the highly significant signal conflict across theriimorpha for monotreme placement. This seems especially reasonable, given that Hu *et al.* (1997) and Ji *et al.* (1999) respectively demonstrated that the spalacotheriid, *Zhangheotherium* and the triconodontid, *Jeholodens* were both small terrestrial predators, and so probably were subject to very similar selection pressures for locomotory traits. In fact, for explaining monotremes grouping with trechnotheres and multituberculates (to the exclusion of the eutriconodonts, *Jeholodens* and *Gobiconodon*) in their cladistic analysis, Ji *et al.* (1999) appeared to favour parallelism between trechnotheres and eutriconodonts, rather than reversal of monotreme upper appendicular characters.

The evidence that Ji *et al.* (1999) provided in support of parallelism between *Jeholodens* and trechnotheres was from phylogenetic inference, and this relied on multituberculates and monotremes being consecutive sister groups to Trechnotheria. With the affinities of these highly derived taxa (multituberculates and monotremes) both being highly controversial, this evidence carries little weight (apparently with Ji *et al.* also). However, a number of lines of evidence suggest the advanced shoulder girdles of eutriconodonts and trechnotheres were in fact inherited from their last common ancestor.

In their review of mammalian character evolution, Hopson and Crompton (1969) suggested that parallel evolution of complex characters (even traits with functions that are highly interrelated) is usually conspicuous by the mosaic nature of character states being acquired at different rates and in different sequences. This certainly appears to be the case for both parallel and convergent evolution among cynodonts and/or mammals for dental (Butler 1990; Muizon and Lange-Badré 1997) and basicranial (Lou 1994) characters. Further, reduction and loss of non-dentary mandibular elements has occurred independently among eutriconodonts (Ji *et al.* 1999 supp. info.; Wang *et al.* 2001), spalacotheriid symmetrodonts (Cifelli and Madsen 1999) and eupantotheres (Krebs 1971; Heinrich 1998). However, little, if any, upper appendicular mosaic evolution appears to have occurred between early trechnotheres and eutriconodonts.

Even with an extra eutriconodont (*Gobiconodon*) and eupantothere (*Henkelotherium*) included with the background phylogeny (as for the expanded taxon sampling of Chapter 3), none of the 22 upper appendicular characters unequivocally support parallel acquisition of apomorphies between eutriconodonts and trechnotheres. In contrast, ten of these characters are unequivocal apomorphies for the branch leading to the eutriconodont/trechnothere LCA. The incompletely known *Gobiconodon* upper appendicular elements (Jenkins and Schaff 1988; Lou *et al.* 2001a supp. info.) may provide further insight. A hypothesis that an advanced shoulder girdle was inherited by eutriconodonts and trechnotheres from their common ancestor would gain further support if

gobiconodontids and triconodontids are paraphyletic with respect to trechnotheres, as is favored in Chapter 3, and by a number of other recent studies (Ji *et al.* 1999; Kielan-Jaworowska and Dashzeveg 1998; but see Wang *et al.* 2001). In this case, upper appendicular traits such as coracoid reduction, reorientation and shaping of the glenoid, expansion of the scapula supraspinous fossa, and trochlea articulation for the humero-ulnar joint (dorsally) would each have to be postulated to have evolved independently at least three times, rather than as synapomorphies of the triconodontid, gobiconodontid and trechnothere clade they support (among the generalized insectivores).

In summary, there is perhaps more contradiction than support for the idea that the highly significant conflict across theriimorpha for U\*LB and U\*MVD (Figure 4.7) can be explained by correlated parallelism of upper appendicular characters, between taxa of the background phylogeny. Hence it is of particular interest to explore the possibility of correlated reversal of upper appendicular characters occurring along the monotreme stem lineage during the transition from a small generalized insectivore, to a larger animal, with a fossorial and probably aquatic niche.

#### 4.3.4 *Correlated reversal of monotreme upper appendicular characters*

Despite the potential selective advantages, the trend towards parasagittalism that occurs among mammals has of course not been taken up universally among terrestrial vertebrates. Generalized lizards have retained a sprawling, and symmetrical diagonal gait (one forelimb followed by the opposite hindlimb, then the other forelimb followed by its opposite hindlimb: see Gambaryan 1974). These lizards are typically short-range ambush predators for which burst acceleration seems to have been selected for (Bennet 1982), rather than endurance and direction change agility. Among mammals (including monotremes) the proximal limb elements make reciprocatory (back and forth) movements during which momentum is somewhat discontinuous (Pridmore 1985). In contrast, generalized lizard limb motion is coupled with the massive tail musculature and lateral flexion of the thorax, with proximal limb elements moving such that the elbow and particularly the knee trace a somewhat circular path (see Pridmore 1985). This rotational limb cycle enhances momentum conservation (Bakker 1971, Hamley 1990) compared to the acceleration-deceleration limb cycles of mammals. A more parasagittal gait among these lizards would tend to uncouple the forelimbs from the hindlimb and tail elements, disrupting the simple-harmonic-motion-like momentum conserving locomotion.

As with generalized lizards, monotremes have very different niche requirements to those of the Mesozoic small mammalian terrestrial insectivores of this study. Monotreme forelimbs are

adapted for operating in high-density soil, or water environments (see Augee and Gooden 1993; Grant 1995), unlike the forelimbs of generalized terrestrial predators, which push off a hard substrate and swing through air during locomotion. Hence, it should not be surprising that monotreme upper appendicular evolution has not followed the phylogenetic trends (associated with parasagittalism) that occurred among the non-specialized mammals and mammal-like reptiles.

In this section I argue that the upper appendicular trends that may be beneficial among cursorial and scansorial mammals, in fact translate as constraints for fossorial and aquatic mammals. Following on from this, I develop a hypothesis that the apparently primitive monotreme upper appendicular characters are instead, a reflection of specialized fossorial/swimming habits that incorporate hypertrophied humeral long-axis rotation.

Cost of transport data (Fish 1992; 2000) and biomechanics (Williams 1999) reveal trade-offs between terrestrial and aquatic locomotion. A similar trade-off between terrestrial locomotion and fossorial adaptations is also expected. Rather than long limbs with proximal muscle attachments providing for long stride per muscular effort, the use of forelimbs for digging/swimming through dense mediums places the emphasis on mechanical advantage, which requires stout forelimbs with relatively distal insertions for the shoulder and breast musculature. These latter features are common to monotremes and therians that use forelimbs for fossorial/swimming habits. A prominent feature of monotreme forelimbs that is shared with numerous fossorial therians (see Kielan-Jaworowska and Gambaryan 1997; Gasc *et al.* 1986) is an olecranon process (see Figure 4.11) that provides a massive insertion site for the triceps musculature at the proximal end of the ulna. From this, a role may be inferred for powerful forearm extension during digging/swimming.

Gambaryan and Kielan-Jaworowska (1997) noted that the primitive features that monotremes share with fossorial therians include: a distinct, rounded radial condyle (talpids, chrysochlorids and myospalacidae); humeral torsion that is greater than for Mesozoic theriomorphs (chrysochlorids); and expanded epicondyles (talpids, chrysochlorids and myospalacidae). Marsupial moles (Notoryctids) are poorly known, though Warburton (pers. comm.) has found that these also possess a distinct, rounded radial condyle and expanded epicondyles. However, marsupial moles and chrysochlorids share a number of characteristics that are notably different from those of monotremes. The fossorial behavior of these “sand swimming” moles involves shovel-like head digging, tail buttressing, and relatively parasagittal forelimb movement (Gasc *et al.* 1986). As mediolateral torsional forces depend largely on humeral abduction during locomotion (Jenkins 1974) the relatively parasagittal forelimb movement might explain the high pectoral mobility, and hence limited transverse pectoral bracing among these taxa. In contrast, fossorial and fossorial/semiaquatic members of the talpid mole family are like monotremes in that they typically

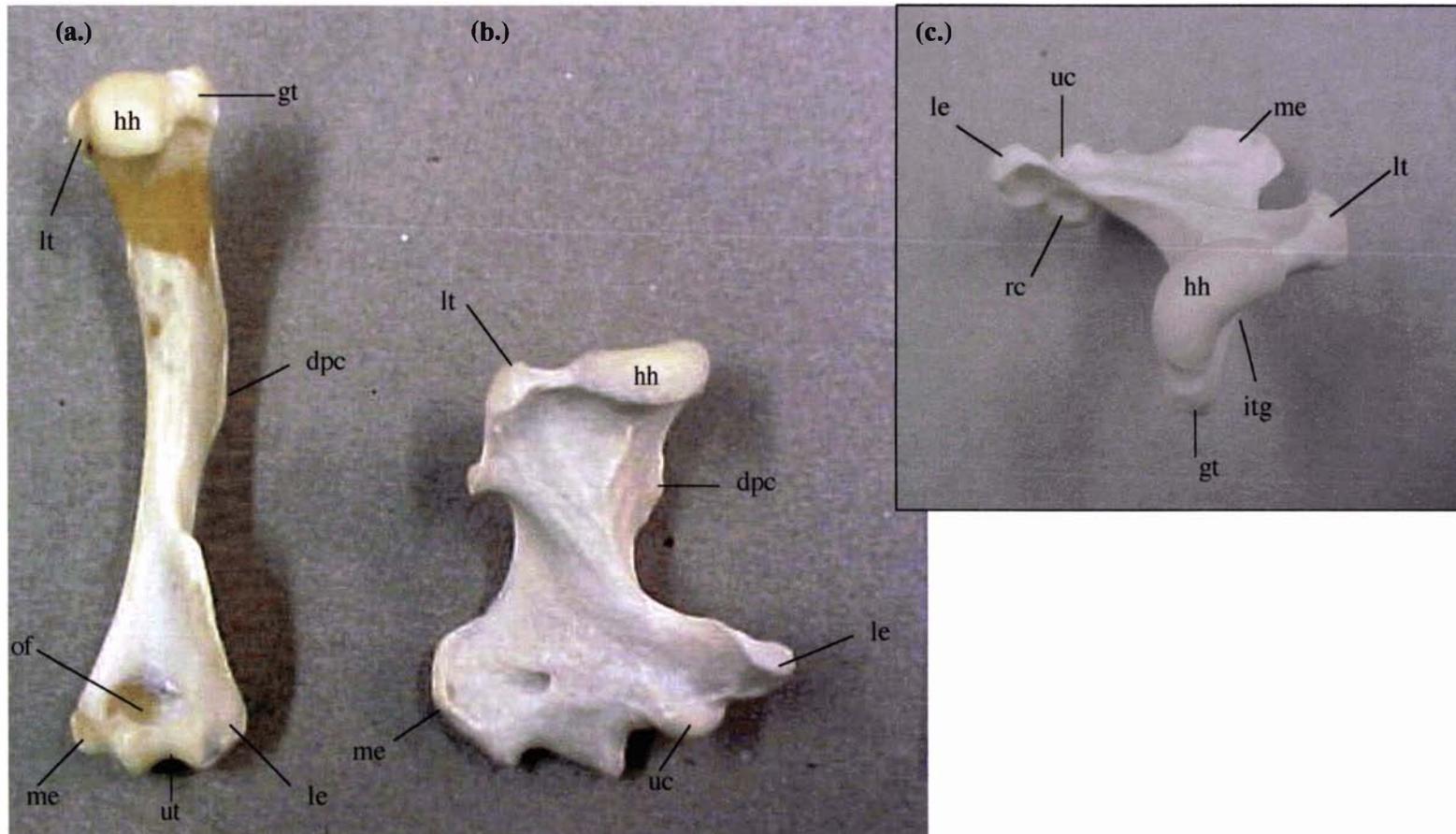
dig in more solid soils and mostly by the use of the forelimbs. These talpids also share with monotremes a highly abducted forelimb posture (Yaldon 1966), and considerable humeral long-axis rotation (but at a claviculo-humeral joint, rather than the shoulder glenoid; see Gambaryan and Kielan-Jaworowska 1997).

Semi-aquatic therians that have largely retained the adaptations associated with a parasagittal terrestrial gait also employ their limbs in parasagittal paddling. Whether utilizing only the forelimbs (e.g. polar bears, ferrets), mainly the hindlimbs (e.g. muskrats, mink), or both sets of limbs (e.g. desmans, giant otters) for propulsion, parasagittal paddling is inefficient, with much energy lost to drag while repositioning the limbs during the recovery stroke (Fish 1993; Baudinette and Gill 1985). Among aquatic placentals that retain little or no requirement for terrestrial locomotion, whales, sirens, true seals and walruses barely employ their front limbs for propulsion (Gordon 1983; Fish 2000), while sea lions combine lift-based oscillations with (non-parasagittal) rowing motions (Feldkamp 1987) for a highly derived, and aerobically efficient forelimb propulsion. Terrestrial locomotion is also of relatively limited importance for platypuses, though their forelimbs, unlike those of seals, whales and sirens, are used for digging massive tunnels into creek and river banks (Grant 1995).

Platypus forelimbs are the primary digging tool and also deliver swimming propulsion that Fish *et al.* (1997) show is, in terms of energetic cost of transport, similar to whales and seals, and much more efficient than semi-aquatic placentals such as muskrats and minks, or even sea otters. This efficiency was largely attributed to the specialized platypus rowing mode.

Forelimb propulsion among generalized therians involves anterior to posterior movement of the distal end of the humerus (mostly distal elevation), relative to the proximal end, as well as rotation of the scapular in a vertical plane (Muizon 1998). Cynodont and morganucodontid forelimb propulsion has been inferred from their glenoid orientation (Jenkins 1971b; Jenkins and Parrington 1976) and elbow joint anatomy (Jenkins 1973). These studies show that advanced cynodonts and morganucodontids appear to have employed considerable horizontal and vertical components of anteroposterior movement of the distal (relative to proximal) end of the humerus, as well as humeral long-axis rotation. It is an important point that monotremes do not conform to either therian or cynodont patterns.

Jenkins (1970b) and Pridmore (1985) used cineradiographic analysis to study forelimb movement during monotreme terrestrial locomotion. It was shown that while horizontal retraction of the humerus plays some role in terrestrial propulsion (at least for the platypus), long-axis rotation of the humerus is the major forelimb propulsive component for both echidnas and platypuses. With



**Figure 4.9** Right humeri of a non-specialized therian, *Dasyurus maculatus* (a) and a monotreme, *Tachyglossus aculeatus*, (b,c). Dorsal views are shown in (a.) and (b.), while a proximal view of the humeral head is shown in (c.). dpc, deltopectoral crest; gt, greater tubercle; hh, humeral head; itg, intertubercular groove; le, lateral epicondyle; lt, lesser tubercle; me, medial epicondyle; of, olecranon fossa; rc, radial condyle; uc, ulnar condyle; ut, ulnar trochlea.

more posterior and ventral shoulder glenoid orientations (than in monotremes), long-axis rotation of the humerus must have played a considerably less substantial role in generating propulsive forces in cynodonts, morganucodontids and indeed theriimorphs. Hence, compared to their mammalian backbone lineage ancestor, the magnitude of humeral long-axis rotation as a forelimb component of propulsion in monotremes may be considered to be hypertrophied (relative to humeral anteroposterior retraction/elevation), and a specialization at least of the crown group. Even among squamates that employ considerable humeral long-axis rotation, including varanid lizards (Jenkins and Goslow 1983), and *Sphenodon* (Pridmore 1985), it is anteroposterior movement of the humerus (distal relative to proximal) that is the major forelimb component of propulsion.

Cineradiographic analysis has not been published for platypus swimming or digging, but may be more useful for understanding monotreme upper appendicular evolution, given that the stresses involved with the slow walking of monotremes could hardly account for the massive shoulder girdle musculature and bracing. Nevertheless, the study of Howell (1937) and my personal observations of swimming platypuses are consistent with humeral long-axis rotation also being a major component of platypus aquatic propulsion. In fact, the articular surfaces of the humeral head and the shoulder glenoid of monotremes indicate that both the platypuses and echidnas are far more specialized for propulsion via long-axis rotation of the humerus, than are cynodonts and perhaps any other mammals. Among monotremes, the glenoid is track-like in its concavity, and is rotated through by the somewhat longer (anteroposteriorly) humeral head, which is ventrally concave (see Figure 4.9 for comparison with a typical marsupial, *Dasyurus*). The shoulder joint among other known Mesozoic cynodonts/mammals (e.g. Jenkins 1971b; Jenkins and Parrington 1976; Kemp 1982; Sun and Li 1985; Jenkins and Schaff 1988) is instead, of a ball and socket type, with the humeral head being at least hemispherical (not ventrally concave as in monotremes) and the glenoid being more open, especially ventrally.

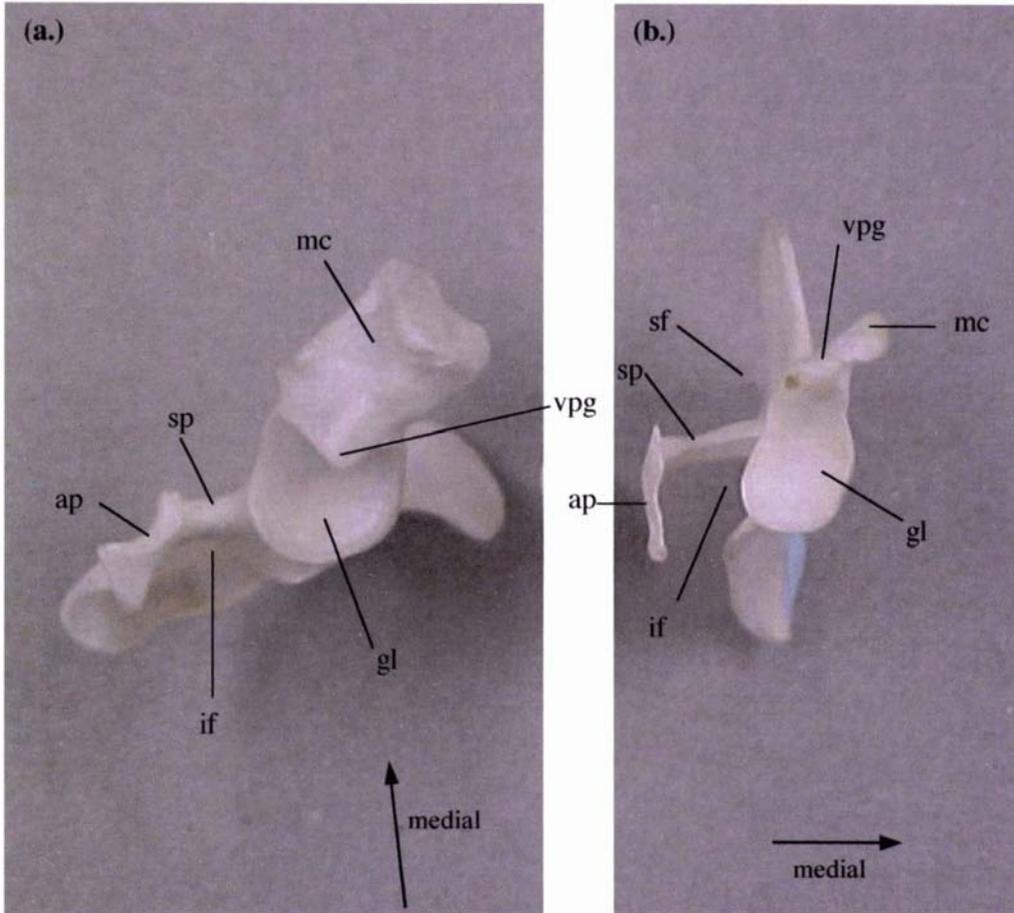
Further evidence for the importance of humeral long-axis rotation for monotreme locomotion and digging, comes from studies of muscle attachments. Jenkins and Weijs (1979) showed that the major muscles active during the propulsive phase of generalized therians, like *Didelphis*, were *m. pectoralis* and *m. latissimus dorsi*. As expected for near-parasagittal walking, these muscles respectively insert on the deltopectoral crest of the humerus, and just medial to this on the shaft. In monotremes (as shown for the echidna by Walter 1988) *m. pectoralis* and *m. latissimus dorsi* insert respectively on the greater tubercle of the humerus, and the medial epicondyle, so that their combined action would rotate the humerus. This rotation would be aided by action of *m. subscapularis*, which Walter (1988) showed to arise from the posterior half of the lateral side of the scapular (a particularly massive area in both the platypus and echidna) and insert on the lesser

tubercle. Gambaryan and Kielan-Jaworowska (1997) suggest *m. proscapulohumeralis*, which inserts on the lesser tubercle, also to be involved with humeral rotation.

Jenkins (1970a) asserted that specializations of monotreme shoulder girdle anatomy, including the highly abducted and transverse humeral posture (which can be related to hypertrophied humeral long-axis rotation, as discussed later) are associated with fossorial habits. Neither being privy to recent molecular work, or knowledge of platypus-like monotreme fossil discoveries from the Paleocene and Early Cretaceous, Jenkins may not have considered that a semiaquatic ancestry may have had a large influence on the evolution of both platypuses and echidnas. Functional analyses of platypus swimming and digging (among echidnas also) will be vital for testing hypotheses on the relationship between monotreme niche specializations and their upper appendicular morphology. For the moment though, it is interesting that the only extant mammal groups (monotremes and talpid moles) for which a departure from a generalized insectivore niche resulted in fossorial and swimming niches occurring among its members, are also the only extant mammal groups in which locomotion involves a transverse and highly abducted humerus and considerable humeral long-axis rotation. Such conditions (except partial humeral abduction) are not present among extant fossorial therians in which a semiaquatic habit is neither inferred for ancestors, nor present among close relatives (such as digging rodents, golden moles and marsupial moles).

Talpidae (see: Nowak 1991; Whidden 2000) includes swimming specialists such as desmans, tunnelers such as *Talpa*, and combined fossorial/semiaquatic members such as the star-nosed mole, *Condylura*. Given the parallels with monotremes, it is interesting that both a sprawling posture and humeral rotation (at the humero-clavicular joint) has evolved from a standard therian near-parasagittal posture and gait among talpids (Castiella *et al.* 1992; Gambaryan and Kielan-Jaworowska 1997). These apparent adaptations may be considered as further evidence for the possibility that with an escape from the constraints placed by running and climbing, and the occupation of a niche that combines swimming and digging, a reversal of the trend among generalized insectivores toward parasagittalism may be selected for.

Whether or not the primitive aspects of monotreme upper appendicular regions are plesiomorphic, or the result of reversal from more advanced states, evolutionary trends among other Mesozoic mammals toward parasagittalism were not selected for among monotremes. As noted earlier, shoulder joint structure, humeral orientation, the size and location of muscle attachments, as well as evidence from cineradiographic movement studies, all point towards hypertrophy of humeral long-axis rotation being the major locomotory specialization that occurred along the monotreme stem lineage.



**Figure 4.10** Right scapula of a monotreme, *Tachyglossus aculeatus* (a.) and a non-specialized therian, *Dasyurus maculatus* (b) in distal view. ap, acromion process; gl, glenoid; if, infraspinous fossa; mc, metacoracoid (=coracoid process); sf, supraspinous fossa; sp, scapular spine; vpg, ventral process of glenoid.

Pridmore (1985) provides a starting point from which to search for potential homoplastic correlation among monotreme upper appendicular characters. He showed that if platypus and echidna postures were corrected for their glenoid orientation and humeral torsion, both would have a posture similar to generalized therians. Long-axis rotation of the humerus is translated into forward (as opposed to lateral) momentum more efficiently with a more transverse humeral orientation. Hence, the near-lateral facing glenoid of monotremes can be related to hypertrophied humeral long-axis rotation providing the major (forelimb) component of propulsive force. In turn, with transversely (or even obliquely) orientated humeri, a combination of humeral torsion and condylar (rather than trochlear) humeroulnar joint structure, allows the manus to trace a path in a more or less parasagittal plane (Jenkins 1973). Furthermore, inspection of Figure 4.10 shows that the relationship between the orientation of the glenoid, the metacoracoid (coracoid process), the scapular spine, and the acromion is the same for monotremes and generalized therians, with this complex of characters simply co-varying with the orientation of the glenoid, with respect to the outline of the thorax (which is indicated by the scapula blade). Hence, the orientation of this suite of characters (the glenoid complex) appears to be developmentally and functionally non-independent.

In order to explain the highly significant conflict across theriomorpha for U\*LB and U\*MVD (Figure 4.7) I propose that the apparently primitive monotreme upper appendicular characters, instead of reflecting phylogenetic signal, are a reflection of specialized fossorial/aquatic habits. It is my hypothesis that the evolutionary non-independence of upper appendicular characters (in relation to monotreme affinities: Figure 4.8a) may be explained by developmental and functional correlation that is associated with hypertrophied humeral long-axis rotation and mechanical advantage for pulling the manus through soil or water.

The framework for assessing monotreme affinities in terms of homoplasy options offers some useful rules for testing whether the hypothesized correlated reversal among upper appendicular characters is consistent with the transition of (generalized insectivore) stem monotremes into a larger animal with fossorial and probably aquatic habits. Comparison of the conditions in monotremes, with phylogenetic trends (among the generalized insectivores) for the functional relations of shoulder and forelimb characters provides a basis for this test.

Table 4.4 provides a summary of my inspection of the ten upper appendicular characters for which the character maps exclude monotremes from Theriimorpha. Where applicable, the monotreme condition is noted as:

- more primitive, or more advanced, than in morganucodontids, or otherwise considerably modified.
- potentially correlated with hypertrophied humeral long-axis rotation and/or provision of mechanical advantage, directly (L), or indirectly by provision of bracing (B), or by co-varying with the orientation of the glenoid (G).
- potentially resulting from outgroup-attraction via character coding asymmetry.
- potentially resulting from outgroup-attraction via paedomorphosis.

A more detailed account for each character, of trends among the generalized insectivores and the condition in monotremes follows, with character numbers referring to the dataset of Ji *et al.* (1999). For these accounts, unless otherwise stated, I refer to Romer and Price (1940), Jenkins (1971b), Jenkins (1973), Kemp (1982, 1983) and Rowe (1988) for pelycosaurs and cynodonts, Sun and Li (1985), Sues (1985) and Rowe (1988) for tritylodonts, Jenkins and Parrington (1976) for Morganucodontids, Ji *et al.* (1999) and Jenkins and Schaff (1989) respectively for the eutriconodonts, *Jeholodens* and *Gobiconodon*, Hu *et al.* (1997, 1998) for the symmetrodont, *Zhangheotherium* and Krebs (1991) and Rougier (1993) respectively for the eupantotheres, *Henkelotherium* and *Vincelestes*. Monotreme muscle attachments follow the study of Walter (1988) on echidnas.

**Character 13.** The **clavicle/sternal apparatus joint** becomes increasingly mobile among the generalized insectivores. From near-mammalian cynodonts such as tritylodonts, through to the Triassic-Jurassic boundary mammals, such as *Morganucodon*, the interclavicle lateral processes (and their contact with the clavicle) are reduced. This trend continued with this joint being mobile in *Jeholodens* and trechnotheres. In these taxa the clavicle only slightly overlaps the lateral processes of the interclavicle (or the sternum, within which the interclavicle is incorporated in cladotheres). This trend must have been reversed along the monotreme stem lineage, as in both the echidna and platypus, the interclavicle is fused with the clavicle almost along its full length, such that monotremes have a less moveable, more stable clavicle/sternal joint than *Morganucodon*, or perhaps any other known synapsid. This stability is consistent with bracing against high compressional forces acting against the thorax, which as discussed earlier are expected for locomotion that involves substantial humeral long-axis rotation of transversely oriented humeri. Such forces might be particularly high among monotremes, given the massive shoulder and forearm musculature.

**Table 4.4** Summary of the states of the 10 upper appendicular characters that place monotremes outside Theriimorpha. The monotreme condition is indicated as either more advanced (toward therians), considerably modified, or primitive (and so reversed) compared to the condition in Morganucodontids. How the monotreme condition might be associated with swimming/digging is noted as niche related correlation directly with hypertrophied humeral long-axis rotation and/or provision of mechanical advantage (L), or indirectly by provision of bracing (B), or by co-varying with the orientation of the glenoid (G). Potential for outgroup attraction of monotremes is indicated by character coding being asymmetric (with the primitive state having the larger morphological range), and by the monotreme adult condition being present among marsupial neonates. An asterisk (\*) denotes where pedomorphosis along the monotreme stem may be inferred, as monotreme adults, and marsupial neonates share similarities for positional relations of the character that do not occur among adults of non-monotreme mammaliaformes.

Character (ji <i>et al.</i> , 1999)	State in monotremes and cynodont outgroup	Primitive state in Theriimorpha	monotremes compared with morganucodontids	niche related correlation	character-coding problem	marsupial neonate character
<b>13:</b> clavicle, sternal apparatus joint	immobile	mobile	more primitive, but modified	B	no	no
<b>15:</b> scapula, supraspinous fossa	absent	weakly developed	more primitive	G	no	Yes *
<b>16:</b> scapula, acromion process	not extending below glenoid	extends below glenoid	similar or more advanced (?)	G	no	yes
<b>18:</b> Procoracoid as separate element in adult	present	absent	modified	B, L	no	Yes *
<b>19:</b> Metacoracoid	large with posterior process	small, without posterior process	modified	G, B, L	no	Yes *
<b>22:</b> orientation of glenoid relative to scapula long axis	near parallel – faces posterolaterally	oblique – faces more posteriorly	more primitive, but modified	G, L	no	Yes *
<b>23:</b> shape and curvature of glenoid	saddle-shaped, oval and elongate	uniformly concave, rounded in outline	modified	B, L	asymmetry	?
<b>24:</b> medial surface of scapula	convex	flat	modified	G	asymmetry	?
<b>30:</b> humero-ulnar articulation	bulbous unlar condyle	cylindrical trochlea, condyle anteriorly	modified	L	asymmetry	?
<b>32:</b> humerus: entepicondyle and ectepicondyle	robust	weak	more primitive, but modified	L	asymmetry	?

**Character 15.** The **scapula supraspinous fossa** increases in its relative contribution to scapular muscle attachment from its incipient development in *Morganucodon*, to running much of the length of the scapular spine in eutriconodonts, and expanding further (anteriorly) in *Zhangheotherium* and cladotheres. Monotremes completely lack a supraspinous fossa, although a relatively small *m. supraspinatus* does originate on the anteroventral edge of the scapular, just dorsal to the glenoid and inserts on the greater tubercle of the humerus, the typical insertion site for therians (Jenkins and Weijs 1979). The monotreme condition appears particularly primitive, given that an incipient supraspinous fossa and a less medially originating acromion (inferred from the drawings and text of Jenkins and Parrington 1976) than in monotremes occur in *Morganucodon* and *Megazostrodon* and have even been suggested for the tritylodont, *Bienotheroides* (Sun and Li 1985). In fact, Howell (1937) suggested the monotreme suprascapular musculature appeared to be “arrested far short of the conditions indicated in Permian dicynodonts, or even short of cotylosaurs”. Although the primitive level of supraspinous development among monotremes is not known to occur among other mammaliaform adults, Broom (1899) and by inference from his drawings, Klima (1987) show that a supraspinous fossa is completely lacking in marsupials until later intrauterine embryological stages.

Selection for a laterally facing glenoid (which allows humeral long-axis rotation to be efficiently translated into forward momentum) may explain the primitive condition of the supraspinous musculature among monotremes. Given the monotreme orientation of the covarying glenoid complex of characters (see Figure 4.10), the supraspinous fossa would be medial to the infraspinous fossa, so would both be restricted by the rib cage and have its musculature largely obstructed from the forelimb by the anteriorly facing spine.

**Character 16.** The **acromion process of the scapula** (the lateral articulation for the clavicle) of non-theriimorphs, including monotremes, is a laterally reflected extension of the anteroventral margin of the scapula that reaches no further than the dorsal margin of the glenoid. Among theriimorphs, the acromion process is long, extending from the scapula spine (which is homologous with the anteroventral margin of the scapula of non-theriimorphs: Cheng 1955; Shrivastava 1962; Klima 1973) and offers a small, mobile articulation for the clavicle. With the monotreme orientation of the covarying glenoid complex of characters (see Figure 4.10), the scapular spine is oriented anteriorly rather than laterally, so that unlike the conditions among theriimorphs, no extension of the acromion is required for articulation with the clavicle. Additionally, the particularly broad and interlocking nature of the acromio-clavicular joint of platypuses and echidnas (more so than in other known cynodonts and Mesozoic mammals) is further evidence for the importance of shoulder girdle bracing among monotremes.

**Character 18.** The **procoracoid** undergoes size reduction and becomes excluded from the glenoid along the lineage from cynodonts to Triassic/Jurassic boundary mammals (such as *Morganucodon*). Among therians the procoracoid is often completely lost during development. However, as has been shown by McKenna (1961) and Klima (1987), in many marsupials (and probably for early trechnotheres for which procoracoids/praeclavia have either not been preserved or identified) the procoracoid persists as cartilage, or bone that communicates between the anterolateral edge of the sternum and the tip of the clavicle (as the praeclavium). For some marsupials (e.g. *Didelphis marsupialis*: Klima 1987) this element is ossified and extends over much of the edge of the portion of the sternum that is derived from the unpaired chondral element of the interclavicle. Among eutherians, similarly derived praeclavia (in some insectivores and rodents), or ossa suprasternalia (including in humans) have also been reported (see Klima 1987 for numerous references), and are homologous with the procoracoid.

For this character, Ji *et al.* (1999) coded monotremes for the same primitive state as non-theriimorph mammals, though the monotreme procoracoid differs from those of the non-theriimorphs in a number of ways. In non-theriimorph mammals the procoracoid is pierced by a foramen, and is sutured to both the metacoracoid and the scapular, such that if it extends anteriorly (as in *Probainognathus*: Jenkins 1971b) it nears the mid to lateral portion of the clavicle. In contrast, among monotremes there is no procoracoid foramen, and the relatively large procoracoid is widely excluded from the scapula. It also differs from non-theriimorph procoracoids in being more medially sutured to the metacoracoid, such that its anteromedial extension overlaps the dorsolateral edge of the posterior process of the interclavicle, up to where the interclavicle abuts the clavicle. It may be significant that these conditions of the monotreme procoracoid are unknown in any adults from other extinct or extant mammal groups, but occur during marsupial ontogeny (Klima 1987).

The monotreme procoracoid provides massive additional ventral attachment for muscles, including for mm. biceps, supracoracoideus and coracobrachialis, which respectively insert distally on the radius, on the greater tubercle, and on the lesser tubercle, with moment arms delivering considerable mechanical advantage. Furthermore, the interlocking of the procoracoid with the metacoracoid and interclavicle provides ventral bracing and resistance to the compressional forces associated with powerful digging/swimming with transversally oriented humeri (Jenkins 1970a).

**Character 19.** The **metacoracoid** of non-theriimorphs is relatively large and extends posteromedially and somewhat ventrally. This element is homologous with the processus coracoidus of therians (Broom 1899; Cheng 1955), which is much reduced and bends medially from just above the anterior lip of the glenoid. As Figure 4.10 shows, the position of the proximal

part of the metacoracoid with respect to the other elements of the glenoid complex is the same in monotremes and therians, covarying with the orientation of the glenoid. Two distinct trends are observable in metacoracoid evolution, along the mammalian backbone lineage from cynodonts through to modern therians, reduction and a less posterior inclination of the distal end. With regard to these trends, the metacoracoids of monotremes extend posteriorly and somewhat ventrally (as for non-theriimorphs) and are perhaps even more massive than was typical for cynodonts.

Jenkins and Parrington (1976) considered the ventral stability of the monotreme shoulder girdle to be largely due to the medial extension of the metacoracoids, which pass over the posterolateral edge of the interclavicle and abut the sternal manubrium anterolaterally. Although there are difficulties in reconstructing shoulder girdle structure from fossils, among adult cynodonts and mammals, these conditions appear to be unique to monotremes (Jenkins and Parrington 1976). However, Broom (1897) noticed that during marsupial development the metacoracoid and the sternum are articulated. In fact, in all marsupial species for which Klima (1987) examined developmental series, the metacoracoid grew posteromedially to abut the sternum anterolaterally before fusing with it. This fusion disintegrated in early pouch young (or earlier in bandicoots) with the lateral portion of the metacoracoid forming the processus coracoidus.

As well as monotreme metacoracoid orientation being related (by co-varying with the glenoid complex) to specialized fossorial/swimming habits, its size and medial positioning allow for massive attachments for the *m. biceps* and *m. coracobrachialis* (compared to therians, see Argot 2001), and considerable mechanical advantage for their respective insertions on the radius and lesser tubercle. As noted above, the unique (among adults) interlocking of the metacoracoid with the procoracoid and interclavicle, as well as with the sternal manubrium, provides ventral bracing and resistance to the compressional forces associated with powerful digging/swimming with transversally oriented humeri.

**Character 22.** The **orientation of the shoulder glenoid** also shows a phylogenetic trend along the mammalian backbone lineage. The glenoid orientation progressively evolves from facing laterally in primitive synapsids, to facing posterolaterally and slightly ventrally in advanced cynodonts and morganucodontids, more posteroventrally (though still somewhat lateral) in eutriconodonts, *Zhangheotherium* and eupantotheres, right up to the lateral inclination essentially being absent among early marsupials and placentals. Monotreme shoulder glenoids are difficult to place along this continuum partly due to their considerable concavity. For echidnas, the shoulder glenoid faces laterally and slightly ventrally, perhaps most similar to pelycosaurs (Romer and Price 1940; Jenkins 1970a), while for the platypus it faces more posteriorly (though not as much as in cynodonts and morganucodontids), and uniquely among synapsids, slightly dorsally. These

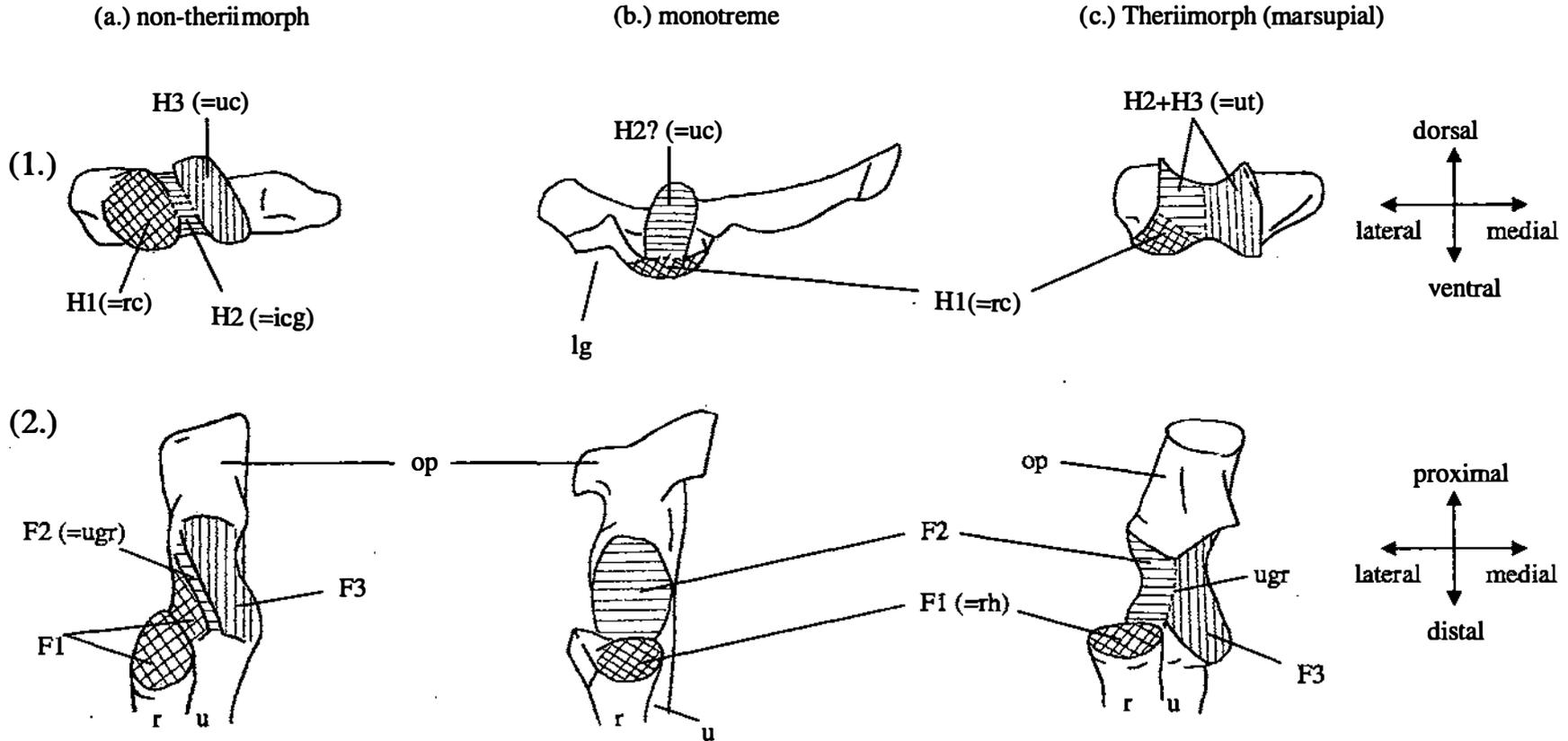
differences illustrate the difficulty of lumping platypuses and echidnas together with all other non-theriimorphs for a single primitive glenoid orientation, as Ji *et al.* (1999) have done (“nearly parallel to the long axis of the scapula and facing posterolaterally”).

Regardless of the position among Mammaliaformes that monotremes arose from, the evolution of the lateral (echidna), or near-lateral and slightly dorsal (platypus) facing orientation of the shoulder glenoid requires a reversal of the earlier initiated trend toward more posterior and ventral facing shoulder glenoids. This conclusion is in agreement the assertion of Jenkins and Parrington (1976) that the orientation of the monotreme shoulder glenoid is a specialization of the group. Notably, a near laterally facing glenoid (similar to the echidna condition) is found during marsupial ontogeny (Broom 1899), though not in the adults of any (non-monotreme) Mesozoic Mammaliaformes.

Having the same relationship with the metacoracoid, scapula spine, acromion, and infraspinous fossa in monotremes and therians, the glenoid orientation covaries with the entire glenoid complex (see Figure 4.10). A reversal towards a more lateral and near-horizontal glenoid orientation results in more efficient translation of humeral long-axis rotation into forward (as opposed to lateral) momentum. It also allows the expansion of the lesser tubercle of the humerus, which is restricted by the thorax for near-parasagittally oriented humeri. Large tubercles that are widely expanded from the humeral head allow large muscle insertions with great mechanical advantage for humeral long-axis rotation (Gambaryan and Kielan-Jaworowska 1997).

**Character 23.** The **shape and curvature of the shoulder glenoids** among the generalized insectivore taxa can be categorized at least superficially into two categories. Non-theriimorph glenoids are quite oval and to some extent saddle-shaped, while theriimorph glenoids are typically more rounded in outline and more uniformly concave. In these regards monotreme shoulder glenoids fit into the non-theriimorph category. Among non-theriimorphs, the glenoid is divided into scapula (dorsal) and coracoid (ventral) portions. There was a trend along the mammalian backbone lineage towards reduction of the coracoid portion, which appears to still persist in primitive theriimorphs, including *Gobiconodon*. Visible sutures in juveniles of *Tachyglossus* (Serenio and McKenna 1995; pers. obs.) show that the coracoid portion fully takes up the ventral half of the glenoid.

In spite of the similarities with the non-theriimorphs, the monotreme shoulder glenoid is highly modified in being wide and deeply concave between protruding upper and lower lips. Jenkins and Parrington (1976) suggested these specializations to be adaptations for increased stability of the joint during digging. Looking at this structure in distal view (Figure 4.10) reveals extraordinary similarity in the outline and orientation of the scapular portion of the shoulder glenoid between



**Figure 4.11** Distal view of the left humerus (1.) and anterior view of the right ulna and radius (2.) of (a.) a non-theriimorph mammal (generalized morganucodontid), (b.) a monotreme (*Tachyglossus aculeatus*) and (c.) a theriimorph, the non-specialized therian (*Dasyurus maculatus*). Three articulation interfaces are hatched: H1/F1 (cross-hatch); H2/F2 (horizontal-hatch); H3/F3 (vertical-hatch). icg, intercondylar groove; op, olecranon process; r, radius; rc, radial condyle; rh, radial head; lg, lateral groove; u, ulna; uc, ulnar condyle; ugr, ulnar guiding ridge; ut, ulnar trochlea.

monotremes and therians. It is in the broad extension of the coracoid part of the monotreme glenoid that this structure is similar to non-theriimorphs. However, in monotremes and therians, this ventral (anteroventral in therians) portion of the glenoid ends in a pointed process, which in monotremes wraps right around the humeral head, due to the extreme concavity of the glenoid.

The highly concave, somewhat saddle-shaped monotreme shoulder glenoid provides a valley-like track for the ventrally concave humeral head to rotate through. This is unlike either theriimorph or non-theriimorph mammals, which have ball and socket type shoulder joints with a near-hemispherical (non-theriimorphs) or near-spherical (non-specialized theriimorphs) humeral head that rotates about a near-central point (of the head). The monotreme glenoid condition would provide considerable resistance to dislocation, by comparison with the more open joints of other mammals and cynodonts. Hence, the specialization (wide and deeply concave) of monotreme shoulder glenoids can be related to the high forces that the hypertrophied humeral long-axis rotation places on the shoulder joint during rotation (while swimming or digging).

**Character 24.** The **medial surface of the scapula** is coded by Ji *et al.* (1999) as flat for theriimorphs, and convex for non-theriimorphs and monotremes. However, lumping monotremes with non-theriimorphs for this character is problematic. The medial convexity of non-theriimorph scapulae relates to the concavity on the lateral side of the blade that is the infraspinous fossa. In contrast, the medial convexity of monotreme scapulae relates to the inflection of the anterior border and acromion, which are homologous with the scapular spine and acromion of theriimorphs (which are similarly inflected; see Figure 4.10). In fact, given the monotreme orientation of the covarying glenoid complex of characters (Figure 4.10), the medial surface of monotreme and therian scapulae are not homologous, so should not be examined as such.

**Character 30. Humero-ulnar articulation.** In most mammals (not monotremes) this articulation involves three major interfaces. In the following discussion and Figure 4.11 I refer to the humeral facets as H1, H2, and H3 and to the corresponding forearm (radius + ulna) facets as F1, F2, and F3. The humero-ulnar articulation of non-theriimorph mammals and cynodonts involves the convex surface of the ulnar condyle (H3, at the distal end of the humerus), fitting into a concave articular surface (F3) on the medial side of the proximal end of the ulna. As shown in Figure 4.11a for a generalized morganucodontid, the ulnar guiding ridge (F2) separates this medial surface on the ulna from a lateral facet. Ventral to this lateral facet is the radial notch, which articulates with the head of the radius and so is obscured in Figure 4.11.

The radial condyle of the humerus (H1) mostly articulates with the head of the radius, but in non-theriimorphs (Figure 4.11a) also has a substantial articulation with the lateral facet on the ulna. As

such, the ulnar guiding ridge fits in the intercondylar groove (F2 in Figure 4.11a) that separates the ulnar and radial condyles of non-theriiform mammals. The widening of this groove (Haines 1946) and retention only of the lateral half of the ulnar condyle (Jenkins 1973) are considered to be the major steps in the evolution of the ulnar trochlea of theriiforms (see Figure 4.11c). Among the cynodont and non-theriiform mammals, the dorsal (extensor) surface of the ulnar condyle is more laterally positioned (closer to the radial condyle) than is the ventral (flexor) surface of the ulnar condyle. This allows the forearm to move through approximately the same plane (suggested to be parasagittal for Jurassic mammals by Jenkins (1973) while it extends, and the humerus adducts, elevates and rotates.

In eutriconodonts (at least *Gobiconodon* and *Jeholodens*), *Zhangheotherium* and eupantotheres, the ulnar condyle is retained at least ventrally, while a trochlea is formed dorsally, and would articulate with the ulna when the forelimb is extended. Argot (2001) interprets the stronger dorsal development of the trochlea of cursorial (as opposed to arboreal) marsupials to relate to the importance of stabilizing forearm extension during running or leaping. As such, development of an ulnar trochlea on the dorsal (extensor) surface of the distal humerus might indicate that non-theriiforms were more agile, in terms of running and leaping than their non-theriiform ancestors were. This would also be consistent with the increased differentiation among thoracic and lumbar vertebrae apparent even in early theriiforms, by comparison with cynodonts and *Morganucodon*. Nevertheless, ventral retention of the ulnar condyle suggests that compared to therians, the locomotion of eutriconodonts, *Zhangheotherium* and eupantotheres (at least when the forearm is considerably flexed) involved a more abducted humerus (Hu *et al.* 1997) and probably greater humeral long-axis rotation.

A full trochlea (as in therians) essentially restricts ulnar movement relative to the humerus, to flexion/extension. An ulnar condyle also allows the ulna to adduct/abduct relative to the humerus (Gambaryan and Kielan-Jaworowska 1997), allowing the long axis of the ulna to move essentially through a single (possibly parasagittal) plane, that would otherwise not be possible with simultaneous elbow flexion/extension and strong humeral long-axis rotation (see Jenkins 1973). There is relatively little humeral rotation during locomotion among non-specialized therians, and its potential as a component of propulsion is limited by humeral orientation that is mostly posteroventral (Jenkins 1971a). However, its implication as a more important propulsive component for primitive theriiforms is also consistent with their retention of a lateral component of glenoid facing orientation, as well as considerable (25-40°) torsion between the proximal and distal epiphyses of the humeri (Rougier 1993; Hu *et al.* 1998; Ji *et al.* 1999).

Like non-theriimorphs, the monotreme humero-ulnar articulation involves a convex ulnar condyle. However, there are a number of notable differences between the humero-ulnar articulation of monotremes and that typical of non-theriimorphs. Firstly, the ulnar condyle of platypuses and echidnas (H2 In Figure 4.11b) is not separated from the radial condyle. As such there is no intercondylar groove and no ulnar guiding ridge. Szalay and Trofimov (1996) regarded this condition to be unique among mammals. Another unique feature of the monotreme ulnar condyle, as noted by Jenkins (1973) is that rather than its dorsal (extensor) surface being lateral to its ventral (flexor) surface, the opposite is the case. Furthermore, the ulnar condyle of monotremes is dorsal (Figure 4.11b), rather than medial to the radial condyle. This condition is unknown among cynodonts and non-therian mammals, and results in the head of the radius being placed anterior to, rather than anterolaterally to the ulna. A similar condition to that of monotremes is however found (Kielan-Jaworowska 1978; Jenkins 1973) in various eutherian groups, including Macroscelididae (elephant shrews), Tupaiidae (tree shrews), Geomyidae (pocket gophers), and zalambdalestids, as well as in a number of marsupials (according to Tornier 1886).

A further unusual feature of the distal end of monotreme humeri is a deep and wide groove that is confluent with and lateral to the radial condyle (see Figure 4.11b). This lateral groove articulates with the margin of the radial head and appears to provide a set path for the forearm to follow during flexion/extension. This groove would also add stability (countering disarticulation) to the elbow joint, which may be particularly important given the absence of an ulnar guiding ridge, and otherwise only the radial condyle articulating with the crus (forearm and manus) ventrally. A similar groove for articulation with the margin of the radial head is shown on the humeri of the zalambdalestid, *Barunlestes* and the macroscelidid, *Elephantulus* (Kielan-Jaworowska 1978). In both of these (and monotremes) the ventral aspect of the humero-ulnar articulation is dominated by the radial condyle (=capitulum in therians) articulating with the radial head, which lies anterior to the ulna.

Seemingly due to the absence of an ulnar trochlea, it has been assumed that the ulnar condyle of monotremes is derived from those of mammals outside of the Theriimorpha, from a level similar to *Morganucodon* (Jenkins 1970a; Gambaryan and Kielan-Jaworowska 1997) or even more primitively (Szalay and Trofimov 1996). However, the highly derived nature of the monotreme elbow joint casts doubt over the homology of the ulnar condyles of monotremes with those of non-theriimorphs (or indeed theriimorphs).

Figure 4.11 indicates the matching humeral and ulnar/radial articulating facets for a non-theriimorph mammal (generalized morganucodontid), a monotreme (*Tachyglossus*) and a non-specialized therian (*Dasyurus*). Each possesses interface 1, the articulation of the radial condyle

(H1) with the radial head (F1). Non-theriimorphs and the therians also share interfaces 2 and 3. Homology of these faces among non-theriimorphs and theriimorphs is in agreement with the conclusion of Jenkins (1973) that the trochlea of therians is homologous with the lateral side of the ulnar condyle and an expanded intercondylar groove (an perhaps part of the medial side of the radial condyle). In monotremes, either interface 2, or 3 has been lost.

The simplest derivation of the monotreme humero-ulnar joint from the non-theriimorph type would require dorsal and lateral migration of the ulnar condyle onto the dorsal surface of the radial condyle, with the radial condyle retreating somewhat ventrally. Deriving the monotreme condition from a condition similar to therians (even generalized modern therians) is not necessarily more difficult. The largely ventral orientation of the radial condyle was already present among the early marsupials (Argot 2001) and eutherians (Kielan-Jaworowska 1978), and the lateral portion of the trochlea (H2 in Figure 4.1 1c) is positioned relatively dorsal to the radial condyle. Hence, the monotreme condition would be approximated by the loss of the medial faces of the ulna trochlea and trochlea notch (H3 and F3 in Figure 4.1 1c) as well as convex swelling of the lateral portion of that trochlea (H2).

Regardless of how the monotreme articulation between the humerus and the crus was derived, as Jenkins (1970a, 1973) noted, its specializations are consistent with adaptation for digging/swimming. Whether swimming or walking (or perhaps digging), forward momentum is most efficiently transferred by pulling the manus through a more or less sagittal plane, so minimizing the medial ground-force vector. Moving the manus back through a more or less sagittal plane is also important for swimming during the recovery stroke, in order to reduce drag. With an abducted humerus rotating as the elbow flexes, achieving such planar movement of the manus, requires adduction/abduction at the elbow joint. Such movement for the ulna (perpendicular to elbow flexion) is largely prevented by a trochlea, but is important among non-theriimorph mammals (see Jenkins 1973) and probably even more so for monotremes, because of their more abducted posture. Two monotreme specializations, (a) confluent ulnar and radial condyles (with no intercondylar groove) and (b) the dorsal position of the ulnar condyle relative to the radial condyle, are consistent with this requirement for reduced restriction of forearm movement away from the plane of elbow flexion/extension. These however would reduce the stability of the joint, which must be especially problematic given that monotreme locomotion is essentially “front wheel drive” and for their size, massive forces are generated by the upper appendicular musculature (Griffiths 1978; Augee and Gooden 1993). As noted earlier, a further monotreme specialization, the extensive lateral groove that is confluent with the radial condyle, would add stability to the elbow joint.

**Character 32.** The **entepicondyle** and **ectepicondyle** (or medial and lateral epicondyles) are respectively the medially and laterally projecting muscle attachment sites at the distal end of the humerus. From pelycosaurs, through cynodonts, to *Morganucodon* and up to Cretaceous therians, the epicondyles (particularly the medial epicondyle) show a general phylogenetic trend, tending to become relatively less massive. Some taxa do not fit this trend, such as *Gobiconodon*, which appears to have possessed relatively larger epicondyles than morganucodontids, though this may partly be an allometric scaling effect (given the large size of gobiconodontids relative to morganucodontids). Consideration of the trend alone would have monotremes, with their massive epicondyles (see Figure 4.9) placed more primitively even than pelycosaurs. This implies that the trend along the mammalian backbone lineage for epicondyle reduction was reversed along the monotreme stem lineage. As for fossorial therians (see Gasc *et al.* 1986; Castiella *et al.* 1992), such evolution of massive epicondyles is consistent with the requirement to pull the manus through dense mediums (soil/water).

#### 4.3.5 *Monotreme upper appendicular characters: is phylogenetic signal confounded by ecological specialization?*

The above accounts (which are summarized in Table 4.4) permit comparison of two competing hypotheses for explaining the highly significant ( $p < 0.0001$ , Table 4.2) apparent phylogenetic signal among the upper appendicular characters for excluding monotremes from Theriimorpha (Figure 4.6a).

1. That the signal results from ancestry that trechnotheres and eutriconodonts share to the exclusion of monotremes.
2. That the apparently primitive monotreme upper appendicular characters are instead, a reflection of specialized fossorial/semiaquatic habits that incorporate hypertrophied humeral long-axis rotation, and that this confounds their phylogenetic affinities.

Inspection of Table 4.4 reveals that for nine of the ten characters placing monotremes outside Theriimorpha (eutriconodonts and trechnotheres, from among the generalized insectivores), the monotreme state is either more primitive than that of morganucodontids (indicating homoplasy), or highly autapomorphic. This leaves only one of these characters (16: acromion process) as a potentially reliable reflection of monotreme affinities. Consideration of the monotreme condition (in view of trends among the generalized insectivores) does not support the hypothesis that the strong signal across theriimorpha within U (Figure 4.8a, column B) is indeed phylogenetic signal. This places in question the reliability of studies that depended heavily on upper appendicular

characters (e.g. Rowe 1993; Sereno and McKenna 1995) for favouring monotremes being excluded from Theriimorpha.

Comparison of the monotreme condition with trends among the generalized insectivores provides considerable support for the hypothesis that the apparently primitive monotreme placement indicated by the upper appendicular characters, is a reflection of specialized fossorial/semiaquatic habits. As indicated in Table 4.4, the monotreme state of each of the ten characters that place monotremes outside Theriimorpha can be correlated either directly with hypertrophied humeral long-axis rotation and/or provision of mechanical advantage (L), or indirectly, by provision of bracing (B), or co-varying with the orientation of the glenoid (G). That the monotreme state for each of these characters (including 16: acromion process) is functionally or developmentally correlated has an important consequence. It provides an explanation for the non-independence of homoplastic evolution that is required by the CMI analysis for incompatibility across the theriimorpha node.

Given that monotremes are more closely related to theriimorphs than are morganucodontids, the characters noted in Table 4.4 as being more primitive among monotremes than morganucodontids, must be monotreme reversals (or be parallelisms between morganucodontids and theriimorphs). More generally in fact, the terrestrial locomotion of monotremes is less advanced with respect to trends along the mammalian backbone lineage toward parasagittalism, than was *Probainognathus*. This eucynodont (and others) had somewhat posteriorly and ventrally oriented humeri (abducted approx. 45° according to Jenkins 1971a). By comparison, monotremes and pelycosaurids have near-horizontally oriented humeri that are short, more massive shoulder girdle bracing, and wider humeral tubercles and epicondyles. As discussed earlier, this combination of characteristics is associated with locomotion that primarily involves transversely oriented humeri, which among monotremes (and probably pelycosaurids: Jenkins 1973) allows efficient propulsive transfer of forces generated by humeral long-axis rotation. The adaptive significance of these conditions and phylogenetic inference for the antiquity of the monotreme condition (with respect to the mammalian backbone lineage) point unambiguously to reversal along the monotreme stem lineage, rather than parallelism between morganucodontids and theriimorphs.

While pelycosaur-level to cynodont-level character states indicate that monotreme upper appendicular evolution has involved numerous reversals, it is more difficult to assess what state these characters have reversed from. It may be the highly modified characters rather than the apparently plesiomorphic (but reversed) characters that provide a better indication of monotreme affinities among the generalized insectivores. These include the deeply concave shoulder glenoid with protruding upper and lower lips, the ventrally concave humeral head, and the unique humero-

ulnar/humero-radial articulation. One might speculate that these specializations evolved as escapes from constraints placed by theriimorph ancestry. Certainly this would explain why the articulation pattern between the distal humerus and ulna/radius of monotremes (Figure 4.11b) would be approximated by the loss of the medial interface (H3 and F3) of the ulna trochlea and trochlea notch of therians (Figure 4.11c). Similarly, the resemblance of the monotreme shoulder glenoid shape to that of generalized therians (but with the ventral lip extended and upturned, see Figure 4.10) would be explained. However, it is uncertain how non-theriimorph upper appendicular characters might be expected to be modified upon a transition to a fossorial/semiaquatic niche.

A detailed investigation of the early development of the monotreme humerus might provide further insight into the homology of the characters that are highly modified among monotreme adults. The recent discovery of a putative monotreme humerus from the Early Cretaceous of southern Australia (Rich and Vickers-Rich 2000) also promises to provide some answers. The authors point out that the humerus has an olecranon fossa (missing among extant monotremes), which indicates greater extension of the elbow joint, so possibly a more upright posture than modern monotremes have. Furthermore, in my brief examination of a cast of the fossil, I observed many typically monotreme features, including a large, ventral radial condyle, but also noted a shallow trochlea dorsal to this. If this humerus is indeed monotreme, as suggested by its size and general attributes (Pridmore and Rich, in prep.), it corroborates my earlier speculation that the humero-ulnar articulation of monotremes is not modified directly from a non-theriimorph (or even pelycosaur) level. Rather, that its condition, along with much of the shoulder girdle and forelimb anatomy of monotremes might be better understood in terms of escaping from constraints placed upon the evolution of fossorial/swimming adaptations, by an ancestry that included near-parasagittal forms.

Correlated reversal of upper appendicular characters reconciles the signal attributable to U, with monotremes arising within Theriimorpha, as is favoured by LB, MVD and is the best estimate from molecular data (Chapter 2). The hypothesis that the incongruence across theriimorpha results from correlated reversal within U, that is associated with fossorial/swimming habits that incorporate hypertrophied humeral long-axis rotation, is consistent with each of the six points derived as a framework for assessing monotreme affinities in terms of homoplasy options. The following statements address these points (as listed at the end of Section 4.3.2).

1. The specialized fossorial/swimming habits of monotremes can explain the non-independence of homoplastic character evolution within U. Each of the characters that place monotremes outside Theriimorpha is functionally/developmentally correlated with hypertrophied humeral long-axis rotation and/or provision of mechanical advantage.

2. The proposed correlated homoplastic evolution involves transformations along the monotreme stem lineage and does not require correlated hidden-homoplasy (among the background phylogeny).
3. The reversal (among monotremes) of the phylogenetic trend (among the background phylogeny) towards parasagittalism can be related to a change in the functional relationships of monotreme upper appendicular characters (the transition along the monotreme stem lineage, from small terrestrial insectivores to mid-sized, fossorial/semiaquatic mammals).
4. Reversal of monotreme upper appendicular characters is consistent with the expectation that any correlated hidden-homoplasy among the background phylogeny would be parallelism or convergence, so would tend to pull (bias) monotremes toward the outgroup.
5. The proposed correlates of reversal among monotreme upper appendicular characters (hypertrophied humeral long-axis rotation and provision of mechanical advantage) are consistent with the transition along the monotreme stem lineage, from small terrestrial insectivores to mid-sized, fossorial/semiaquatic mammals.
6. Many upper appendicular characters (including at least four of the ten with character maps that are excluded from Theriimorpha: see Table 4.4) place monotremes more primitively than morganucodontids, or even eucynodonts. These characters must be homoplastic.

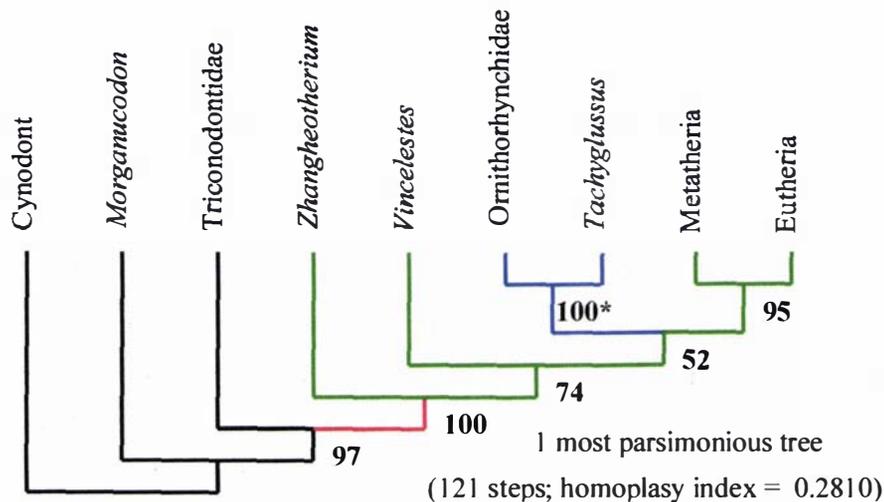
Given this consistency with the framework for assessing monotreme phylogenetic affinities, correlated reversal within U (associated with specialized fossorial/swimming habits that incorporate hypertrophied humeral long-axis rotation) is sufficient to explain the incongruence across theriimorpha. However, it would be premature to conclude that conflict between U and the other meta-regions (Figure 4.7a,b) is fully explained by homoplasy within U. The condition of the upper appendicular characters of the monotreme-theriimorph LCA remains uncertain and the possibility of homoplasy among LB and MVD contributing to conflict across the theriimorpha node requires further examination. Unfortunately the form-function relations of monotreme LB and MVD characters are less well understood than for U. However, the possibility of LB and MVD being unreliable for inferring monotreme affinities is highlighted by  $CMI_o[LB*MVD]$  being significantly greater than expected, across the trechnotheria and cladotheria nodes (Figure 4.7c).

#### 4.3.6 Outgroup-attraction of morphological long-branches

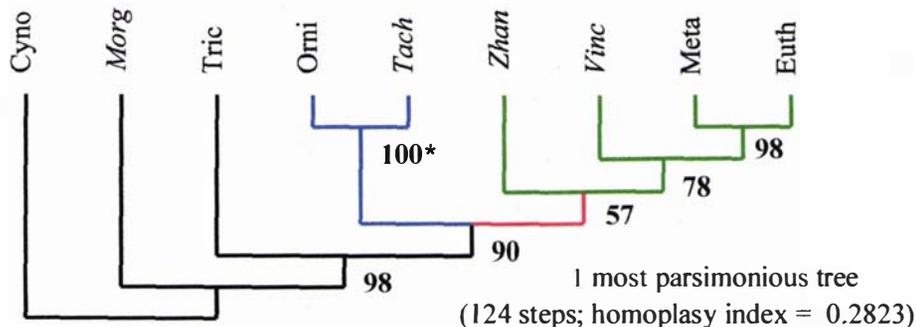
Figure 4.12 shows the phylogenetic position of monotremes among the generalized insectivores for LB+MVD (all characters minus U), with ornithorhynchid character states (a) preferentially designated by fossil material (the order of preference is *Steropodon* > *Obdurodon* > *Ornithorhynchus*), and (b) limited to the extant *Ornithorhynchus*. Only dental, mandibular and

cranial characters have been described for monotreme fossils older than the Late Miocene, with the most useful information being provided by the mid Miocene platypus (*Obdurodon dicksoni*) skull and dentary (Archer *et al.* 1992, 1993; Musser and Archer 1998), as well as the Early Cretaceous *Steropodon* (Archer 1985) dentary. These fossils allow for the re-coding of 14 characters (see Appendix D), of which two are basicranial, one is mandibular and 11 are dental, including 8 characters that could only be coded as uncertain for the modern taxa.

(a.) including *Obdurodon/Steropodon* characters for Ornithorhynchidae



(b.) *Ornithorhynchus* characters only for Ornithorhynchidae



**Figure 4.12** Maximum-parsimony trees for the placement of monotremes among the background phylogeny of generalized insectivores. In (a.), only the condition of the modern platypus, *Ornithorhynchus* is considered for designating ornithorhynchid character states. In (b.), ornithorhynchid character states are preferentially designated by fossil material. The order of preference is *Steropodon* > *Obdurodon* > *Ornithorhynchus*. Bootstrap support values correspond to the clade above each of them. Branches are colour-coded as green for Trechnotheria, red for the trechnothere stem, and blue for monotremes. \*Note that monotreme monophyly is constrained (unconstrained it is supported in 57% and 16% of replicates in a. and b. respectively).

It is noteworthy that without including the monotreme fossil information, the monophyly of Monotremata is only supported in 16% of bootstrap replicates. The potential importance of the fossil information is underpinned by its inclusion resulting in this support rising to 57%. Within the context of this study, a more important result is that with the fossil information included (Figure 4.12a), monotremes are sister to therians, but with this fossil information discarded, monotremes are pulled back to being outside Trechnotheria, two internodes closer to the outgroup. This implies that for at least the dental, mandibular, basicranial, and (as discussed earlier) upper appendicular characters, the phylogenetic manifestation of the autapomorphy of modern monotremes is attraction towards the outgroup.

The prevalence of dental characters among those for which exclusion of fossil data resulted in outgroup-attraction is not surprising. Musser and Archer (1998) identified three trends among ornithorhynchids that gave rise to the highly modified dentition of *Obdurodon*. These were: 1. elaboration and multiplication of the transverse shearing blades; 2. progressive reduction of the roots of the molars; and 3. an increased role for the oral epithelium in dental function, through production of horny pads. It is interesting to speculate that the modification of ancestral monotreme molars may also involve a loss of protocone/talonid basin occlusion. This is the most parsimonious explanation for lack of tribosphenic molar wear patterns among early monotremes, if Lou *et al.* (2001a) are correct in their claim that *Ausktribosphenos* and *Ambondro*, which have tribosphenic molars, are consecutive sister taxa for monotremes. Additionally, the oldest described monotreme upper molar, from *Monotrematum* (Pascual *et al.* 1992a; 1992b) has a cusp on the lingual border of the valley between the putative paracone and metacone. Not only is this cusp in the position of the protocone, but given the occlusal relations of *Obdurodon* molars (see Archer *et al.* 1992, 1993), it would have occluded with the unbasined talonid, possibly with the cristid obliqua. This cusp might represent a reduced protocone.

As a highly modified taxon (examined among more conservative forms), the long-branch outgroup-attraction that is apparently affecting monotreme affinities may be quite common among morphological datasets. Molecular phylogenetics provides anecdotal evidence to support this, and should encourage rigorous statistical investigation of the potential for long-branch outgroup-attraction among morphological data. Arguments over the affinities of cetaceans (whales) present a recent, high profile example. Molecular studies (e.g. Gatesy *et al.* 1999; Nikaido *et al.* 1999) have shown that whales are nested well within the Artiodactyla, as sister to hippopotamids. However, these highly derived aquatic mammals are traditionally considered to stem from basal ungulates (e.g. Thewissen 1994; O'Leary and Geisler 1999), outside the Artiodactyla and removed from hippopotamids (towards eutherian outgroups) by at least two internodes. Similarly, molecular studies (e.g. Kumazawa and Nishida 1999; Zardoya and Meyer 2001) have shown that turtles are

nested within the diapsid reptiles, as sister to archosaurs. In contrast, most recent morphological studies place the anapsid turtles as sister to diapsids (e.g. Gauthier *et al.* 1988; Lee 1996), while they have traditionally been placed outside all other extant amniotes (e.g. Williston 1917; Gaffney 1980). These placements are respectively, at least (more with the inclusion of extinct groups) one and two internodes closer to vertebrate outgroups by comparison with the molecular results. DNA hybridization (Kirsch *et al.* 1998) and now nuclear and mitochondrial DNA sequences (Teeling *et al.* 2000) have shown the plant-feeding megachiropteran bats to be nested within insectivorous microchiropteran bats, as sister to Rhinolophoidea (horseshoe and ghost bats). In contrast, morphology has favoured megachiropterans being basal among bats (see Simmons and Geisler 1998).

It is not surprising that ecological long-branches (as functionally divergent taxa) will be difficult for morphological studies to place phylogenetically. Arnold (1990) suggests that ecological niche transitions are perhaps the most problematic factor for phylogeny reconstruction. Perhaps analogous to the monotreme situation, phylogenetic bias among morphological data for the placement of whales (Gatesy and O'Leary 2001) and tropical salamanders (Parra-Olea and Wake 2001) have respectively been related to ecological transitions to aquatic and fossorial niches. Why, though, should apparent affinities of ecological long-branch taxa be biased toward more basal placements (outgroup-attracted)?

As noted in the introduction, biases among molecular sequence data have received considerable attention. Meanwhile, biases among morphological data remain little studied, aside from ecological niche-related convergence (still not as statistical analyses), which for example has occurred between many placentals and marsupials with similar foraging strategies (see Springer *et al.* 1997b), such as golden moles and notoryctid moles. Nevertheless, for a specified character, parallelism may be more likely between two closely related (but non-sister) taxa that have retained the niche of their LCA, than between one of these taxa and its sister taxon that has entered a alternative niche, for which the functional relationship of the character with the environment is different. Such parallelism could result in the highly derived taxon being pushed out of its true sister group relationship and closer to the outgroup. Of particular concern here, are evolutionary trends that were initiated before the LCA of mammals and have continued (perhaps to varying degrees) in many of the generalized insectivore lineages, but clearly have not in monotremes.

Trends toward parasagittalism impact a suite of lower appendicular characters (Lewis 1983; Szalay 1993b; Kielan-Jaworowska and Gambaryan 1994; Gambaryan 2001) that may be synapomorphies of trechnotheres and multituberculates. These include: (a) at least moderate superposition of the astragalus over the calcaneum, (b) extension of the calcaneal tubercle and (c)

dorsal orientation of the femoral greater trochanter. Contrary to framework point 6, the first of these characters is more advanced in *Morganucodon* and *Oligokyphus* than among monotremes (see Szalay 1993b). Additionally, the latter two characters are anomalous in being more advanced (toward the therian condition) in multituberculates (Kielan-Jaworowska and Gambaryan 1994) and Spalacotheriids (pers. obs.), than in the eupantotheres *Henkelotherium* (Krebs 1991; Gambaryan 2001) and *Vincelestes* (Rougier 1993; pers. obs.).

In his study of marsupials, Muizon (1998) considers the dorsal elongation of the greater trochanter, and the extension of the calcaneal tubercle to be adaptations for running and, or leaping activity. Such activities were likely to have been integral to the ecology of early trechnotheres (Hu *et al.* 1997, 1998) and multituberculates (Kielan-Jaworowska and Gambaryan 1994), but clearly are not for extant monotremes. Hence, it is important to be aware of the possibility that trends associated with lower appendicular characters have resulted in parallelism among the generalized insectivores, and in turn, outgroup-attraction for monotremes.

Outgroup-attraction of highly derived taxa would be further encouraged by autapomorphies erasing (what were) synapomorphies shared with sister taxa. For example, the highly derived (or lost) dentition of modern platypuses and echidnas represents autapomorphic erasing of numerous potential synapomorphies of monotremes with trechnotheres. This is most clearly evidenced by the dentition of the Cretaceous monotreme, *Steropodon galmani* (see Archer *et al.* 1985; Kielan-Jaworowska *et al.* 1987).

In addition to the mechanisms noted above (parallelism among conservative taxa, and autapomorphy erasing synapomorphies), I have identified two novel biases that may exist among morphological data and would tend to draw ecological long-branches (monotremes in this case) toward outgroup taxa. These are described below.

#### 4.3.6a *Asymmetry between the morphological ranges of “primitive” and “advanced” character states*

Tree steps, or character state transformations are the fundamental units for comparison of phylogenetic hypotheses under the cladistic methodology (Hennig 1966). The identification of synapomorphies (with ordering typically defined implicitly by the use of an outgroup) takes a central role in cladistic analysis, as they act to reduce tree length and are the basis of support for clades. Hence, the major emphasis of critical review is typically directed at the conditions for qualification as shared derived states (synapomorphies). This might be expected to result in derived states often being defined more explicitly than primitive states. If for example, a primitive

character state is simply the absence (or universal complement) of a more precisely defined derived state, the morphological range of the primitive state will be greater than that of the derived state.

As the condition of a character becomes more derived, its likelihood of fitting within one or other pre-defined states that are universally complementary (so do not provide for autapomorphy, e.g. presence versus absence) becomes more random, and depends less on the ancestral state from which it was derived. Hence, as the condition of a character becomes more derived along a lineage, the probability of that condition being erroneously assigned to the wide morphological range of a general character state increases, relative to that of being erroneously assigned to the narrower morphological range of a specific character state. In light of this, a tendency for primitive states to encompass a wider morphological range than derived states would result in a phylogenetic bias, where a highly modified (long-branch) taxon, analysed among conservative taxa, would tend to be pulled towards the outgroup. Despite their ecologically/functionally highly modified nature, Ji *et al.* (1999) did not classify monotremes as autapomorphic for any of the 101 characters in their dataset.

Outgroup-attraction is indicated for monotremes by (apparent) correlated reversal among the upper appendicular characters, and by the exclusion of the fossil information for the non-upper appendicular characters (see Figure 4.12). For both sets of data there is potential for asymmetry between the morphological ranges of character states to contribute to outgroup-attraction. Discrete characters with larger morphological ranges for the primitive states than for derived states appear to be common for both the upper appendicular and basicranial characters. For example, the advanced scapula (ch 24) state of being flat medially can only occur in one way (the whole medial surface being flat), whereas the primitive state (medially convex) allows for the convexity to be developed in different (and non-homologous) parts of the scapula. Even with a little coding license for the shape of the humeral head (ch 25), the derived state (spherical) clearly encompasses a narrower morphological range than the near-infinite array of possibilities for the primitive state (subspherical). On this basis monotremes group with early mammals with near-hemispherical humeral heads, such as *Morganucodon* (Jenkins and Parrington 1976). Perhaps the unusual ventrally concave and anteroposteriorly elongated monotreme condition would better be considered autapomorphic.

Among the basicranial characters, derived states such as a post glenoid depression on the squamosal (ch 57) and an elongate (state 1), or bulbous (state 2) petrosal cochlea housing (ch 59) have a more specific morphological scope than the respective primitive states (simply the absence of these conditions). To be considered to have the primitive state, the homologous ossifications could theoretically be of any form other than those specified for the advanced states. Upon

inclusion of the fossil information, the most parsimonious solution requires that these basicranial characters reversed to the primitive state in the modern platypus (ch 57) and echidnas (ch 59).

The degree of humeral torsion (ch 28) provides an example of asymmetry between the morphological ranges of character states for a continuous character. The morphological ranges of the derived characters, 1 and 2 are respectively 0°-15° and 15°-30°, while the primitive state range includes any degree of torsion above 30°. Pridmore (1985) and I measure platypus humeral torsion at approximately 75°, though other measurements range from 70°-85° (respectively: Gambaryan and Kielan-Jaworowska 1997, and Simpson 1928:). For all other taxa assigned to the primitive state, humeral torsion is between about 40° and 50°, a range that more precisely describes the apparent ancestral state for mammals. As with the golden moles which have humeral torsion ranging from 60°-120° (respectively: Gambaryan and Kielan-Jaworowska 1997, and Gasc *et al.* 1986), the platypus state is well outside the range bounded by the background phylogeny taxa assigned the primitive state, and so might be considered autapomorphic. Certainly the condition in golden moles (members of the Afrosoricida; see Stanhope *et al.* 1998) is autapomorphic, as they are derived from generalized insectivores with very little humeral torsion. Perhaps a conservative approach in which autapomorphy is recognised should be encouraged. Simply expanding the morphological scope of the primitive state to accommodate highly divergent conditions will tend to result in outgroup-attraction for highly modified taxa.

Other examples of asymmetry between the morphological ranges of character states are noted in the character descriptions of Appendix D. Some cases are clearer than others. Morphometrics might provide an objective assessment of the asymmetry of character states, though the rapidly improving understanding of the interaction between genetic mechanisms and developmental trajectories (see Lovejoy and White 1999) may provide a great boost for assessing potential phylogenetic bias in terms of character space distribution. Nevertheless, for all but one of the characters among the current dataset for which asymmetry between the morphological ranges of states is suggested, it is the primitive state that appears to have the wider morphological range. This warns at least of the potential for long-branch related outgroup-attraction to effect the placement of monotremes among the generalized insectivores they are analyzed alongside.

#### 4.3.6b *Paedomorphosis*

Paedomorphosis, or “reversal heterochrony” (involving neotony, post-displacement, or progenesis: see Gould 1977; Klingenberg 1998) involves the early developmental states of ancestral species being retained in later (e.g. adult) stages of descendant species. Hence, whenever ontogeny recapitulates phylogeny among the ancestors (which is often the case, as much of evolution involves addition to, or replacement of terminal stages) paedomorphosis results in characters being reversed to states of even more distant ancestors (Alberch *et al.* 1979). The switching on of ancient, but recently dormant developmental programs (or Atavism) can produce a similar result. I will lump the possibility of atavism in with paedomorphosis for the current purposes, as these two processes are difficult to distinguish without an excellent temporal fossil series. Experimental manipulation of *Hox* genes has showcased some of the potential of paedomorphosis/atavism to produce the adult characteristics of distant ancestors. For example, by disabling *Hoxa-2*, Rijli *et al.* (1993) showed that instead of developing the incus auditory ossicle of normal mice, a quadrate similar to those of mammal-like reptiles resulted.

The onset, rate of change and retention of characters during development is largely determined by developmental genes that are subject to heritable variation (Atchley 1984; Cowley and Atchley 1992). Thus, if increased retention of a juvenile phenotype increases fitness, paedomorphosis should be expected. As will be discussed further, examples of paedomorphosis are typically associated with niche shifts. For an organism that is making an ecological transition, truncation of development can offer a relatively fast mechanism for dumping deleterious specializations that evolved as adaptations to the ancestral niche (McKinney 1999). This follows von Baer’s principle that ontogeny proceeds from more general to more specialized, with most recent innovation restricted to later developmental stages (see Gould 1977; Fink 1982).

Selection for paedomorphosis falls into at least two categories in the literature. The first is where juvenile (and ancestral adult) characters are directly selected for because they offer a “pre-adaptive” fitness advantage upon a niche transition. This transition may involve a return to an earlier niche, such that the function of the paedomorphic traits is in fact similar to those of distant ancestors (and possibly juveniles). Aquatic salamanders are well documented examples (Roth *et al.* 1993; Voss and Shaffer 1998), with gills and tails retained in conjunction with arrested development of lungs, brains and often legs. Alternatively, the function of the paedomorphic characters may differ from those of distant ancestors (and juveniles), and still be directly selected for the “pre-adaptive” fitness advantage they offer. For example, it is unlikely that the theropod lineage leading up to modern birds included aquatic forms, yet Livezey (1992, 1995) demonstrated

shoulder girdle paedomorphosis of flightless sea birds that use their wings as rudders, or for aquatic propulsion.

The second category is “indirect” selection for paedomorphosis, for which increased retention of a juvenile (and ancestral adult) phenotype increases fitness by truncating the later development of specialized characters that are deleterious subsequent to a niche transition. Such indirect selection for paedomorphosis is thought to have played an important role in the origin of brachiopods (Morris and Peel 1995) and sand dollars (Mooi 1990). Similarly, McKinney (1999) noted that early ideas on paedomorphosis centered on its potential for removing late developmental stage “burdens”, so providing a clean state for evolutionary innovations. However, any initial selection must relate specifically to the phenotype resulting from the developmental truncation. The evolution of evolvability may be a consequence of such selection, but cannot in itself be selected for (Poole *et al.* in press).

Whether selection for paedomorphosis involves the first (direct) or second (indirect) categories of selection, ecological shifts typically seem to be associated. Indeed, heterochrony (particularly paedomorphosis) was suggested to have played an important role in earlier cited examples of trend reversals that are coincident with major ecological shifts. Paedomorphosis was implicated in the evolution of flightlessness among birds (Livezey 1992, 1995; Cubo and Arthur 2001), arborealism among tree kangaroos (Grand 1990), and a benthic to planktonic habitat shift among adult female chaenopsid fish (Emerson and Hastings 1998). Furthermore, paedomorphosis among brachiopods (McNamara 1997), trilobites (Cronier *et al.* 1998), and bivalves (Jones and Gould 1998) has for each been attributed to adaptation to ecological shifts associated with differences in water depth.

By accessing forms and functions that exist during development, *de novo* evolution of complex co-adapted groups of characters is not required. This is a particular advantage of paedomorphic evolutionary mechanisms. McKinney (1999) also points out that this sort of heterochronic evolution can potentially proceed very rapidly. For some taxa, such as those that undergo metamorphosis, early developmental character complexes are independently operable (rather than being “non-functional” in an egg, or uterine environment). Such independent operability might be expected to confer an increased probability of early developmental characters being functionally viable if retained as (paedomorphic) adult stages. Hence, it is not surprising that major evolutionary transitions have been suggested to involve paedomorphic reversal to larval stages. An impressive example is the origin of vertebrates, which are apparently derived from a sea squirt (Urochordata) like filter feeder in which the free-swimming larval stage was retained to adulthood (Romer and Parsons 1977).

Monotremes and marsupials are born at such an early stage (relative to placentals and reptiles) that Griffiths (1978) considers monotreme and marsupial early neonatal development as essentially larval. Whether or not this is justifiable, the *ex uteri* maintenance of functional integration of many anatomical and physiological systems of non-eutherian mammals at very early developmental stages, might be expected to extend the scope for paedomorphosis among the ancestors of monotremes (and marsupials), compared to placentals. Such retention of early developmental traits is still of course likely to require that these confer a selective advantage.

As noted in Table 4.4 (also see the individual character accounts at the end of Section 4.3.4), the positional or functional relations of a number of monotreme (adult) shoulder girdle characters (15, 18, 19, 22) more closely approximate those of newborn marsupials, than those of any other adult mammaliaform, or any known synapsids (chs. 18, 19). Thus, it is reasonable to postulate that paedomorphosis has played a role in the evolution of monotreme upper appendicular characters. This hypothesis is made more attractive by the potential for paedomorphosis (via developmental correlation) to further explain the non-independence of the apparent phylogenetic signal among the upper appendicular characters.

For each of the above noted characters it could be argued that paedomorphic reversal (in monotremes) of phylogenetic trends that occurred along the mammalian backbone lineage would have provided a selective advantage for fossorial/swimming habits (see Table 4.4: niche-related correlation column). Advantages include increased mechanical advantage, augmented bracing against compressional forces on the thorax, and a lateral glenoid orientation, that together allow for the unique forelimb propulsion (incorporating hypertrophied humeral long-axis rotation) that provides a very efficient swimming (and perhaps digging) stroke (Fish *et al.* 1997: Fish 2000). Furthermore, monotremes stand out (*a priori*) candidates among extant mammals for access to cynodont level ontogenetic trajectories. This is conferred by their unique combination of bearing “larval” neonates, and having undergone a major ecological shift very early (during the Late Jurassic or Early Cretaceous). Nevertheless, further testing is required for determining the role of paedomorphosis in the apparent reversal among monotremes, of the upper appendicular trends that occurred among non-specialized Mesozoic mammals. In this regard, and also with a view to documenting the anomalous humeroulnar articulation of monotremes, comparative tracking of the ontogeny of the proximal and distal epiphyses of monotreme, marsupial, and sauropsid (outgroup) humeri may be particularly fruitful.

It is noteworthy that the level of shoulder girdle paedomorphosis required to explain the proposed monotreme reversal has not occurred among therians that have made fossorial or aquatic ecological transitions. However, the development of therian taxa that are most ecologically similar to

monotremes, such as talpids (for which ancestries involving transitions to fossorial and semi-aquatic niches have occurred), is very different from that of monotremes. Among these moles and desmans, the neonates are precocial rather than “larval”. Additionally, compared to monotremes, their later ecological transition to fossorial/swimming habits provided as much as a further 100 million years for crucial aspects of ontogenetic trajectories to be modified or erased, so resulting in cynodont-level character complexes being non-viable. For example, the paired desmal elements that form the lateral processes of the interclavicle of monotremes, and apparently those of non-cladothere mammals (as indicated by *Morganucodon*: Jenkins and Parrington 1976, multituberculates: Meng and Wyss 1995 and *Zhangheotherium*: Hu *et al.* 1997, 1998), are not present at all during the ontogeny of marsupials and placentals (Klima 1987). It is the hypertrophy (full extension along and fusion with the clavicles: see Klima 1973) of these elements in monotremes, to which the anteroventral bracing of their shoulder girdle is largely owed.

A scenario in which an early fossorial/semiaquatic transition along the monotreme stem lineage selects for reversal from a more parasagittal posture, to a highly abducted posture, parallels ideas on the evolution of crocodylians. Crush (1984) considered the late Triassic relatives of modern crocodylians, such as sphenosuchids, to be terrestrial and to have employed a relatively parasagittal, even digitigrade gait. Upon examination of the facultative galloping of *Crocodylus johnstoni*, Webb and Gans (1982) suggested that the sprawling posture typical of modern crocodylians was secondary, and probably related to a shift to semi-aquatic habits. This gained support from Carrier (1987), as it allowed him to explain a number of anatomical and physiological traits of crocodylians that would instead be expected in endothermic tetrapods with high stamina. As a further parallel with the reversal scenario for monotremes, Müller and Alberch (1990) found a number of the derived skeletal patterns of crocodylian limbs to be paedomorphic.

As Gardiner *et al.* (1998) discuss with regard to vertebrate limb development, a further consideration is that genetic and morphological changes do not usually involve a one to one relationship. Selection for heterochrony for one trait may result in other traits being heterochronic, simply as a consequence of genetic/developmental non-independence. For example, Kluge (1989) suggested that selection for small size explains the apparently correlated evolution of a number of other paedomorphic characters among small species of boid snakes. Could the outgroup-attraction of monotremes for non-upper appendicular characters indicated by Figure 4.12 be in part related (as pleiotropy) to selection for paedomorphic evolution of upper appendicular characters? Regardless of this, modification related to a fossorial/semiaquatic transition, extends too much (perhaps all) of monotreme skeletal morphology (Griffiths 1978; Lewis 1983; Augee and Gooden 1993; Grant 1995). Hence, selection for paedomorphosis among a diversity of monotreme morphological traits should not be surprising, given the apparent link between paedomorphosis and

modifications that relate to major ecological transitions. Ontogenetic comparison of a diversity of anatomical regions at a scale similar to the shoulder girdle studies of Klima (1973, 1987) may shed further light on the involvement of heterochrony in the evolution of monotremes, as well as their phylogenetic position.

Heterochrony has clearly been an important mechanism for the evolution of mammalian basicrania. For example, Zeller (1993) showed that the monotreme tympanic bone, tympanic cavity and ossicles develop slowly, relative to other monotreme cranial features (when compared to marsupials and placentals). Furthermore, the pila antotica, which is resorbed in juvenile marsupials, is mostly resorbed in monotremes, though not till adulthood (Wible 1991).

How much, if any of the basicranial heterochrony among mammals is the result of paedomorphosis along the monotreme stem lineage is equivocal. Nevertheless, specialization for hearing high frequency airborne sounds increased along the mammalian backbone lineage (Allin 1975; Meng and Wyss 1995) at the expense of lower frequency vibration detection. The evolution of this specialization is also represented in marsupial ontogeny (Zeller 1993; Sánchez-Villagra *et al.* 2002). Reversal of these trends would have enhanced transmittance and transformation of groundborne and waterborne vibration (Griffiths 1978; Aitkin and Johnstone 1972), for which the highly derived monotreme bill and basicranial region are specialized (Griffiths 1978). Although tenuous, some soft tissue evidence points to reversals of the ear region characters among monotremes. The sharp change in hair bundle angle in the cochlea of therians is thought to have evolved in concert with the extreme spiral curvature of that organ. Ladhams and Pickles (1996) show that despite the cochlea curving only gradually, the sharp change in hair bundle angle also occurs among monotremes, and they wonder if this represents a ghost of monotreme evolutionary past. Further, although monotreme ossicles cannot efficiently transmit high frequency sound, Ladhams and Pickles (1996) showed that the organ of Corti contains the specialized outer hair cell bundles that provide for higher frequency hearing among therians.

In summary, the novel phylogenetic biases postulated in this section are expected to result in outgroup-attraction for morphological long-branch taxa (when analyzed among more conservative taxa). Bias resulting from character coding asymmetry may apply to any characters with unequal probabilities of transformations among states. Paedomorphosis biases may apply to any (non-neutral) characters for which (at least ancestrally) ontogeny has recapitulated phylogeny. As such, these biases are potentially of general importance for determining the affinities of highly modified taxa.

#### 4.4 Concluding remarks and Future Study

Character-map incompatibility (CMI) has three key advantages over previous character compatibility analyses for examining apparent phylogenetic signal relating a specific taxon of interest to a background phylogeny.

1. With CMI, the observed character-map incompatibility ( $CMI_O$ ) count represents only the homoplasy that could be attributed to the stem and crown lineages of the taxon of interest. Previous analyses have measured compatibility over all taxon/character interactions and can only infer such homoplasy indirectly, by taxon inclusion/exclusion.
2. Incompatibility tests traditionally (e.g. Le Quesne 1969; Meacham 1994; Day *et al.* 1998) determine expected levels of incompatibility from the numerical distribution of character states across the taxa. However, these character states, and hence, that most-parsimonious placements of the taxon of interest will be non-randomly distributed over the background phylogeny, due to character covariation among the background taxa. To dissociate the pattern of monotreme placements from the character covariation among the background taxa, expected character-map incompatibility ( $CMI_E$ ) values are derived by random shuffling of character-maps (RSCM) across the background phylogeny. The character-maps preserve both the number and relative topological association of the most-parsimonious positions of the taxon of interest on the background phylogeny as either leaves, node-groups, connected node-groups, terminal node-groups, or compound character-maps (see methods and Figure 4.3). Inspection of Table 4.1 reveals that both the numerical distribution, and the relative association of the most-parsimonious positions of the character pairs affect the combinatorial probability of incompatibility considerably.
3. Not only does CMI allow the examination of apparent phylogenetic signal for a taxon of interest, with respect to the background phylogeny as a whole, but also for the position of that taxon with respect to any given node on that phylogeny. Examination of incompatibility across specific nodes allows the topological distribution of conflict to be isolated. Additionally, relative character-map incompatibility ( $CMI_R$ ) provides a relative measure of signal strength (homogeneity), or conflict, that can be compared between partitions and across different nodes. For example, with respect to monotreme affinities indicated by the current data, the greatest conflict (relative to that expected under RSCM) occurs between U and MVD, across the theriimorpha node. Over the entire background phylogeny, LB is the meta-region with the least homogenous signal.

A future modification that may be considered for estimating  $CMI_E$  is a correction for differences in length among the various internal and external branches of the background phylogeny. In the

extreme situation of two identical taxa being included among the background phylogeny, a most parsimonious placement (of the taxon of interest) with one, will necessarily also be shared with the other, and their stem. As noted by Day *et al.* (1998) and Fu and Murphy (1999), this issue of non-independence that relates to uneven distributions of branch-lengths is also a problem for previous analyses of incompatibility and other significance tests for phylogenetic signal. RASA (Lyons-Weiler *et al.* 1996), which measures cladistic similarity relative to phenetic similarity, may be an exception. Determining combinatorial probability of incompatibility with respect to branch length estimates (rather than the unit branch-length assumption) would be an important advance when the branch-length distribution is very uneven, though it may be computationally expensive with large datasets. Branch length unevenness is not expected to be greatly affecting the results of the current analysis, as among the 11 background phylogeny branches (including internodes), the standard deviation of their lengths (6.74 steps) is somewhat less than the average (9.46 steps), for minimum-evolution distances.

One current shortcoming of the CMI analysis is the use of the standard deviation of the binomial sampling distribution for estimating variance associated with expected character-map incompatibility ( $CMI_E$ ) values. By doing so, instead of the number of assumed independent characters providing the sampling number, the number of pairwise comparisons provides the sampling number. The non-independence will tend to result in  $CMI_E$  variance being underestimated. However, the non-homogeneity of combinatorial probabilities of incompatibility among pairwise comparisons within or between partitions will tend to result in  $CMI_E$  variance overestimates. This will to some extent counterbalance the problem of non-independence among pairwise comparisons. As such, future examination of character-map incompatibility may be enhanced by the use of Monte Carlo simulations to estimate variance for  $CMI_E$ . However, given the very high binomial probability significance levels ( $p < 0.005$ ) used for considering deviations from  $CMI_E$  to be significant, estimating sampling error from simulations is unlikely to alter any of the conclusions of the present study.

The current CMI analysis provides quantitative measures of signal strength (homogeneity), or conflict, for monotreme placement with respect to the background phylogeny overall (and across specific nodes), both within and between the data partitions. This allowed for a more thorough assessment (than other tests provide) of how the evolutionary independence of characters and the anatomical region incongruence relate to monotreme placement. Results from partition homogeneity testing (PHT) in Chapter 3 showed that the inclusion of monotremes with the generalized insectivores induces significant incongruence between the anatomical meta-region partitions. The PHT does not indicate the topological distribution of the incongruence, or test for signal within partitions (so when not rejecting the null hypothesis of congruence, it is unable to

discriminate signal from noise). CMI analysis allows greater interpretation of the nature of this homoplasy, in that it tests the null hypothesis,  $H_0$ : that the distribution among characters, of signal for monotreme placement is random within, and/or between partitions, across any of the nodes of the background phylogeny of generalized insectivores.

An additional problem with the PHT is inflation of apparent congruence when partitions contain different numbers of characters (Dowton and Austin 2002), as congruence among characters within larger partitions can swamp incongruence between partitions. Such partition size differences cannot affect this CMI analysis, because within-region and between-region pairwise character comparisons were analysed separately.

The important findings of the CMI analysis are: 1. Pairwise comparison of characters reveals signal across each node for monotreme placement within each meta-region (U, LB, and MVD), except across trechnotheria for  $CMI_0[LB]$ , for which signal does not differ significantly from random (Figure 4.8) and 2. In contrast, significantly greater than expected conflict for monotreme placement occurs across at least one node, for pairwise comparisons between each of the three meta-regions (see Figure 4.7). From these two points it can be inferred that homoplasy relating to monotreme placement has evolved non-independently across characters, at the level of anatomical regions (or meta-regions), rather than being randomly distributed among characters. If the sister group of monotremes among the generalized insectivores is Trechnotheria, then both U and MVD must be homoplastic. Likewise, if monotremes stem from within Trechnotheria, or outside Theriimorpha, the minimum meta-region-level homoplasy required would be U and LB, or LB and MVD respectively.

Until further monotreme fossil material (especially basicranial/post-cranial) is found, embracing the most parsimonious compromise among the partitions cannot be justified on the basis of teasing phylogenetic signal from “noise”. In fact this amounts to a loss of information on the nature of phylogenetic signal within and between the data partitions. Currently, a framework within which monotreme affinities are viewed as competing options for anatomical region-level homoplasy may best serve the objective of determining monotreme placement among the background phylogeny. Within this framework, the most likely placement is the one that is the most consistent with what can be established on the nature of the incongruence among the anatomical regions.

Within the framework for assessing monotreme affinities as competing options for homoplasy, a number of points were derived from the CMI analysis (Figures 4.7 and 4.8), the partition homogeneity testing (Chapter 3), and parsimony analysis (Figure 4.6). Key among these, are that any proposal of monotreme affinities must explain the non-independence of homoplastic character

evolution at the anatomical region (or meta-region) level, and that the correlated homoplasy is expected to largely involve transformations along the monotreme stem lineage. Given these conditions and that the anatomical regions approximate functional units, the incongruence is expected to be associated, either with phylogenetic trends that are apparent among the background taxa, or with the ecological (and so, functional) transition along the monotreme stem lineage. This transition involved the evolution of a mid-sized, fossorial and probably semiaquatic predator of mostly invertebrates, from the small terrestrial (or scansorial) insectivore/carnivore that was the last common ancestor of monotremes with the background phylogeny. A further condition of the framework (derived from molecular data: Chapter 2) is that monotremes arose from the mammalian backbone lineage prior to the placental/marsupial split, but more recently than morganucodontids. Any character in conflict with this is homoplastic.

Although considerable efforts have been made in describing monotreme osteoanatomy, there is currently a lack of knowledge of how the form/function relations of monotreme characters differ from those of other mammals. This limits the potential for using the framework that was derived for determining monotreme affinities in terms of homoplasy options. The lack of access to fossil material, at least for this study, is a further restriction. Nevertheless, the preliminary comparison of phylogenetic trends (among the generalized insectivores) for the functional relations of shoulder and forelimb characters, with the conditions in monotremes, was instructive. This showed that for nine of the ten characters that place monotremes outside Theriimorpha, the monotreme state is either more primitive than that of morganucodontids (indicating homoplasy), or highly autapomorphic. Hence, the apparent phylogenetic signal attributable to monotremes by the upper appendicular region cannot be considered reliable. However, the hypothesis that this signal instead results from correlated reversals that are associated with specialized fossorial/swimming habits, is consistent with each of the six points of the framework for assessing monotreme affinities in terms of homoplasy options.

Numerous monotreme upper appendicular characters are more primitive than those of morganucodontids, or even Triassic cynodonts, indicating that trends along the mammalian backbone lineage were reversed along the monotreme stem lineage. I argued that the proposed reversals are consistent with the evolution among monotremes of unique forelimb propulsion (incorporating hypertrophied humeral long-axis rotation) that provides a very efficient swimming (and perhaps digging) stroke. The (homoplastic) evolutionary non-independence of the upper appendicular characters can be explained via functional/developmental correlation among characters for the provision of increased mechanical advantage, augmented bracing against compressional forces on the thorax, and a lateral glenoid orientation. Each of these correlates is associated with the hypertrophied humeral long-axis rotation of monotremes.

Reversal of upper appendicular character states along the monotreme stem lineage is consistent with monotreme affinities being either within, or as sister to the Trechnotheria (as supported respectively by MVD and LB). I remain cautious with respect to favouring either of these placements, though a more diverse range of evidence may favour monotremes arising from within Trechnotheria. With the inclusion of the fossil information for non-upper appendicular characters, monotreme affinities within Trechnotheria are favoured (Figure 4.12a). Furthermore, that this favoured placement moves outside (as sister to) Trechnotheria upon exclusion of the fossil information (Figure 4.12b) is consistent with outgroup-attraction (of morphological long-branches) being the dominant phylogenetic bias associated with apomorphies of the crown group monotremes. Molecular data (Chapter 2) also suggests that monotremes arose from close to the last common ancestor of modern therians, possibly even hinting at a monophyletic clade of tribosphenic mammals. The advantage of such a grouping of australosphenidans (including monotremes) and boreosphenidans, is that the complex anatomy and occlusal relations of tribosphenic molars are required to have evolved only once.

Despite the attractiveness of a monophyletic grouping of tribosphenic mammals (including monotremes) such a clade may be in conflict with recent suggestions (see Kielan-Jaworowska *et al.* 1998; Lou *et al.* 2001a) that early australosphenidans retained jaw-attached postdentary bones (middle ear bones). Rich *et al.* (2001b) remain skeptical about the nature of the mandibular grooves that the above authors consider as troughs for postdentary bones. Only the holotype of *Ausktribosphenos nyktos* has a “surangular facet” that is separated from the meckelian groove, but this facet is much reduced (and not overhung by a medial ridge), in comparison to primitive mammals in which the ear bones were jaw-attached. In the second *A. nyktos* specimen (Rich *et al.* 1999) and related *Bishops whitmorei* (Rich *et al.* 2001b) only one shallow groove exists, somewhat ventral to the mandibular foramen, while such grooves are not at all apparent in the earliest known monotreme, *Teinolophos* (Rich *et al.* 2001a). Further confusion over the significance of the alleged jaw-attached postdentary bones comes from the fact that such bones have been suggested for a number of eupantotheres (e.g. Allin and Hopson 1992; Heinrich 1998), while they were completely detached from the primitive mammaliaform *Hadrocodium* (Lou *et al.* 2001b).

An additional issue with jaw-attached dentary bones that needs explanation is that in ausktribosphenids, the groove suggested to contain postdentary bones is well anterior to the angular process. The angular (tympanic) develops in close association with the posterior aspect of the angular process in therians (Rowe 1996; Sánchez-Villagra *et al.* 2002), and by inference from the adult condition, in more primitive synapsids also (Allin 1986; Crompton and Lou 1993). Hence, if the angular process of australosphenids is homologous with that of other mammals, it is difficult to

imagine the mandibular groove(s) containing the tympanic bone. Even the most anteriorly positioned postdentary bone of mammal-like reptiles, the prearticular (which becomes part of the malleus in mammals), develops dorsally to the angular (tympanic) in monotremes (and therians) so is not an obvious candidate for containment within the anteriorly positioned grooves of ausktribosphenids. A possible solution to this though is that the middle ear bones of *Ornithorhynchus* develop more medially with respect to the dentary, than do those of marsupials and placentals (Maier 1993). The nature of the mandibular grooves of the ausktribosphenids deserve further consideration, especially in light of the recent discovery of ossified meckelian cartilage (possibly for attachment of pterygoid musculature) associated with medial mandibular grooves in gobiconodontids (Wang *et al.* 2001).

The CMI analysis provides further reason for being cautious about assuming that the reliability of determining monotreme affinities among the generalized mammals, is increased simply by excluding the upper appendicular characters. The signal attributable to at least one of MVD or LB must largely be a reflection of homoplasy that has evolved non-independently, at the anatomical region (or meta-region) level. Comparison of phylogenetic trends (among the generalized insectivores) for the functional relations of characters among LB and MVD, with the conditions in monotremes, may reveal that correlated homoplasy among only one of MVD and LB is consistent with the framework for assessing monotreme affinities. Though until the form/function relations for monotreme characters from the MVD and LB meta-regions are better understood, any conclusion for monotreme placement on the background phylogeny that is based on these data should be treated with caution.

An important point that comes out of this study is the need to explore the potential effects of phylogenetic biases associated with the inclusion of taxa that are highly modified relative to others in an analysis. For such modified taxa, outgroup-attraction in particular may be common for phylogeny reconstruction from morphological data. This is suggested from the current examination of monotreme affinities, and from phylogenetic inference from molecular data for whales, turtles and megachiropteran bats.

Parallelism related to trends that were initiated before the LCA of a clade, is likely to be more frequent between taxa within that clade, that have retained the most similar functional relationship with the environment. This could lead to a highly derived taxon being pushed out of its true sister group relationship and closer to the outgroup. Lack of evidence for correlated homoplasy among the background taxa (except perhaps among a few mandibular characters) argues against such parallelism having a substantial effect on monotreme placement. However, asymmetry between

the morphological ranges of character states, and paedomorphosis (and / or atavism) are suggested to have encouraged apparent outgroup-attraction of monotremes.

Apomorphies of highly modified taxa are more likely to fall within the bounds of a pre-defined character state, the wider the morphological range of the state is. It is argued that the plesiomorphic (or outgroup) state often has the widest morphological range, and so highly modified taxa will tend to be outgroup-attracted. Paedomorphosis allows access to (often more generalized) functionally and developmentally co-adapted character complexes that in a new niche, may provide a fitness advantage over specializations evolved as adaptations for the niche occupied prior to an ecological transition. The developmental correlation among characters that is inherent in paedomorphic evolution is a particularly attractive feature for this study, given (a) the need to explain the non-random distribution of homoplastic evolution within the meta-regions and (b) that a number of aspects of monotreme shoulder girdles more closely resemble conditions in marsupial neonates, than among any known mammaliaform adults.

The functional/ecological derivation of monotremes is conspicuous when they are compared to the more conservative generalized insectivores. More generally however, deciding what constitutes a morphological long-branch is problematic. As outgroup-attraction may involve autapomorphic characters being erroneously considered as plesiomorphic, such functional/ecological long-branches cannot simply be assumed to be detectable as long branches on a cladogram or phylogram. This problem could however be solved by re-coding characters as having continuous states. This has the further benefit of eliminating the problem of asymmetry between the morphological ranges of discrete character states. As discussed by Wiens (2001), re-coding characters as continuous quantitative traits is possible for almost any morphological character, and certainly this deserves further attention within the context of examining morphological long-branch problems, including outgroup-attraction. However, simply re-coding discrete characters for continuous states would not nullify biases associated with paedomorphosis, or parallelism among taxa that retain ancestral niche characteristics.

In summary, the CMI analysis and consideration of monotreme shoulder girdle and forelimb traits (in view of trends among the background phylogeny), were used to derive a framework for assessing monotreme affinities in terms of homoplasy options. Correlated reversal among monotreme upper appendicular characters (associated with specialized fossorial/swimming habits that incorporate hypertrophied humeral long-axis rotation) is consistent with the six points of the framework and sufficient to explain the incongruence across theriomorpha. In turn, this reconciles U, LB, MVD and molecular data with monotreme affinities that lie at least within Theriomorpha. A better understanding of the affinities and evolution of monotremes may require renewed efforts

in examining the developmental and form/function relations of their characters. Although cladistics provides a useful and repeatable statistical framework, phenomena such as long-branch outgroup-attraction and niche-related convergence are important reminders that phylogeny reconstruction is ultimately limited by our understanding of evolutionary processes.



## **Chapter 5**

### **The timing and ecological implications of the interordinal diversification of modern mammal groups**

## **5.1 Growing up with Dinosaurs: molecular dates and the mammalian radiation**

Bromham, L., Phillips, M. J. and Penny, D. (1999)

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## Growing up with dinosaurs: molecular dates and the mammalian radiation

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**Dates of divergence derived from molecular data have been used to place the beginning of the radiation of modern mammalian orders in the Cretaceous, long before the final extinction of the dinosaurs. These molecular dates have been used to challenge the idea that the ordinal diversification of mammals was triggered by the availability of 'empty niches' left vacant by the disappearance of the dinosaurs. However, the broad discrepancies between molecular date estimates from different studies warn that molecular dates should not be accepted uncritically. Consideration of the wide confidence intervals around molecular date estimates, and the potential for geographic bias in the fossil record, could lessen the discrepancy between molecular and palaeontological data but might still prompt a re-evaluation of the timing and causes of the mammalian radiation.**

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The study of mammal phylogeny has been revolutionized by molecular systematics<sup>1</sup>. Novel relationships have been suggested, such as the whales as the sister group to hippos<sup>2</sup>, and marginal hypotheses supported, such as grouping marsupials and monotremes into Marsupionta to the exclusion of the placentals<sup>3</sup>. Also, the presumed stochastic nature of molecular evolution has led to the prediction that changes should accumulate approximately linearly with time, so the divergence time between taxa can be estimated from their genetic distance. Some of the earliest applications of 'molecular clocks' were to mammalian evolution<sup>4,5</sup> and the technique continues to be widely applied.

Recent molecular phylogenetic studies<sup>3,6-9</sup> have presented surprisingly old dates for the origin of many mammalian orders. These dates have been proclaimed as the death knell of the idea that the radiation of modern mammals occurred in the early Tertiary, as mammals evolved into niches left vacant by the end-Cretaceous extinction of the dinosaurs. A radiation of mammalian orders after the Cretaceous-Tertiary (K-T) boundary 65 million years ago (Mya) has been supported by the relatively low diversity of Mesozoic (245-65 Mya) mammals, with few fossils unambiguously attributed to modern orders. This low Mesozoic diversity combined with the increase in mammal fossil diversity in the Palaeocene (65-60 Mya) following the apparently sudden disappearance of the dinosaurs from the fossil record

at the K-T boundary, and the 'bush-like' phylogeny of mammals, were interpreted as the signature of a rapid radiation.

Although some researchers in palaeontology accept a late Cretaceous origin of many lineages of modern mammals<sup>10</sup>, fossil evidence of modern orders before the K-T boundary is restricted to only a few lineages: monotremes, marsupials, insectivores and, possibly, primates. The molecular dates are controversial because they suggest that most mammalian orders crossed the K-T boundary, and that the divergences between these lineages are surprisingly deep<sup>11</sup> (Fig. 1). Are the molecular dates accurate, and can they be reconciled with the fossil record?

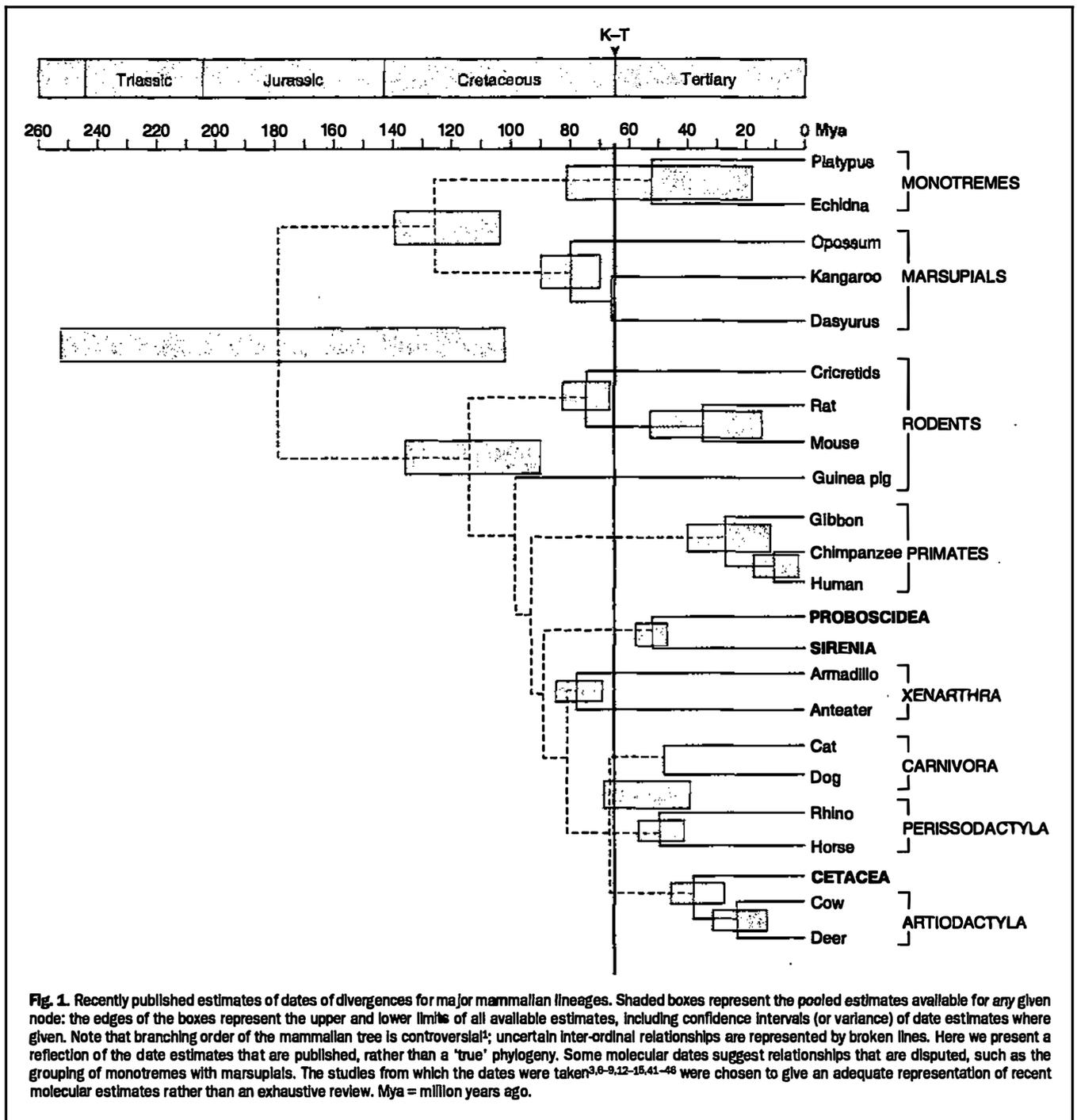
### Dating mammalian origins

The time-honoured way of estimating the age of a lineage uses the first appearance of a taxonomic group in the fossil record (Box 1). This not only marks the minimum age of that taxon, but also puts a minimum age on its sister groups (or outgroups). Fossil-based divergence dates are subject to various sources of error, owing to the temporal incompleteness of the fossil record, uncertainty in identifying specimens and difficulty in establishing absolute age. The size of the gap between the origin of a lineage and its first identifiable fossil will vary with location, age and type of organism. Despite these difficulties, fossil dates are irreplaceable as the primary means of assigning a minimum age to a taxon.

Molecular dates are obtained by converting estimated genetic distance between lineages to time since divergence using a calibration rate (the expected substitution rate; i.e. amount of molecular change per unit time), which has been calculated for some lineage with a 'known' date of origin (usually a palaeontological estimate). Molecular dates should mark the actual point where lineages split, and so are often earlier than the corresponding palaeobiological estimates, but not always – for example, the considerably younger estimates for the divergence of the great apes<sup>12,13</sup> and monotreme families<sup>14</sup>. Molecular data can be used to circumvent some of the natural biases of the fossil record, by inferring the presence of lineages in periods for which the fossil record of that taxon is poor or non-existent. Using sequences from extant taxa removes the difficulties associated with identifying early members of a lineage (such as stem groups that do not have all the defining features of the crown group: Box 1) from fragmentary evidence. However, molecular dates, like fossil dates, are subject to various sources of error. The accuracy of molecular date estimates depends on the data and methods used, which is why estimates of the same node from different studies can differ so dramatically (Table 1).

### Variation in molecular date estimates

The degree of variation in molecular date estimates is striking. Published estimates of the same node by different researchers can vary by as much as 100% (Table 1). The variation between dates indicates that differences in the data or methods used can substantially alter the results. Unfortunately, because studies differ in many aspects of data and methods, it is difficult to isolate specific causes of the discrepancy. For example, two recent studies<sup>3,7</sup> give disparate estimates of the marsupial-eutherian split (Table 1), and this discrepancy could be because of differences in the choice of sequences (concatenated mitochondrial protein genes versus many individual nuclear sequences), calibration date (artiodactyl-whale split at 60 Mya versus bird-mammal split at 310 Mya) or phylogenetic method (maximum likelihood and mt-REV distances versus 'Poisson corrected' distance). Note that because some sources of error will produce consistently biased results, these estimates might not be normally distributed about the true value, so averaging all published estimates will not necessarily give a more reliable estimate. One potential source of consistent bias in molecular studies, which is a particular cause for concern in dating mammalian



divergences, is the difficulty in establishing an appropriate calibration rate.

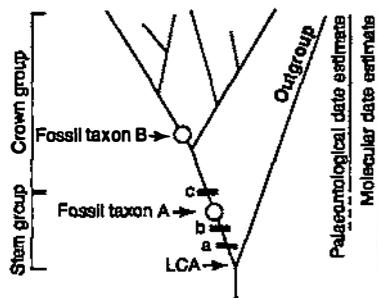
The accuracy of fossil dates used for calibration has been a focus of criticism<sup>13,15</sup>, because different calibration dates produce different estimates of rate of substitution<sup>16,17</sup>. The importance of the calibration date to the accuracy of date estimates is illustrated by comparing the dates for hominoid divergence times obtained in two studies using similar data and methods but different calibration dates (Table 2). A biogeographical estimate of the age of a radiation of seal species (*Phoca*) gave date estimates that were approximately half the age of those

given by a fossil-based estimate of the whale-artiodactyl split. To minimize the error resulting from fossil calibration dates, two opposite strategies can be adopted: use as many fossil dates as possible<sup>9</sup>, to spread the risk of error, or place your faith in one date deemed to be especially reliable. For example, Arnason *et al.*<sup>14</sup> suggested that primate fossil dates were so unreliable that it was preferable to use a well constrained fossil date from another taxon to date primate divergences. Hedges *et al.*<sup>6,7</sup> used a similar argument in adopting the split between mammals and birds (310 Mya) as a calibration for dating mammalian ordinal

divergence times. In contrast, Easteal *et al.*<sup>13</sup> aimed to avoid the error inherent in fossil dates by developing a fossil-free approach, applying a range of possible rates to pairwise distances and selecting the one that gave the most reasonable answers for the nodes tested. However, this approach served to illustrate the difficulty in using a single substitution rate to date all mammalian divergences. The intermediate rate chosen was much slower than previous estimates of the rate in rodents and much faster than previous primate estimates<sup>13</sup>, with the result that the rodent divergence dates were surprisingly old and the primate dates unusually

**Box 1. Stem and crown groups**

Estimates of molecular and fossil dates mark different points in the history of a lineage, which can complicate their comparison. A taxonomic group might be defined as: (1) all of the taxa that show a defining set of characters; or (2) all of the members of a lineage. Molecular dates mark the point at which two lineages last shared a common ancestor (LCA), so estimate the age of the stem + crown group. The palaeontological age of a taxon marks the appearance in the fossil record of a specimen with the diagnostic characters of that group, which could be a member of the crown group or the stem lineage. In the example below, a taxon is defined as a monophyletic group (i.e. descended from a single ancestral taxon) consisting of species with three defining characteristics (a, b and c). Fossil B marks the known minimum age of the crown group because it has all the defining features. Fossil A has some but not all of the features, so might be recognized as a member of the ancestral lineage of the taxon. The palaeontological age might also represent an earlier fossil of a known sister group. In this review, we refer to the origin of a mammalian order as the point at which it diverges to form a separate lineage (i.e. stem→crown).



(Online Fig. 1)

**Table 1. Comparison of some molecular date estimates published in the last five years for three mammalian divergence events of varying 'depth'<sup>a</sup>**

Node	Estimated date of split (Mya) <sup>b</sup>	Refs
Marsupial–Eutherian	133–175	12
	218–257	13
	120–160	3
	104	17
	161–185	7
Primate–Rodent	118–150	12
	112–133	13
	88–202	6
	97–126	42
	106–141	3
Human–Chimpanzee	109–116	7
	8.5–16.2	12
	5.2–6.9	15
	3.2–4.5	13
	5.3–5.7	7

<sup>a</sup>Depth is a broad term for the approximate age of a lineage.  
<sup>b</sup>Mya = million years ago.

**Table 2. Comparison of estimated dates of hominoid divergences calculated from two different calibration dates**

Divergence	Calibration date	
	A/C-60 <sup>a</sup> (Mya)	<i>Phoca</i> standard <sup>b</sup> (Mya)
Human–Orangutan	24.5 ± 2.7	18.1 ± 1.6
Human–Gorilla	18 ± 2.9	8.4 ± 1.1
Human–Chimpanzee	13.5 ± 2.5	6.1 ± 0.9
Common chimp.–Pygmy chimp.	7 ± 1.8	3 ± 0.6

<sup>a</sup>Fossil-based estimate of the split between cetaceans and artiodactyls at 60 million years ago (Mya): whole mitochondrial genomes for 14 species analysed at the nucleotide and amino acid level by maximum likelihood and neighbour joining<sup>12</sup>.  
<sup>b</sup>Radiation of *Phoca* (seal) species at 2.7 Mya, inferred from climetological date: whole mitochondrial sequences for eight species, neighbour-joining branch lengths<sup>15</sup>.

young. This pattern is consistent with previous claims that rodents have a faster rate of evolution than primates<sup>18–20</sup>. Such lineage-specific rate variation casts doubt on the use of a single calibration rate, whether produced by cross-taxon calibration or other means, to date all mammalian divergence times. A corollary of this observation is that the adequacy of a calibration rate cannot be established by referring to the reasonableness of the date estimates it gives for some nodes.

**Rate variation affects date estimates**

The common reliance on rate uniformity is one of the greatest stumbling blocks in the production of accurate molecular date estimates for mammalian orders. The existence of established cases of lineage-specific rate variation, whether for specific taxa (e.g. between hominids and murid rodents<sup>18–20</sup>), particular sequences

(e.g.  $\psi\eta$  globins<sup>21,22</sup>) or related to life history characters (e.g. generation time<sup>18,23</sup>), indicates that rate uniformity cannot be taken for granted for any given set of sequences.

One way of dealing with the potential for inaccuracy caused by rate variation is to use a 'clock test' to select data that approximates rate uniformity. Clock tests are commonly based on the relative-rates test (which compares the amount of change in each of two ingroup sequences with respect to an outgroup<sup>5,24</sup>). Maximum likelihood can also be used to test how well a rate-constant model fits the data<sup>25,26</sup>. However, these clock tests are limited in power when applied to short sequences and are unlikely to detect moderate levels of rate variation for sequences of less than 1000 variable positions. Regression of observed genetic distance through palaeontological estimates to establish a linear relationship between distance and time<sup>7,9</sup> has the advantage of using a range

of fossil dates and species in the calibration. However, it is limited by the frequent use of nonindependent comparisons, which can give an overconfident impression of rate constancy.

The problems associated with selecting rate-constant sequences can be illustrated by a common approach to dating the frequently estimated primate–rodent split<sup>6,7,13</sup> (Table 1). A relative-rates test can be used to select rate-constant sequences with which to estimate the date of the split by multiplying half the pairwise distance by the calibration rate. However, relative-rates tests cannot reliably reject shorter sequences that show a realistic level of rate variation (e.g. rodent sequences with a rate 1.5 to 3 times faster than primate sequences<sup>18–20</sup>). Failure to reject sequences that evolve faster in rodents could result in an overestimation of the age of the split between rodents and primates, because the calibration rate will overestimate the number of years

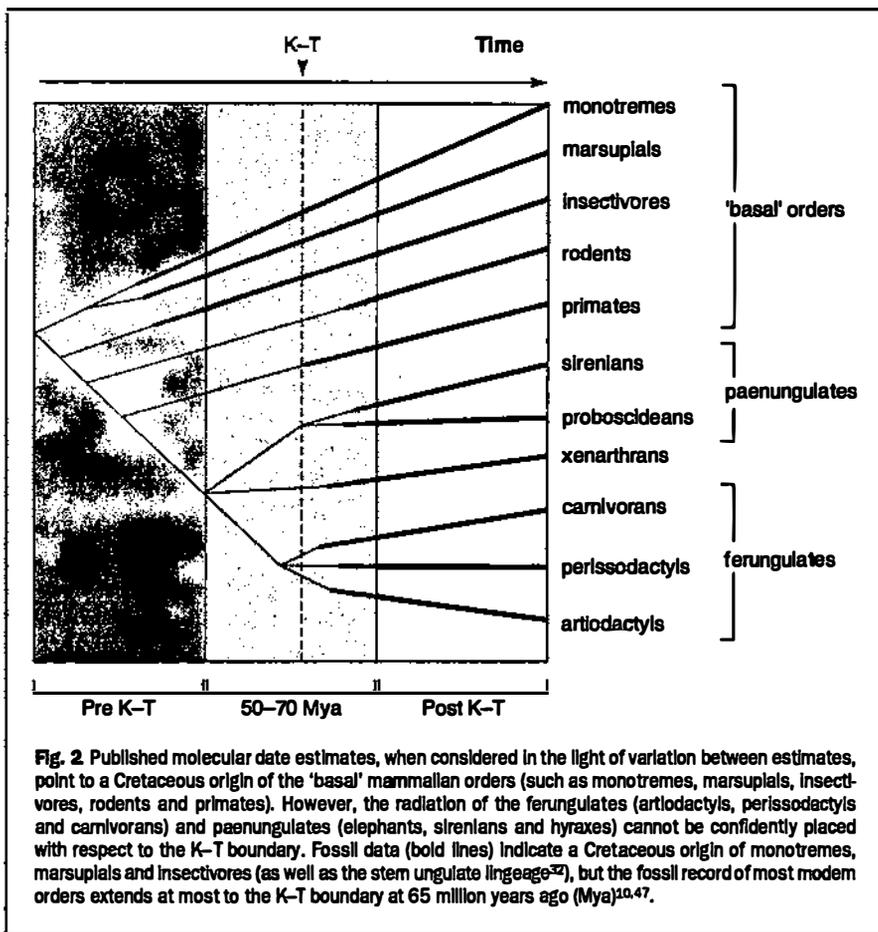


Fig. 2 Published molecular date estimates, when considered in the light of variation between estimates, point to a Cretaceous origin of the 'basal' mammalian orders (such as monotremes, marsupials, insectivores, rodents and primates). However, the radiation of the ferungulates (artiodactyls, perissodactyls and carnivorans) and paenungulates (elephants, sirenians and hyraxes) cannot be confidently placed with respect to the K-T boundary. Fossil data (bold lines) indicate a Cretaceous origin of monotremes, marsupials and insectivores (as well as the stem ungulate lineage<sup>29</sup>), but the fossil record of most modern orders extends at most to the K-T boundary at 65 million years ago (Mya)<sup>10,47</sup>.

required to produce the observed distance between rodents and primates. This discrepancy is expected whether the calibration rate is calculated from cross-taxon calibration (e.g. the artiodactyl-whale split<sup>12</sup>) or an external calibration point (e.g. the bird-mammal split<sup>6,7</sup>, although the discrepancy will be even greater if birds have a slower rate of molecular evolution than mammals<sup>27,28</sup>). If lineage-specific rate variation is a widespread feature of mammalian sequence evolution<sup>23</sup>, and if clock tests with limited power are used to screen sequences with relatively low numbers of variable sites, then failure to reject rate-variable sequences could potentially be a common cause of inaccuracy in molecular date estimates of the origins of mammalian orders.

### A sloppy clock is better than no clock

Given the effect of choices of data and methods and the potential for error in molecular dates as a result of undetected rate variation, it might be unwise to expect a molecular clock analysis to give precise point estimates of divergence dates<sup>29</sup>. However, acknowledging the imprecision of molecular date estimates does not deny their usefulness in evolutionary biology.

Molecular dates considered in terms of confidence intervals, which reflect their imprecision, can be used to assess the compatibility of molecular dates with a given hypothesis<sup>26</sup>.

The variation in the date estimates presented in Fig. 1 and the potential inaccuracy resulting from rate variation prevent us from confidently placing the radiation of the ferungulates (artiodactyls, whales, perissodactyls and carnivorans) and paenungulates (elephants, sirenians and hyraxes) on either side of the K-T boundary because most of these dates are in the region of 50-70 Mya. There is also insufficient resolution to determine if the radiation of mammalian orders occurred in a gradual, step-like or rapid, bush-like radiation. However, even allowing for a large margin of error in the range of dates in Fig. 1, molecular data support placing the divergence of primates from rodents, and monotremes from marsupials, before the K-T boundary. This also implies that any sister groups to these nodes, such as lagomorphs (rabbits and hares), insectivores and the lineage leading to the ungulate orders, must also have originated in the Cretaceous (Fig. 2).

Because the potential for error in molecular date estimates cannot be ignored, these estimates should never be

accepted uncritically but should be examined in the light of other sources of historical information in biology, particularly palaeontology, biogeography and phylogeny. For example, biogeographical information can be used to put upper and lower bounds on the divergence of mammalian lineages by considering the presence of lineages on specific continents and the periods in which migration of mammals between continents was possible. Springer *et al.*<sup>9</sup> used the geographic isolation of Africa from South America from 80 Mya ago until after the K-T boundary to support a Cretaceous origin of an African clade of paenungulate lineages inferred from molecular data.

In particular, the proposed early diversification of modern mammals must be reconciled with the fossil record, which shows low diversity of modern orders in the Cretaceous, followed by a burst of diversity after the K-T boundary<sup>30</sup> (Fig. 2). Is it plausible to suggest that so many modern mammalian orders could have a long history before the K-T boundary but have so far largely failed to turn up in the Cretaceous fossil record?

### Mammals in the Cretaceous

Fossil evidence for the history of modern mammals before the K-T boundary<sup>31,32</sup> is rare and often complicated by the difficulty of recognizing ancestral members of a lineage, which might not show the defining characteristics of the crown group – a problem compounded by the fragmentary nature of the specimens. Many species are known only from dental characters (sometimes only a single tooth), the phylogenetic stability of which can be questioned<sup>33</sup>.

Could early mammals have been hidden from the fossil record by consistent geological bias? Biogeography might hold the key to assessing the potential for a 'hidden' period of early mammalian evolution. For example, recent molecular phylogenies have promoted an 'African clade' deep within the mammalian tree, placing the origin of many lineages (such as paenungulates and some insectivore lineages) within the African continent<sup>9,34</sup>. But, as yet, there are no known African late Cretaceous beds of terrestrial vertebrate fossils that could provide information on the early members of these mammalian lineages. Similarly, recent finds suggest the presence of eutherian mammals in Australia as early as 120 Mya (Ref. 31), but there are no terrestrial vertebrate fossil-beds known from Australia between 100 and 55 Mya. Mammal fossils from stages just before the K-T boundary (Campanian-Maastrichtian, 83-65 Mya) are primarily from fossil beds of the western interior of North America<sup>35</sup> and the

central latitudes of Asia<sup>36</sup>, so if early mammal evolution occurred elsewhere, it could be effectively invisible to the fossil record.

Could the post-K-T burst in mammal diversity represent the movement of mammals from other continents into North America and Asia, rather than their rapid radiation within that region? The drop in sea levels in the late Cretaceous<sup>37</sup> could have brought together previously isolated faunas, and the Palaeocene burst in diversity in North America for terrestrial taxa other than mammals, such as amphibians and lizards<sup>38</sup>, might reflect a common biogeographical pattern of faunal movement. If the early history of modern mammals occurred in a region with an impoverished late Cretaceous fossil record, such as in Africa, then the absence of fossil evidence to support the molecular dates might be explained.

### Radiation of the mammals

So what do molecular dates tell us about the evolution of modern mammalian orders? If the molecular estimates are true, then many more modern orders had their origin in the Cretaceous and began radiating long before the dinosaurs disappeared. This might suggest that mammalian diversification was not primarily driven by release from the constraint of the presence of the dinosaurs, although the evolution of mammals into larger size and more conspicuous niches (such as large diurnal grazers and large carnivores) might have been limited until the demise of the larger dinosaurs, perhaps explaining the later radiation of the paenungulates and ferungulates. A Cretaceous divergence of mammalian orders also implies that many of the characteristics considered 'key innovations' of the mammalian radiation, such as aspects of brain function, reproduction or metabolism, evolved long before the extinction of the dinosaurs.

The coexistence of modern mammals with dinosaurs raises interesting questions about the nature of their interaction. If Cretaceous mammals, as a whole, were ecologically diverse as well as phylogenetically diverse (evidence from fossil dentition suggests possible mammalian insectivores, herbivores, granivores, carnivores and scavengers<sup>39,40</sup>), then it seems possible that the spectrum of resource requirements could have overlapped with that of the dinosaurs. It is intriguing to note that the projected rise of the modern mammals coincides with the disappearance of the smaller dinosaurs, those most likely to have been in direct competition with the new mammals (M.J. Phillips, unpublished).

### Prospects

Phylogeny has an important role to play in resolving the tempo and mode of the radiation of the mammals. Higher resolution of internodes would distinguish between two modes of diversification: explosive radiation versus stepwise divergence. Phylogeny can also allow the inference of past states, by providing relative timing of evolution of traits based on lineages sharing homologous characters. In addition, the estimation of divergence dates from molecular data will play a critical role in forming hypotheses concerning the origins of mammal diversity, particularly as the accuracy and precision of molecular phylogenetic methods develop. If date estimates with appropriate confidence intervals that reflect the uncertainty in molecular dating are combined with palaeontological data (as the only direct evidence of past forms) and biogeography (which gives a global view of mammal evolution), molecular data could help us understand the early evolution of modern mammals.

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## Longhurst Areas

### Ecological Geography of the Sea

by A. Longhurst

Academic Press, 1998.  
\$79.95 hbk (xiii + 398 pages)  
0 12 455558 6

We were all waiting for this book. As pointed out in its introduction, a 'geography of the sea' – that is, a rigorous definition of 'provinces' suitable for describing, in standardized fashion, the distribution of all marine organisms – did not exist despite a history of oceanographic research starting with the Challenger Expedition (1872–1876). Numerous maps did exist in which this or that oceanographic parameter or the distribution of a few organisms had been used to draw provinces or 'Large Marine Ecosystems' (LME) of some sort. However, no test had been conducted of the ability of these proposed maps to predict distributions other than those from which they were derived: circularity reigned supreme.

Reasons for this are easy to imagine, from the excessive preoccupation of various specialists with their favorite taxonomic groups, to the absence, before the recent computer revolution, of analytical tools up to the task. However, the real reason is probably that developing a truly synoptic vision of the ocean was impossible before the advent of satellite-based oceanography.

Satellites cannot see very deep into the sea, nor can they see very much – at least as far as those satellites are concerned that civilians know about. However, what satellites do see is the very stuff that generates

fundamental differences between ocean provinces: sea surface temperatures and their seasonal fluctuations, and pigments such as chlorophyll, and their fluctuations. Marine systems differ from terrestrial ones in that their productivity is essentially a function of nutrient inputs to illuminated layers. This gives a structuring role to the physical processes that enrich surface waters with nutrients from deeper layers, such as wind-induced mixing, fronts, upwelling, etc. Thus, the location, duration and amplitude of deep nutrient inputs into different oceanic regions (as reflected in their chlorophyll standing stocks, and described in *Ecological Geography of the Sea*) largely define the upper trophic level biomasses and fluxes that can be maintained in these regions. This is the reason why satellite images reflect fundamental features of the ocean, whereas maps based on the distribution of various organisms – even 'indicator' organisms – can only reflect second-order phenomena.

Alan Longhurst is among the very first to have fully realized these implications of satellite oceanography, and to have followed up on them. This led to an estimate of global marine primary production much superior to earlier attempts<sup>1</sup>, based on a stratification by 'provinces' defined in another major contribution<sup>2</sup>. Then Alan Longhurst went into retirement to run an art gallery in southern France with his wife.

The book presented here was written thereafter, largely by popular demand: many colleagues adopted the provinces proposed in these earlier works as standard for work now published<sup>3</sup>, or still in preparation, but wanted more details on what it was obvious to call 'Longhurst Areas'<sup>4</sup>. For example, Longhurst Areas will provide the architecture for several projects in which I am involved, whose products are expressed on a global basis. This will involve stratifying

global marine fish biodiversity in forthcoming releases of FishBase<sup>5</sup> (<http://www.fishbase.org>). It will also involve replacing the 18 'FAO Statistical Areas' currently used for raising upper trophic level biomass fluxes from local ecosystem models to regional estimates, and thence to the global ocean<sup>6,7</sup> (<http://www.ecopath.org>).

This work will be aided not only by the existence of Longhurst's classification of ocean provinces – whose reliability can be assessed in the first 98 pages of the book, which discusses conceptual and methodological issues – but also by the detailed description of the 51 neritic and oceanic provinces presented in the next 300 pages. The work of various research groups will undoubtedly modify these descriptions. However, most of this follow-up work will only add details to one or other Longhurst Area. The excellence of this book guarantees that the overall structure will remain, and that the well deserved eponym will stick.

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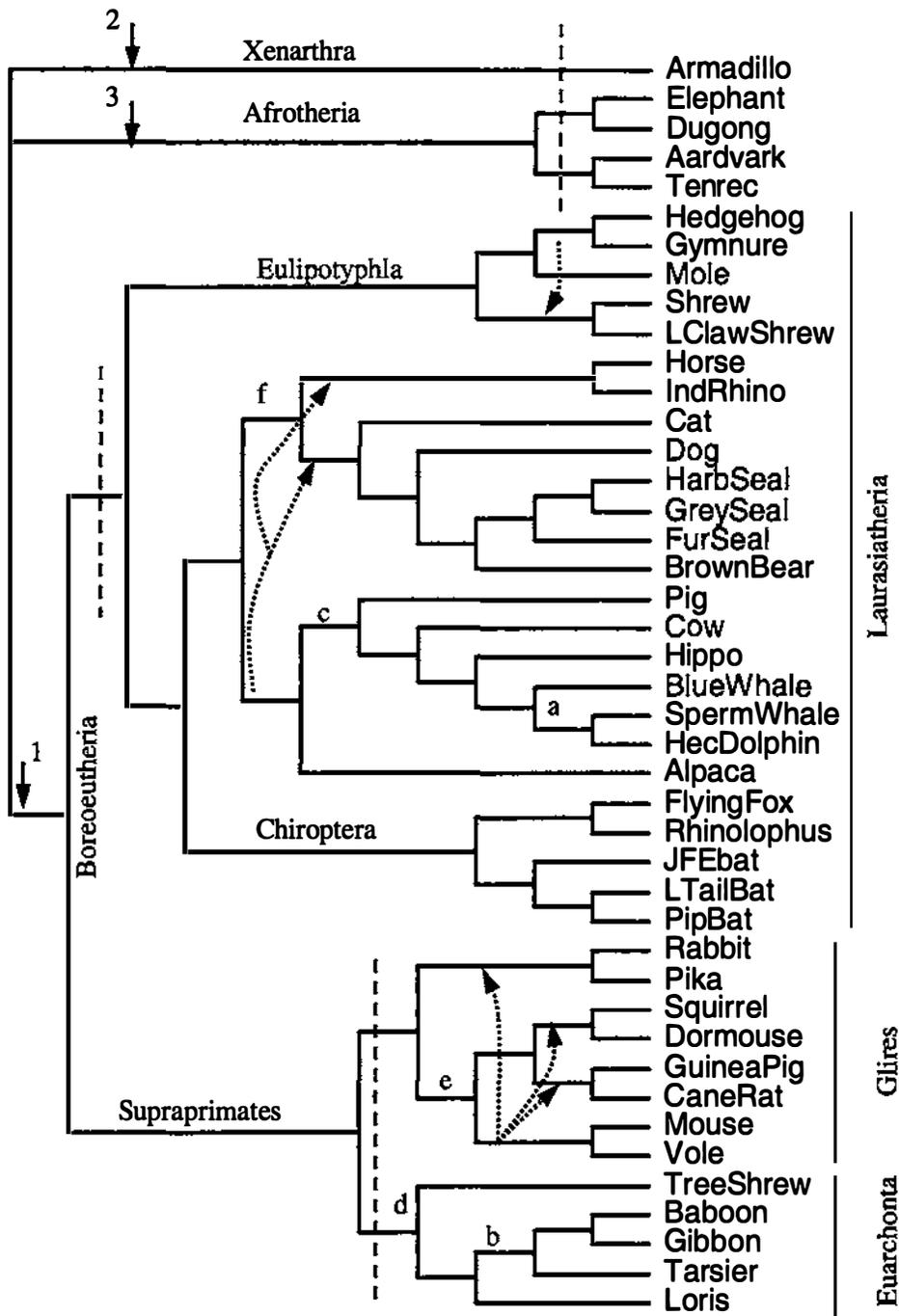
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## 5.2 Addendum

Section 5.1 (Bromham *et al.* 1999) focused on two apparently conflicting facts; firstly, that molecular-based divergence date estimates suggest that much of the interordinal diversification of placental mammals occurred prior to the K-T boundary, and secondly, that no pre-Tertiary fossil can unambiguously be attributed to a crown group placental. Three years on, the situation remains essentially the same. However, a number of recent studies have a considerable bearing on our understanding of the nature of the mammalian interordinal diversification. Perhaps the most important development is that the relationships among the extant orders of placental mammals have largely been resolved.

In reflection of analyses of mt and nuclear sequence data that were presented at a mammal phylogeny symposium in Japan, Waddell *et al.* (1999c) proposed a novel phylogeny for Placentalia. They argued that the four groups stemming from the three most basal nodes are: Xenarthra (consisting of the armadillos, sloths and anteaters), Afrotheria (consisting of elephants, sirens, hyraxes, elephant shrews, the aardvark, tenrecs and the golden moles), a clade (Supraprimates) consisting of primates, flying lemurs, tree shrews, rodents and lagomorphs) and the Laurasiatheria (bats, carnivorans, artiodactyls, perissodactyls, and the remaining insectivorans, which have been named Eulipotyphla). This four-way split and most of the interordinal relationships within these have since been endorsed with substantial statistical support by studies of long (>4000bp) nuclear genes or nuclear gene concatenations (e.g. Madsen *et al.* 2001; Murphy *et al.* 2001; Killian *et al.* 2001). Some of the implied relationships conflict with the results from most analyses of mt protein-coding genes. However, with increased sampling of complete mt genomes, analyses of these data (Lin *et al.* 2002) are converging upon the relationships that are supported by the nuclear datasets. Indeed, my analysis of the mt data, which utilized the conservative RY-code of the sequence, recovers a tree (Figure 5.2.1) that is entirely consistent with the conclusions from the nuclear studies (see Appendix G for details of the analysis).

The finding that the root of the placental tree associates with one (or both) of the Gondwanan groups (Xenarthra, Afrotheria) has the effect of reducing the “fossil gap” between the earliest placental divergence and the K-T boundary. Many earlier studies (e.g. Hedges *et al.* 1996; Penny *et al.* 1999) tended to root the placental tree along the hedgehog branch, or among the rodents, and suggested that the placental last common ancestor (LCA) existed more than 120 mybp. The apparent placental LCA was pushed back on these trees by primates, rodents, the hedgehog (especially in mt studies) and often lagomorphs being (erroneously) placed basal to xenarthrans and/or afrotherians (which have both tended to group with the laurasiatherians). How this can affect the estimate of the timing of the placental LCA can be seen in Figure 2 of Section 5.1.



**Figure 5.2.1** Placental tree inferred from minimum-evolution analyses using ML(CF87) distances and the PTN12+RNAstems dataset. The placement of the root was determined by hypothesis testing from maximum-likelihood analyses. See Appendix G for details of the ME and ML analyses and expansions of the abbreviated taxon names. Bootstrap values for nodes (a-f) are also shown in Appendix G. Nodes not denoted by a letter were supported in all analyses in greater than 60% of replicates. The numbered arrows indicate the three most likely positions of the root, based on the ML(CF87+I+Γ<sub>4</sub>) analysis. Vertical dashed lines indicate the (minimum number of) lineages expected to have crossed the K-T boundary.

Recent studies that have placed the root among the Gondwanan taxa, have inferred that the placental LCA existed approximately 80-105 mybp (Eizirik *et al.* 2001; Waddell *et al.* 2001; Murphy *et al.* 2001). The date inferred for this basal placental split in Chapter 2 was 88 mybp (see Table 2.7).

Aside from its use in estimating divergence dates, the tree shown in Figure 5.2.1 also might help to explain the “fossil gap” in terms of the probability of finding and identifying early members of extant placental lineages. Firstly, as noted by Murphy *et al.* (2001), the basal placement of Xenarthra and Afrotheria is suggestive of the placental crown group originating in the Late Cretaceous of one of the Gondwanan landmasses. Late Cretaceous mammal-bearing fossil deposits are an almost totally untapped resource in Africa, Australia, Antarctica and the northern part of South America, which was split from the southern part during much of the Late Cretaceous (see Woodburne and Case 1996).

In the tree presented in Figure 5.2.1, the vertical dashed lines indicate lineages that are expected to have crossed the K-T boundary. This is in fact a minimum estimate for the number of modern mammal lineages originating in the Cretaceous, and was drawn from the molecular dating studies of Eizirik *et al.* 2001, Murphy *et al.* 2001 and the results in Chapter 2. The mammals represented by the internodes that are older than, or crossing the K-T boundary (as shown in Figure 5.2.1) were almost certainly all small-sized and fed mostly of invertebrates (and perhaps small vertebrates and fruit). This may be inferred via parsimony, by coding the modern taxa for their niche characteristics and the conclusion is further strengthened if fossil taxa are included. Hence, Cretaceous placentals do not appear to have occupied niches that differ greatly from those inferred for the non-placental trechnotheres, “triconodonts” and multituberculates that dominated mammal faunas for much of the Mesozoic. Niche conservatism among early placentals adds weight to the assertion of Bromham *et al.* (1999) that even if crown group placentals were found at Cretaceous sites, they may be difficult to recognize.

In the absence of a shared ancestry that includes a major niche transition, phylogenetic inference from morphological data has been unable to successfully group placental orders together. Without molecular data, tenrecs would probably never have been grouped with animals such as dugongs and elephants. Would the teeth, mandibles and skull fragments of the Cretaceous relatives of modern placental groups stand any better chance of being identified as an early relative of paenungulates (elephants, dugongs, hyraxes), or of xenarthrans?

Perhaps the most effective criticism of the early dates for the placental radiation has come from studies in which the speciation and extinction rates of Cretaceous mammals were modeled. Foote

*et al.* (1999a, 1999b) used such models to show that the preservation rate that is required to explain the “fossil gap” between the molecular dates and the K-T boundary was too low to be plausible.

As noted by a number of authors (e.g. Hedges and Kumar, 1999; Tavaré *et al.* 2002), the results of the Foote *et al.* (1999a, 1999b) studies may be invalidated by substantial departures from at least two of their assumptions being probable; (1) that taxa would be recognizable immediately after speciation; and (2) that preservation rates (the probability of finding taxa as fossils) of early placentals would be comparable to those of known Late Cretaceous taxa. The first of these assumptions appears to be particularly problematic given the lack of synapomorphies that morphologists have been faced with for defining superordinal taxa such as Laurasiatheria and Afrotheria. Furthermore, if much of the early radiation of placentals occurred outside of North America and the central latitudes of Asia, the second assumption is highly unrealistic. Hence it is interesting that Tavaré *et al.* (2002) used clade diversification models that did not rely on either of the above assumptions, to estimate the date for the LCA of primates at 72-90 mybp. This is broadly consistent with recent molecular estimates.

Though not accepted by many palaeontologists, Late Cretaceous fossils have been linked with at least three modern placental groupings: *Batadon* and *Paranyctooides* with lipotyphlan insectivores (see McKenna and Bell 1997), “zhelestids” with Ungulatomorpha (Archibald 1996), and zalambdalestids with Glires (Archibald *et al.* 2001). If these phylogenetic statements are correct, much of the difference between the molecular dates and the fossil record would effectively be bridged. However, each of the proposed relationships requires further testing. The molecular evidence clearly shows that Lipotyphla (Eulipotyphla and Afroinsectivora) and Ungulatomorpha (Fereuungulata, Paenungulata and Tubulidentata) are polyphyletic and contain taxa that may have arisen from different sides of the placental root. Thus sister relationships with Lipotyphla and Ungulatomorpha do not clearly discriminate between their respective (proposed) fossil sister-taxa being within, or outside the placental crown group. In the case of the zalambdalestids, the only taxa employed in the study of Archibald *et al.* (2001) that can be placed within the placental crown group with any certainty are a rodent (*Tribosphenomys*) and a lagomorph (*Mimotona*). Thus in the context of the taxon sampling that was employed, a sister relationship with Glires does not require that zalambdalestids are crown group placentals.

Despite uncertainty in the affinities of fossils that are putative close relatives of superordinal clades of modern placentals, their publication is symbolic of a gradual whittling down of differences between morphological and molecular interpretations of the early diversification of modern mammal groups.

As the mammalian tree has become better resolved and rate heterogeneity among lineages accepted in molecular dating studies, the temporal extension back into the Cretaceous of estimates for placental divergences has been reduced. Nevertheless, even with conservative molecular dating estimates, the stem lineages of at least nine modern mammal groups (monotremes, australidelphian and ameridelphian marsupials, Xenarthra, paenungulate and afroinsectiphilian afrotherians, Glires, Euarchonta, and Laurasiatheria) must have crossed the K-T boundary. Among these, only monotremes have been identified in the pre-Tertiary fossil record.

If the earliest radiations of placentals and marsupials occurred outside of North America and the central latitudes of Asia, the “fossil gap” may be resolved by furthering the search for Cretaceous mammals. Alternatively, two suggestions may be useful. Firstly, (as noted in Section 2.4.4) the possible effect on dating estimates of a parallel slowdown in substitution rates among fereuungulate taxa requires further examination, at least for mt data. Clearly though, the use of more conservative reference dates still results in the above-listed nine lineages being inferred (from analyses of both mt and nuclear data) to have crossed the K-T boundary. Secondly, the placental tree derived from molecular data should be employed as a background phylogeny, in order to validate the placement of taxa such as zhelestids and zalambdalestids within the placental crown group. Placing this constraint on analyses of morphological data may also provide synapomorphies that support placement of other fossil taxa with modern groups.



## **Chapter 6**

### **Conclusions**



The primary aim of sequencing the mt genomes of a northern brown bandicoot (*Isodon macrourus*) and a brushtail possum (*Trichosurus vulpecula*) was to test whether bandicoots group with other Australian (Australidelphian if including *Dromiciops*) marsupials. Analyses of shorter mtDNA sequences and DNA-hybridization had tended to place bandicoots with Ameridelphian marsupials, or at the base of Marsupialia. The analysis of the mt protein-coding and RNA-coding DNA sequences (Chapter 1) provide very strong support for grouping bandicoots with the Australian taxa. The result suggests that possession of a continuous lower ankle joint (see Szalay 1982) is a reliable diagnostic trait of Australidelphia. In contrast, the chorio-allantoic placenta and some hindlimb characteristics (such as a robust patella) of bandicoots appear to have evolved independently of similar traits of placental mammals.

The secondary aim of the phylogenetic analysis of the mt sequence data was to further test the Marsupionta (Gregory 1947). This hypothesis, which groups monotremes and marsupials, was resurrected by molecular studies, initially on the basis of analysis of complete mt protein-coding sequences (Janke *et al.* 1996), but has been controversial due to the substantial number of morphological characters that unite marsupials with placentals (Theria).

The analysis of mt protein-coding and RNA-coding DNA sequences in Chapter 2 could not resolve the rooting of the mammal tree with high statistical significance, in favour of either of the three hypotheses (1. Theria, 2. Marsupionta, 3. Monotremata-Eutheria). However, with the most conservative data treatment (RY-coded DNA) and the best fitting ML models that were used (in which the analyses were partitioned between subsets of characters with similar inferred substitution patterns), Theria was the favoured hypothesis. Thus mt, nuclear, anatomical, physiological and reproductive data all support the therian clade.

Although I consider the placement of platypuses and echidnas among extant mammals to be resolved, the placement of monotremes with respect to Mesozoic mammals is far from certain. Indeed, the inclusion of monotremes with the “background phylogeny” of Mesozoic generalized mammalian insectivores induced highly significant incongruence between anatomical regions (Chapter 3). While the background phylogeny was well resolved (see Figure 3.1) and not dependent on the anatomical regions examined, the favoured placement of monotremes among these taxa ranged from sister to Theria for the mandibular, vertebral and dental partitions (MVD), to being sister to Trechnotheria (spalacotheriids and cladotheres) for the basicranial and lower appendicular partitions (LB), to being placed more primitively (with respect to modern therians) than even the Triassic/Jurassic boundary morganucodontids for the upper appendicular partition.

A new technique, character map incompatibility (CMI) analysis was used in Chapter 4 to further investigate signal and conflict relating to the placement of monotremes on the background phylogeny of generalized insectivores. The results of the CMI and cladistic analyses, along with information on niche transitions that occurred during monotreme evolution, and divergence date estimates for the Monotremata/Theria split (Chapter 2) were all drawn upon to derive a framework for assessing the phylogenetic affinities of monotremes. Monotreme shoulder girdle and forelimb traits were then considered in view of trends among the background phylogeny of generalized insectivores. These comparisons indicated that in the context of the framework for assessing monotreme affinities, that correlated reversal of upper appendicular characters (in monotremes) is a likely explanation for much of the incongruence among the anatomical region partitions.

In Chapter 4 it was argued that homoplasy (particularly reversal) that is non-independent across the upper appendicular (U) characters and attributable to the monotreme stem lineage, is consistent with the early transition of monotremes to a fossorial and semi-aquatic niche. More specifically, the condition of monotreme U characters is consistent with the adoption by monotremes of forelimb driven propulsion that involves hypertrophied humeral long-axis rotation. In contrast, the condition of monotreme U characters is not consistent with the common assumption that monotreme U characters can be derived directly from a condition similar to that of *Morganucodon*.

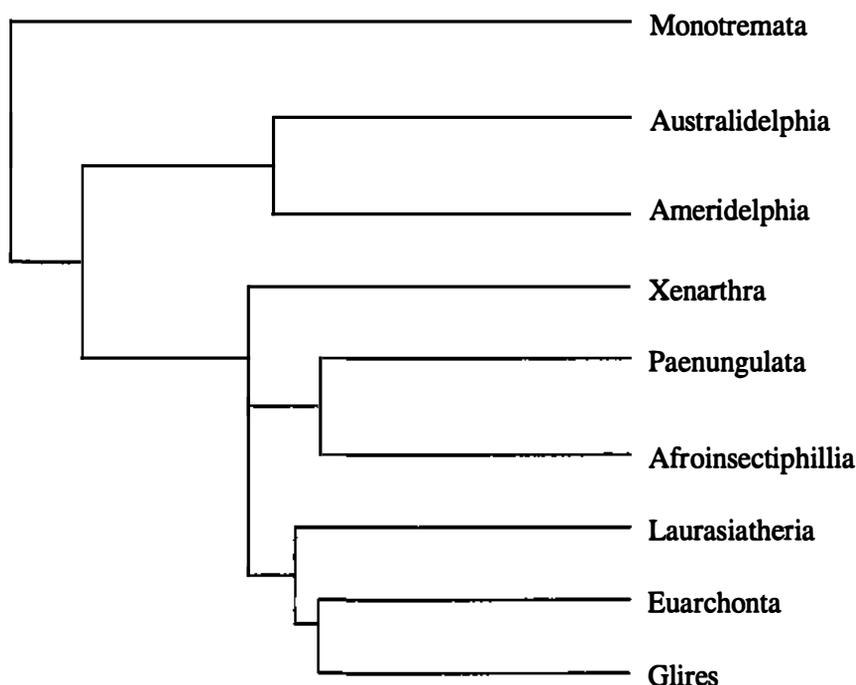
Hypotheses advocating a close relationship between monotremes and trechnotheres appear more likely in light of the apparent correlated reversal of monotreme U characters. Though caution against any solid conclusions regarding the affinities of monotremes is warranted because of significant phylogenetic conflict between the non-upper appendicular anatomical region data partitions (MVD versus LB). However, the small (<20 million years) difference between the dates of divergence that were inferred from the mt data (Chapter 2) for the monotreme/therian split, and the subsequent marsupial / placental split, tends to favour a close relationship between monotremes and trechnotheres. Given also the highly significant conflict among the anatomical region partitions for the placement of monotremes and the apparent reversal of U characters along the monotreme stem lineage, it is premature to rule out (as per Lou *et al.* 2001a, 2002) the possibility that the tribosphenic mammals (Boreosphenida plus Australosphenida) form a monophyletic group.

The uncertainty of the placement of monotremes on the background phylogeny appears to relate mostly to the extreme modification of platypus and echidna morphology, which in turn can be related to their specialized ecological niches. Although the inclusion of monotremes within Australosphenida was not explicitly tested in the current analyses, which were based on the dataset of Ji *et al.* (1999), evidence for the relationship is now substantial (see Lou *et al.* 2001a, 2002; and see Figure 3.7). As more complete specimens of australosphenidans that retained a niche that is

similar to that which is ancestral for mammals are found, the affinities of these Gondwanan mammals (and monotremes) should be progressively resolved.

Returning to extant mammals, the analysis of RY-coded mt protein-coding and RNA-coding DNA (see Figure 5.2.1 and Appendix G) supports all of the interordinal relationships among placental mammals that recent studies of nuclear genes have resolved (e.g. Killian *et al.* 2001; Murphy *et al.* 2001b). Combining phylogenetic inference from Chapters 1, 2 and 5 allows a phylogeny to be reconstructed for the (minimum number of) modern mammal groupings that have stem lineages extending back into the Cretaceous. These are shown in Figure 6.1 with only one unresolved node, the placental root. In order to finalise our understanding of the (phylogenetic) Mesozoic diversification of modern mammal groups three questions are required to be answered.

1. Where is the root placed on the placental tree?
2. Where are caenolestids and *Dromiciops* placed on the marsupial tree (most recent studies, including my unpublished analysis, indicate that caenolestids are the sister group of Didelphimorphia and support *Dromiciops* grouping with the Australian marsupials, possibly as the most basal member of the Australidelphia)?
3. Do divergences within Laurasiatheria, Euarchonta, Glires, Afroinsectiphillia and Xenarthra pre-date the K-T boundary?



**Figure 6.1** The phylogeny of modern mammal groups with stem lineages that cross the K-T boundary, as a consensus of the analyses of RY-coded mt protein and RNA-coding sequences (in Chapters 1 and 2 and in Appendix G). The (minimum) number of modern mammal lineages crossing the K-T boundary is inferred from the dating estimates in Chapter 2.

Answering the remaining questions on the phylogenetic relationships between the modern mammal orders may simply require more data. However, some methodological considerations may be gleaned from the results of both the molecular and morphological analyses employed in the current study. Firstly, for inferring deep-level relationships from complete mtDNA sequences, two suggestions may be useful when standard methods produce results that are inconclusive, or indicate the presence of more than one signal for the affinities of the taxon of interest.

1. RY-coding the DNA sequence
2. Partitioning ML analyses

RY-coding nullifies what is typically the primary nucleotide composition bias among vertebrate mt genome sequences (pyrimidine bias: differences among taxa for T–C frequency). It was shown in Chapter 2 that this bias also translated into an amino acid composition bias. Further, in agreement with earlier studies (e.g. Tarrío *et al.* 2001) corrections such as LogDet appear to only partially account for the effect of the composition bias on apparent phylogenetic signal. Of course if there is little evidence of compositional non-stationarity among data, employing the standard (NT) coded nucleotide sequence, or the amino acid sequence (which are likely to be more informative), may be preferable to RY-coding the data as the former treatments may be more informative. However, due to increased signal retention (or reduced substitution saturation), RY-coding may also reduce the potential for other substitution biases to mislead phylogenetic inference.

Partitioning ML analyses between RNA stem and loop sites, and first and second codon positions for five groups of mt proteins increased the signal for the traditional Theria hypothesis, relative to that for Marsupionta. In earlier studies (e.g. Cao *et al.* 2000a) it had been suggested that partitioning ML analyses could result in more accurate phylogenetic inference because of a closer overall fit between the data and the ML models. In the current study it is hypothesized that an important factor in this is that partitioning ML analyses can uncover biases that are complementary between partitions, such that they are not detected when the data are concatenated.

The disadvantages of RY-coding and partitioning analyses are loss of information and reduced statistical power respectively. Hence it will be important to design criteria for deciding when RY-coding is appropriate and how best to partition sequence data. Preferably an optimal scheme would be identified prior to phylogenetic analyses being carried out. Ultimately though, RY-coding simply “hides” compositional non-stationarity and partitioning ML analysis is an extension of methodology that has been developed for increasing the fit between ML models and data where substitution processes vary across sites (see Swofford *et al.* 1996; Yang *et al.* 1994). It is more critical that further attention is given to accommodating variation in substitution processes that

occur across the tree. Notably, Such non-stationarity (in C versus T preference) was the major concern for inferring the rooting of both the marsupial and the mammal trees in the NT-coded analyses in Chapters 1 and 2 respectively.

Difficulties associated with phylogenetic inference from morphological data may be more entrenched than those associated with molecular sequence data. Nucleotide or amino acid residues are readily coded as discrete characters. In contrast, morphological characters tend to evolve within character space that is more or less continuous (though not random). Unless *a priori* decisions are made on how character conditions should be grouped into discrete states, it may be all too easy for this grouping to reflect investigator bias. A further difficulty is that morphological data is not readily assessable within frameworks provided by evolutionary models, so restricting tree selection to simple ME, or more commonly MP optimality criteria.

Convergence is perhaps the most well documented problem for phylogeny reconstruction from morphological data and it typically involves many (putatively) functionally linked characters. Examples among mammals that have been identified by comparison with molecular analyses include bats and flying lemurs, afrotherian and laurasiatherian ungulates, and bandicoots and placentals. Another, more subtle bias among morphological data is outlined in Chapter 4. This is the tendency for highly modified taxa to be drawn towards the outgroup when analysed alongside taxa that have retained a more plesiomorphic niche. Four likely mechanisms that may promote outgroup-attraction of “ecological long-branches” are identified in section 4.3.6.

1. Greater parallelism between taxa that retain the plesiomorphic niche, than between any of these and the taxon for which the niche transition has occurred.
2. High rates of autapomorphy in the highly modified taxon, which erases synapomorphies with their sister group.
3. Asymmetry between the morphological ranges of “primitive” and “advanced” character states.
4. An increased probability of paedomorphosis among highly modified taxa (relative to more plesiomorphic taxa).

It was suggested that each of these mechanisms that promote outgroup attraction has affected phylogenetic inference for the placement of monotremes among the generalized insectivore background phylogeny. Furthermore, reflecting on comparisons of other molecular and morphological studies, it is curious to wonder whether outgroup-attraction has also affected the placement, in morphological studies, of taxa such as whales, megachiropterans (mega bats) and turtles.

Overcoming convergence and outgroup attraction may be critical for inferring the relationships with modern groups, of many fossil taxa. As an alternative to developing explicit mathematical models for dealing with these problems, an alternative approach was used in chapter 4 to assess the affinities of monotremes. Instead of inferring monotreme placement on the basis of the least homoplasy (MP), monotreme placement was considered in terms of the homoplasy options that different placements create, so maximizing the use of information on the nature of the homoplasy that relates to the placement of monotremes. The character-map incompatibility (CMI) analysis was of central importance in this. CMI analysis examines signal that can be related to the placement of a specific taxon, with respect to each node on a background phylogeny and allows the identification of (potentially homoplastic) co-evolving characters. The most important role for CMI analysis may be for identifying situations where MP-bootstrap analyses may be providing overconfidence in hypotheses.

CMI analysis was initially intended to be used for examining signals among morphological data for the placement of taxa that have evolved under different “rules” to others they are included with. However, CMI analysis may also be used with molecular data. This has not been done in this study, though the problems facing phylogeny reconstruction from molecular and morphological data have more in common than is often appreciated. If there is a change in evolutionary processes along one or both of two sister lineages, reconstructing the internode they share will often be difficult with both types of data. Reconstructing the Theria stem was difficult with mt sequence data, largely because the substitution process differs between marsupials and placentals and is more similar between marsupials and monotremes. Likewise, constraints on the morphological evolution of bats, eulipotyphlans, carnivorans, perissodactyls and artiodactyls (including cetaceans) are very different and so it is not surprising that analyses of morphological data have not been able to reconstruct the Laurasiatheria clade.

Having made a transition to a fossorial and probably semi-aquatic niche during the (Early) Cretaceous, monotremes have a very different niche history to marsupials and placentals. As with changes in substitution regimes among molecular data, niche transitions result in morphological characters evolving under a different set of constraints. The synapomorphies that are diagnostic for clades such as Theriimorpha, Trechnotheria and Cladotheria all evolved in mammals that were generalized insectivores. Such synapomorphies might be more readily lost in monotremes than among modern therians with even the most modified niches. This is because the amount of time for synapomorphies with sister taxa to potentially be canalized (developmentally fixed) under the constraints that are specific to generalized mammalian insectivores was at least 60 million years longer for all marsupials and placentals, than for monotremes. In fact, much of the morphological evolution of some marsupials and placentals (such as the smaller dasyurids and didelphids) is likely

to be subject to essentially the same evolutionary constraints as archaic therians (e.g. eupantotheres). Similarity in evolutionary constraints among the generalized insectivores, but differences from monotremes, are key factors in explaining why dental, vertebral, basicranial, lower and upper appendicular characters, which are congruent for the background phylogeny, are highly incongruent with respect to the placement of monotremes.

Given that both molecular sequence data and morphological data can be subject to variability in evolutionary processes across the tree that effect phylogenetic inference, arguments for the primacy of one type of data over the other should be considered as case specific, rather than general. Nonetheless, the problems associated with analysis of morphological data currently seem less tractable. This should not be taken as discouragement for morphological analyses. In fact, further development of analytical techniques for phylogenetic inference from morphological data is critical, because molecular studies are unable to incorporate information from the bulk of the fossil record. Methods that consider the effects of niche transitions on the evolution of morphological characters would offer a great boost for phylogeny reconstruction and for inferring the evolutionary and ecological history of mammals and other groups.

Although the uncertainty surrounding the relationships of many fossil taxa to modern mammal groups is a limiting factor, phylogenetic inference from the current study provides a number of insights into ecological aspects of the early diversification of modern mammals. For example, it may be inferred that the last common ancestor (LCA) of Marsupialia, of Placentalia, of these two groups together (Theria), and of Mammalia, was in each case a small to medium-sized terrestrial (or scansorial) animal, with a diet consisting of invertebrates and possibly small vertebrates and fruit. In contrast, the LCA of platypuses and echidnas (Monotremata), which post-dates the K-T boundary, was somewhat larger, fossorial and was likely to have been semi-aquatic. The absence of evidence for echidnas in the fossil record until much later (Miocene or Pliocene), might be explained by the LCA of the monotreme crown group being somewhat platypus-like.

As a final note, the presence in the Cretaceous of a minimum of two lineages leading to modern marsupial groups and six lineages leading to modern placental groups has an important bearing on arguments linking the diversification of crown group placentals (or marsupials) to the extinction of dinosaurs at the K-T boundary. The implication is that genetic and developmental mechanisms involved in the ecological/morphological diversification that is observable in the fossil record of the early Tertiary, had evolved earlier, during the Mesozoic. Furthermore, phylogenetic inference from the mammal tree suggests that the taxa that the Cretaceous lineages of modern marsupials and placentals represent, all retained what were essentially archetypical (and plesiomorphic) mammalian niches. Thus the Cretaceous diversification of these groups does not relate to

substantial expansion of mammalian niche-space, so in turn is unlikely to have resulted in competitive exclusion of the dinosaur groups that became extinct near the K-T boundary. Alternatively, phylogenetic inference is consistent with the major ecological/morphological diversification of modern mammal groups being triggered by the extinction of dinosaurs and other large vertebrates at the end of the Cretaceous period.

Further elucidating how the ecological diversification of mammals relates to physical events in the geological record and to interactions between mammals and other taxonomic groups, will require fossil taxa to be correctly placed on the phylogeny of modern mammals. Before this is possible, phylogenetic analyses of morphological data must be robust to biases that result from taxa evolving in different niches (under different evolutionary constraints). Currently I suggest that the two most important steps for determining the placement of fossil groups among modern taxa will be (a) utilizing a background phylogeny derived from the analysis of molecular data and (b) incorporating information on evolutionary processes in phylogenetic analyses of morphological data.

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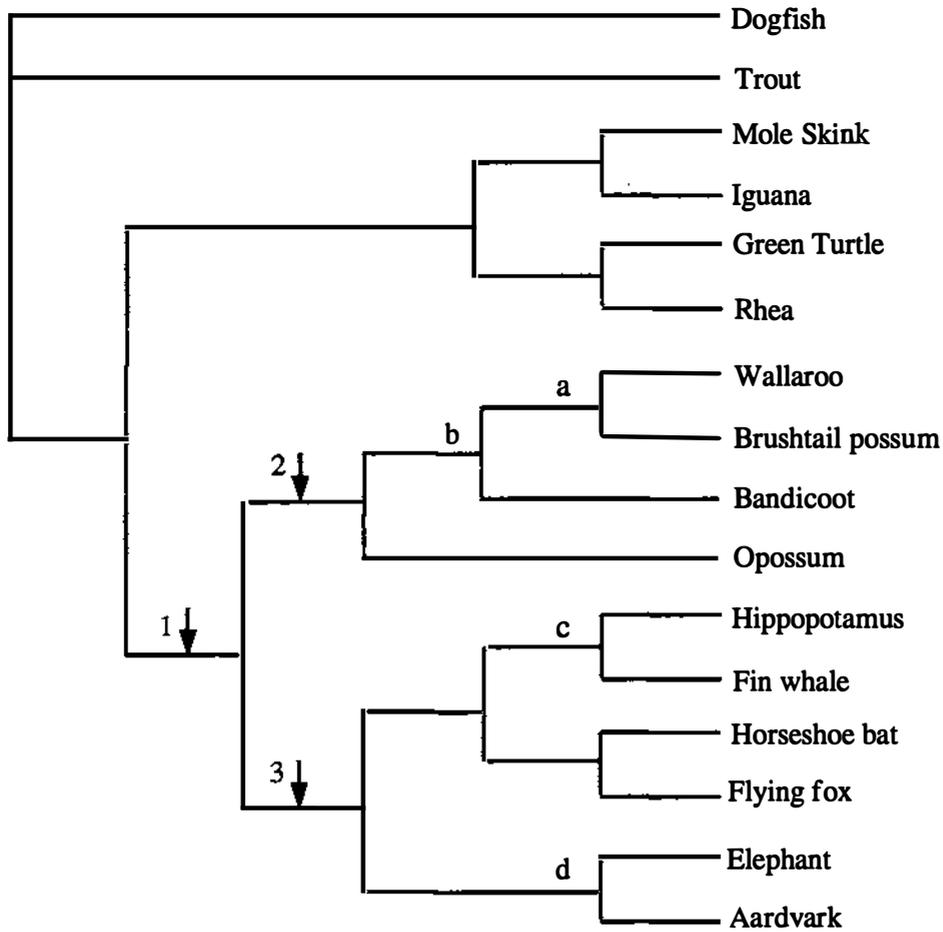
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**Appendix A: Background phylogeny for ML significance testing of monotreme placement hypotheses**



**Figure 1.** The background phylogeny of vertebrates used (in Chapter 2) for testing three hypotheses for monotreme placement (as indicated by arrows): 1. Theria, 2. Marsupionta and 3. Monotremata-Eutheria. This phylogeny was inferred from analysis of the PTN12+RNArt dataset. Bootstrap support (500 replicates) for nodes a-d is shown in the table below for MP and ME (TN93 distance) analyses with the data NT-coded (NT) and MP and ME (CF87 distance) analyses with the data RY-coded (RY). All of the other nodes were supported in 100% of the bootstrap replicates in each of the four analyses.

Analysis	node a	node b	node c	node d
RY-coded MP	99	100	97	86
RY-coded ME (F81)	100	100	100	100
NT-coded MP	100	100	100	79
NT-coded ME (TN93)	100	99	100	99

**Appendix B: Purine composition and RY-constant sites among mtDNA partitions**

**Table 1** Purine base frequency and the observed proportion of RY-constant sites among mitochondrial protein coding genes (first and second codons only) and RNA-coding (stems and loops). These values were determined from the 18-taxon dataset, which was used for the ML analysis.

	Frequency of purine bases (A+G)	Observed proportion of RY-constant sites
NADH1	0.389	0.742
NADH2	0.376	0.528
COI	0.444	0.922
COII	0.459	0.796
ATPase8	0.355	0.406
ATPase6	0.389	0.666
COIII	0.426	0.829
NADH3	0.377	0.668
NADH4L	0.377	0.558
NADH4	0.401	0.648
NADH5	0.433	0.642
NADH6	0.531	0.479
<i>Cytb</i>	0.416	0.791
RNAstems	0.500	0.794
RNAloops	0.569	0.675

## Appendix C: Branch-length estimates for the vertebrate phylogeny

**Table 1** Minimum-evolution (ME) and maximum-likelihood (ML) branch-length estimates for ME and ML trees that were inferred from the PTN12+RNArt data (and used for estimating divergence dates; see Tables 2.7 and 2.8). The ME tree is shown in figure 2.9. The ME analysis used ML (CF87) distances with the proportion of invariable sites (0.700) estimated within SplitsTree2.4 and the ML analysis used the CF87+I+ $\Gamma_8$  model with PAUP\* estimates of invariable sites (0.353) and a shape parameter (0.401) for an 8-category gamma distribution. The standard error values are associated with the ML branch-lengths.

	Minimum-evolution	Maximum-likelihood	
	branch-lengths	standard error	
dogfish (+1st internode)	0.03469	0.04001	0.00306
trout	0.03513	0.03612	0.00292
tetrapod stem	0.01048	0.01834	0.00267
amphibian stem	0.00954	0.01099	0.00284
ceacilian	0.08697	0.10083	0.00601
salamander	0.05796	0.06678	0.00454
amniote stem	0.01423	0.01536	0.00272
sauropsid stem	0.01125	0.01562	0.00272
squamate stem	0.03369	0.04175	0.00372
mole skink	0.03651	0.03882	0.00327
iguana	0.04256	0.04760	0.00354
turtles+archosaurs stem	0.01183	0.01294	0.00254
turtle stem	0.03039	0.03147	0.00299
green turtle	0.00501	0.00675	0.00122
eastern painted turtle	0.02286	0.02242	0.00183
bird stem	0.04397	0.05476	0.00393
rhea	0.01771	0.01475	0.00189
rook	0.02297	0.02849	0.00231
mammal stem	0.06052	0.06961	0.00494
monotreme stem	0.03903	0.04391	0.00343
platypus	0.00658	0.00683	0.00115
echidna	0.01142	0.01179	0.00134
Theria stem	0.00178	0.00374	0.00189
marsupial stem	0.02527	0.03446	0.00312
australidelphian stem	0.00760	0.00944	0.00167
diprotodontian stem	0.00341	0.00789	0.00123

**Table 1 continued**

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wallaroo	0.01005	0.01085	0.00126
brushtail possum	0.01016	0.01023	0.00123
bandicoot	0.01697	0.01536	0.00159
opossum	0.02444	0.02600	0.00220
placental stem	0.03128	0.03791	0.00333
bats + Whippomorpha stem	0.00988	0.01417	0.00193
Whippomorpha stem	0.00596	0.00698	0.00128
hippopotamus	0.01059	0.01113	0.00130
fin whale	0.01635	0.01712	0.00157
bat stem	0.00701	0.00949	0.00139
horseshoe bat	0.01803	0.01664	0.00159
Flying fox	0.01280	0.01574	0.00155
Afrotheria stem	0.00653	0.01005	0.00177
elephant	0.03802	0.03969	0.00280
aardvark	0.02465	0.02827	0.00238

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## Appendix D: Character list for phylogenetic analyses of Mesozoic mammal relationships

The phylogenetic analyses in Chapters 3 and 4 used the dataset of Ji *et al.* (1999). However, a number of alterations were made to the dataset, including the exclusion of ten characters, the inclusion of character states for the echidna (*Tachyglossus aculeatus*) and updates for the character states of other taxa (in light of more recent publications and personal observations). These alterations are noted in the character list below, with the character numbers being those from the Ji *et al.* (1999) dataset. Also noted are characters for which there is clear asymmetry in the morphological range of the states and characters for which fossil information alters the ornithorhynchid state.

### a. Ji *et al.* (1999) characters that are excluded from analyses

Characters were excluded where states are: (a) equivalent to, or dependent on, the state of other characters, (b) uncertain for three or more of the background phylogeny taxa, or (c) subject to considerable variability in modern mammals (so not expected to be reliable at deep levels of phylogeny).

#### 8. The number of thoracic vertebrae:

(0) 13

(1) 15 or more

Excluded because assignment is uncertain for many of the taxa. It is difficult to identify the transition from thoracic to lumbar vertebrae in monotremes and many non-trechnothere mammals. Both states are known to exist among placentals and marsupials (see Slijper 1946; Gregory 1947) and an increase in the number of thoracic vertebrae appears to be associated with fossorial and aquatic behaviour. Furthermore, the state of this character is unknown for the outgroup (cynodonts), Morganucodontids and *Henkelotherium*.

#### 11. Contact relationships in adults between the interclavicle and the sternal manubrium:

(0) the two elements are distinct and the posterior end of interclavicle abuts anterior border of manubrium.

(1) the two elements are distinct and the interclavicle broadly overlaps the ventral side of the manubrium

(2) complete fusion of the embryonic membranous (interclavicle) and endochondral (sternal manubrium) elements

Excluded because state 2 is effectively the same as state 1 in character 10. Furthermore, the state of this character is unknown for the outgroup (cynodonts), Morganucodontids *Gobiconodon*, *Henkelotherium* and *Vincelestes*.

#### 20. Fusion of medial part of the embryonic scapula-coracoid plate with the sternal manubrium:

(0) scapula-coracoid plate remains as a separate element in adults

(1) scapula-coracoid plate fused to sternal manubrium in adults

Excluded because it is difficult to assess the fate of embryonic tissue in fossils (especially without obvious sutures) and even if state 1 is assumed from a small coracoid (relative to the size of the scapula), then state 2 is effectively the same as character 19 state 1. Furthermore, the state of this character is unknown for the outgroup (cynodonts), Morganucodontids, *Gobiconodon* and *Henkelotherium*.

53. Sesamoid bones in flexor tendons:

- (0) absent
- (1) present and unpaired
- (2) present and paired

Excluded because this character cannot be included for a specific anatomical region partition.

66. Foramen for the ramus superior of the stapedial artery:

- (0) laterally open notch
- (1) foramen enclosed by the petrosal
- (2) foramen enclosed between the squamosal and the petrosal
- (3) absent

Excluded because this character is highly variable in extant (and extinct) mammals (see Wible and Hopson 1994; Rougier *et al.* 1996).

70. Orientation of the anterior segment of the Meckelian groove:

- (0) near-parallel to and separated from the ventral edge of the mandible
- (1) converges toward the ventral edge of the mandible
- (2) groove absent

Excluded because state 2 is equivalent to state 2 in character 69.

The following dental characters (77, 78, 79, 95) were excluded because they are highly variable among modern marsupials and placentals. More importantly, this variability appears to correlate with dietary factors that would be expected to act non-independently between these characters. For example, herbivores such as wombats, mole rats and multituberculates all have procumbent, enlarged first incisors (ch 77), a reduced number (2) of lower incisors (ch 78) and do not have canines (ch 79). None of these taxa are closely related, but all eat highly corrosive plant matter.

77. Procumbent and enlarged first lower incisor:

- (0) present ( $\geq 125\%$  longer than the second lower incisors)
- (1) absent (first and second lower incisors are sub-equal)

78. Number of lower incisors:

- (0) more than four
- (1) reduced to two or fewer

79. Canine size:

- (0) Canine present and greatly enlarged ( $>150\%$  of last incisor in crown height)
- (1) Canine present and slightly enlarged ( $100\%$ - $125\%$  of last incisor in crown height)
- (2) Canine greatly reduced ( $<100\%$  of the adjacent incisors) or absent

95. Relative size of the lower molar cusps (therian homologies are paraconid for b and metaconid for c)

- (0) c larger than b
- (1) b larger than c

**b. Ji *et al.* (1999) characters that are included for analyses in Chapters 3 and 4**

The following characters are coded as for Ji *et al.* (1999). An asterisk (\*) indicates states that have the larger scope, for characters in which the morphological range of states is asymmetric (either in its coding, or usage of that coding by Ji *et al.* 1999). More characters than noted may be considered asymmetric, but without a statistics based criterion I have only identified those in which the asymmetry is most obvious. Ji *et al.* (1999) coded ornithorhynchids only for *Ornithorhynchus anatinus*. I provide an explanation below for each character for which my coding differs for Ornithorhynchidae, with (or without) the inclusion of fossil taxa. Character coding for fossil monotremes was obtained from Archer *et al.* (1985, 1992, 1993); Archer and Musser (1998), Lou *et al.* (2001a and 2001b), and Rich *et al.* (2001a) and personal observations the skull (cast) of *Obdurodon dicksoni* (provided by Guillermo Rougier).

**Vertebral characters**

**1. Proatlas neural arch as separate ossification in adults:**

- (0) present
- (1) absent

**2. Fusion of atlas neural arch and intercentrum in adults:**

- (0) unfused
- (1) fused

**3. Atlas rib in adults:**

- (0) present
- (1) absent

**4. Prezygopophysis on axis:**

- (0) present
- (1) absent

**5. Rib of axis in adults:**

- (0) present
- (1) absent

**6. Postaxial cervical ribs in adults:**

- (0) present
- (1) absent

**7. Postaxial cervical transverse canal:**

- (0) absent
- (1) present

**9. Lumbar ribs:**

- (0) unfused to the vertebra
- (1) syntosed to the vertebra to form transverse processes

## Upper appendicular characters

### 10. Interclavicle in adults

- (0) present
- (1) absent

### 12. Cranial margin of the interclavicle (assuming the interclavicle is fused to the sternal manubrium in living therians; e.g. Klima 1973, 1987)

- (0)\* anterior border is emarginated or flat
- (1) with a median process

### 13. Clavicle-sternal apparatus joint (assuming the interclavicle is fused to the sternal manubrium in living therians; e.g. Klima 1973, 1987)

- (0) immobile
- (1) mobile

### 14. Curvature of clavicle

- (0) boomerang shape
- (1) slightly curved

Ji *et al.* (1999) coded *Ornithorhynchus* as (0) for this character. Gauging the curvature of the monotreme clavicle is complicated by its being fused along its full length to the lateral processes of the interclavicle. The lateral processes of the interclavicle of *Ornithorhynchus* could loosely be considered as boomerang shaped, but the clavicles are less so, instead being slightly sinusoidal, more similar to those of generalized therians. This is most clear in juveniles (before the fusion is complete). Thus I have coded *Ornithorhynchus* as (1). The clavicle of echidnas is more boomerang shaped (like *Morganucodon* see Jenkins and Parrington 1976) so I have coded Tachyglossidae as (0).

### 15. Supraspinous fossa of the scapula

- (0) absent
- (1) weakly developed
- (2) fully developed - present along the entire dorsal border of the scapula

### 16. Acromion process of the scapula

- (0)\* absent or weakly developed (leveled to the glenoid)
- (1) strongly developed and extending below the glenoid

### 17. Distinctive fossa for the teres major muscle on the lateral aspect of the scapular plate

- (0) absent
- (1) present

### 18. Procoracoid (as a separated element in adults)

- (0) present
- (1) absent

### 19. Coracoid (metacoracoid)

- (0) large, with posterior process
- (1) small, without posterior process

### 21. Size of the anterior-most element relative to the subsequent sternebrae in the sternal apparatus in adults:

- (0) large
- (1) small

22. Orientation (facing or articular surface) of the glenoid, relative to the plane or the axis of the scapula:

- (0) nearly parallel to the long axis of the scapular and facing posterolaterally
- (1) oblique to the long axis of the scapula and facing more posteriorly
- (2) articular surface of the glenoid is perpendicular to the main plane of the scapular plate

23. shape and curvature of the shoulder glenoid:

- (0) saddle-shaped, oval and elongate
- (1) uniformly concave and more rounded in outline

24. Medial surface of scapula:

- (0)\* convex
- (1) flat

25. Humeral head:

- (0)\* subspherical, weakly inflected
- (1) spherical and strongly inflected

26. Intertubercular groove of humerus:

- (0) pectodeltoid crest separated from lesser tubercle by shallow and broad intertubercular groove
- (1) narrow and deep intertubercular groove

27. Size of lesser tubercle of humerus:

- (0) wider than the greater tubercle
- (1) narrower than the greater tubercle

28. Torsion between the proximal and distal ends of the humerus:

- (0)\* strong ( $\geq 30^\circ$ )
- (1) moderate ( $30^\circ - 15^\circ$ )
- (2) weak

29. Ventral extension of pectodeltoid crest:

- (0) not extending beyond the midpoint of the humeral shaft
- (1) extending beyond the midpoint of the humeral shaft

30. Ulnar articulation on the distal end of the humerus:

- (0)\* bulbous ulnar condyle
- (1) cylindrical trochlea dorsally with condylar articulation ventrally
- (2) cylindrical trochlea without an ulnar condyle

31. Radial articulation on the distal humerus:

- (0)\* distinct and rounded condyle (that does not form a continuous synovial surface with the ulnar articulation in the ventral/anterior view of the humerus)
  - (1) capitulum (radial articulating surface that forms a continual synovial surface with the trochlea)
- Ji *et al.* (1999) coded *Ornithorhynchus* as (0). I accept this, though note that the radial condyle of platypuses and echidnas does in fact form a continuous synovial surface with the ulnar articulation.

32. Entepicondyle and ectepicondyle of humerus:

- (0) robust
- (1) weak

33. Styloid process of radius:

- (0) weak
- (1) strong

## Lower appendicular characters

### 34. Acetabular dorsal emargination:

- (0) open (emarginated)
- (1) closed (with a complete rim)

### 35. Size of the pelvic obturator foramen:

- (0) smaller than the acetabulum
- (1) equal to or larger than the acetabulum

Ji *et al.* (1999) coded *Ornithorhynchus* as (0) for this character. I agree with this coding for *Tachyglossus*. However, the obturator foramen was at least as large as (and usually somewhat larger than) the acetabulum in every platypus specimen I have examined (including male and female individuals from Queensland, Tasmania, Victoria and New South Wales). Thus I have coded *Ornithorhynchus* as (1).

### 36. Sutures of ileum, the ischium and the pubis within acetabulum in adults:

- (0) unfused
- (1) fused

### 37. Inflected head of the femur set off from shaft by a neck:

- (0) neck absent (and head oriented dorsally)
- (1) neck present (and head inflected medially)

### 38. Greater trochanter of the femur:

- (0) directed dorsolaterally
- (1) directed dorsally

### 39. Orientation of lesser trochanter of the femur:

- (0) on the medial side of the shaft
- (1) on the ventromedial or ventral side of the shaft

### 40. Size of the lesser trochanter of the femur:

- (0) large
- (1) small

### 41. Patellar facet ("groove") of femur:

- (0) shallow and weakly developed
- (1) well developed

### 42. Tibial malleolus:

- (0) weak
- (1) distinct

### 43. Fibular styloid process

- (0) weak
- (1) distinct

### 44. fibular contact with calcaneum ("tricontact upper ankle joint" from Szalay 1993):

- (0) extensive contact
- (1) contact is reduced or absent

### 45. Superposition of astagalus over the calcaneum (lower ankle joint):

- (0) little or absent
- (1) weakly developed
- (2) present

46. Astragalar neck:

- (0) absent
- (1) present

47. Astragalar trochlea

- (0)\* absent
- (1) present

48. Calcaneal tubercle:

- (0) short and without terminal swelling
- (1) elongate with terminal swelling

49. Peroneal process and groove of calcaneum:

- (0) forming a laterally directed shelf and without a distinct process
- (1) weakly developed with shallow groove on lateral side of process
- (2) with a distinct peroneal process

50. Contact of the cuboid on the calcaneum:

- (0) on the anterior (distal) end of the calcaneum (the cuboid is aligned with the long axis of the calcaneum)
- (1) on the anteromedial aspect of the calcaneum (the cuboid is skewed to the medial side of the of the long axis of the calcaneum)

51. Relationships of the proximal end of metatarsal V to the cuboid:

- (0) metatarsal V is far off-set to the cuboid
- (1) metatarsal V is so far off-set to the cuboid that it contacts the calcaneum
- (2) metatarsal V is aligned with the cuboid

52. Angle of metatarsal III to the calcaneum (indicating how much the sole of the foot is bent from the long axis of the ankle):

- (0) metatarsal III is aligned with or parallel to the imaginary line of the long axis of calcaneum
- (1)\* metatarsal III is arranged obliquely from the imaginary line of the long axis of calcaneum

54. Tarsal spur:

- (0)\* absent
- (1) present

**Basicranial characters**

55. Cranial moiety of squamosal:

- (0) narrow
- (1) broad

56. Squamosal notches for quadrate and quadratojugal:

- (0) present
- (1) absent

57. Postglenoid depression on squamosal (= external auditory meatus):

- (0) absent
- (1) present

Ji *et al.* (1999) coded *Ornithorhynchus* as (0) for this character. *Obdurodon dicksoni* does have a postglenoid depression on the squamosal (Lou *et al.* 2001b and pers. obs.). As such I have coded Ornithorhynchidae as

(0/1), but in the modern versus fossil analysis (see Figure 4.12) the Ornithorhynchidae was coded (0) for modern and (1) for fossil.

**58. Position of craniomandibular joint:**

(0) posterior or lateral to the level of the fenestra vestibuli

(1) anterior to the level of the fenestra vestibuli

Ji *et al.* (1999) coded *Ornithorhynchus* as (0) for this character. In *Obdurodon dicksoni* the craniomandibular joint is anterior to the level of the fenestra vestibuli (Lou *et al.* 2001b and pers. obs.). As such I have coded Ornithorhynchidae as (0/1), but in the modern versus fossil analysis (see Figure 4.12) Ornithorhynchidae was coded (0) for modern and (1) for fossil.

**59. Pars cochlearis:**

(0)\* without an elongate petrosal cochlear housing

(1) with an elongate and cylindrical petrosal cochlear housing

(2) with a bulbous and oval-shaped promontorium

**60. Cochlea:**

(0) short and uncoiled

(1) elongate and partly coiled

(2) elongate and coiled at least 360°

**61. Crista interfenestralis:**

(0) horizontal and extending to the base of the paroccipital process

(1) vertical, delimiting the back of the promontorium

(2) indistinct from surrounding elements

Ji *et al.* (1999) only used states 0 and 1, but with the inclusion of *Tachyglossus*, an extra state (2) is necessary and I follow the coding of Rougier *et al.* (1996) for this.

**62. Post-tympanic recess:**

(0) absent

(1) present

**63. Caudal tympanic process of petrosal:**

(0) absent

(1) present

**64. Epitympanic recess:**

(0) absent

(1) present

**65. Epitympanic recess flanked laterally by squamosal:**

(0)\* absent

(1) present

**Mandibular characters**

**67. Medial dentary ridge that overhangs the posterior segment of the postdentary trough:**

(0) broad with prominent ridge

(1) trough and ridge absent

**68. Attachment of surangular and prearticular in adults:**

(0) attached to the mandible

(1) detached from the mandible

69. Size of anterior part of the Meckelian groove in adults:

- (0) well developed groove
- (1) weak and faint groove
- (2) absent

71. Angular process of dentary:

- (0) present
- (1) absent

Ji *et al.* (1999) coded *Ornithorhynchus* as (1) for this character. *Obdurodon* has an angular process (Musser and Archer 1998 and pers. obs.) as does *Teinolophos* (Rich *et al.* 2001a) and the angle is observable during the ontogeny of *Ornithorhynchus* (Zeller 1993). As such I have coded Ornithorhynchidae as (0), but in the modern versus fossil analysis (see Figure 4.12) Ornithorhynchidae was coded (0) for modern as the angle is not clearly observable in the adult, and (1) for fossil.

72. Distinctive insertion area for pterygoid muscle on the medial side of the mandible:

- (0) absent
- (1) present
- (2) medial pterygoid fossa has prominent ventral shelf along the ventral border of mandible

73. Coronoid fossa in adults:

- (0) present
- (1) absent

74. A distinct mandibular foramen for the inferior alveolar nerve and vessels:

- (0) absent
- (1) present

75. Mandibular symphysis:

- (0) fused
- (1) unfused
- (2) unfused and further reduced

76. Scar or depression for the splenial bone on the dentary:

- (0) present
- (1) absent

## Dental characters

80. Differentiation of postcanine crowns into premolars and molars:

- (0)\* absent
- (1) present

Ji *et al.* (1999) coded *Ornithorhynchus* as (?) for this character. However, *Obdurodon* has both molars and premolars (Musser and Archer 1998 and pers. obs.). As such I have coded Ornithorhynchidae as (1).

81. Number of the postcanine roots:

- (0) single (undivided)
- (1) divided, but no more than three roots
- (2) multiple roots (more than three)

Ji *et al.* (1999) coded *Ornithorhynchus* as (?) for this character. The most plesiomorphic monotreme mandibles discovered so far (from *Steropodon* and *Teinolophos*) contain molars with two roots. Hence (1) is inferred as the monotreme ancestral state and assigned to Ornithorhynchidae in order to convey this in the analyses. However, in the modern versus fossil analysis (see Figure 4.12) Ornithorhynchidae was coded (2) for modern because the deciduous platypus teeth have multiple roots, and (1) for fossil.

82. Positional correspondence of the upper and lower postcanines:

- (0) absent
- (1) present

83. Interlocking of adjacent lower postcanines:

- (0) absent
- (1) linguolabially compressed molars interlock by cingulum or the cingular cusps
- (2) lingualabially wide molars abut each other

84. Lingual cingula on the lower postcanines:

- (0) present, with well developed cingular cusps
- (1) cingular cusps absent, cingulum vestigial or absent

Ji *et al.* (1999) coded *Ornithorhynchus* as (?) for this character. This coding is retained for the modern platypus in the modern versus fossil analysis (see Figure 4.12). However, the wrapping cingulid (see Lou *et al.* 2001) of *Steropodon* and *Teinolophos* is derived from either an anterior cingulid, or an anterolingual cusp and these most plesiomorphic monotreme mandibles do not have true lingual cingula as do most non-cladotheres. Hence (1) is inferred as the monotreme ancestral state and assigned to Ornithorhynchidae in order to convey this in the analyses.

85. Anterolingual cuspule (“cusp e”) on the lingual cingulum of the lower molars:

- (0) present
- (1) absent

Ji *et al.* (1999) coded *Ornithorhynchus* as (?) for this character, though they noted that (1) would be appropriate for Ornithorhynchidae (presumably in consideration of the lack of “cusp e” in *Obdurodon*). *Steropodon* and *Teinolophos* also lack cusp e. Thus I have coded Ornithorhynchidae as (1).

86. Arrangement of main cusps of upper postcanines:

- (0) in a single longitudinal row
- (1) multiple cusps forming multiple longitudinal rows
- (2) as reversed triangles

87. Upper molar styler shelf (the are between the paracone/metacone and the buccal margin of the crown:

- (0) narrow or absent
- (1) broad

88. Styler cusps between the paracone and metacone of upper molars:

- (0) absent or weak (= crenulations on the buccal cingulum)
- (1) present and enlarged

89. Upper molar protocone:

- (0) absent
- (1) present

90. Alignment of main cusps of lower postcanines:

- (0) single longitudinal row
- (1) multiple cusps in multiple rows
- (2) as reversed triangles

Ji *et al.* (1999) coded *Ornithorhynchus* as (?) for this character. This coding is retained for the modern platypus in the modern versus fossil analysis (see Figure 4.12). However, *Obdurodon*, *Steropodon* and *Teinolophos* all have reverse triangle cusps on the lower molars. Hence I have coded Ornithorhynchidae as (2) for this character.

91. Orientation of protocristid relative to the length of the lower molar:

- (0) longitudinally oriented
- (1) oblique
- (2) more transverse

Ji *et al.* (1999) coded *Ornithorhynchus* as (?) for this character. This coding is retained for the modern platypus in the modern versus fossil analysis (see Figure 4.12). However, *Obdurodon*, *Steropodon* and *Teinolophos* all have near-transverse protocristids. Hence I have coded Ornithorhynchidae as (2) for this character.

92. Orientation of metacristid relative to the long axis of the lower molar:

- (0) longitudinal
- (1) oblique
- (2) transverse

Ji *et al.* (1999) coded *Ornithorhynchus* as (?) for this character. This coding is retained for the modern platypus in the modern versus fossil analysis (see Figure 4.12). However, *Obdurodon*, *Steropodon* and *Teinolophos* all have near-transverse metacristids. Hence I have coded Ornithorhynchidae as (2) for this character.

93. Lower molar talonid:

- (0) no talonid
- (1) simple with a single cusp
- (2) basined and with more than one cusp

Ji *et al.* (1999) coded *Ornithorhynchus* as (?) for this character. This coding is retained for the modern platypus in the modern versus fossil analysis (see Figure 4.12). However, *Obdurodon*, *Steropodon* and *Teinolophos* all have talonids with more than one cusp. Hence I have coded Ornithorhynchidae as (2) for this character.

94. Wear facet on talonid (or posterior cingulid of the lower molar):

- (0) absent
- (1) present

Ji *et al.* (1999) coded *Ornithorhynchus* as (?) for this character. This coding is retained for the modern platypus in the modern versus fossil analysis (see Figure 4.12). However, *Obdurodon*, *Steropodon* and *Teinolophos* all have talonid wear facets. Hence I have coded Ornithorhynchidae as (1) for this character.

96. Occlusion of the principal cusps of the upper and lower molars:

- (0) consistent contact relationships are lacking
- (1) principle lower molar cusp a (homologous with the protoconid) is positioned anterior to a central upper molar cusp A but posterior to an upper molar anterior cusp B of the same tooth
- (2) principle lower molar cusp a (homologous with the protoconid) occludes between an upper molar anterior cusp B and an upper molar posterior cusp C of the preceding tooth
- (3) interdigital occlusion between multiple cusp rows

97. Functional development of wear facets on molars:

- (0) absent for lifetime
- (1) absent at eruption but developed later by wearing of the crown
- (2) upper and lower cusps match upon eruption

Ji *et al.* (1999) coded *Ornithorhynchus* as (?) for this character. This coding is retained for the modern platypus in the modern versus fossil analysis (see Figure 4.12). However, in their supplementary information, Ji *et al.* (1999) recognize that wear facets between upper and lower molars in ornithorhynchids result from cusps matching upon eruption. This is in agreement with Hu *et al.* (1997). Hence I have coded Ornithorhynchidae as (2) for this character.

98. Relationships of wear facets to main cusps:

(0) wear facet absent

(1) a single principal cusp bears two wear facets

(2) two cusps bear a single wear facet

(3) multiple cusps, with each cusp bearing one or two transverse facets

Ji *et al.* (1999) coded *Ornithorhynchus* as (?) for this character. This coding is retained for the modern platypus in the modern versus fossil analysis (see Figure 4.12). However, state (2) occurs in *Obdurodon*, *Steropodon* and *Teinolophos*. Hence I have coded Ornithorhynchidae as (2) for this character.

99. Direction of the lower jaw movement during occlusion:

(0) orthal movement

(1) dorsoposterior movement

(2) dorsomedial movement

Ji *et al.* (1999) coded *Ornithorhynchus* as (1/2) for this character. This coding is retained for the modern platypus in the modern versus fossil analysis (see Figure 4.12). Among fossil monotremes there is little evidence for mastication involving dorsoposterior movement. Among fossil monotremes, dorsomedial jaw movement appears to be important in *Obdurodon* and *Steropodon*, but as Rich *et al.* (2001) note, orthal jaw movement may be primary in *Teinolophos* mastication, and perhaps of some importance (in that dorsomedial movement may not be well developed) in *Obdurodon*, *Steropodon*. Hence I have coded Ornithorhynchidae as (0/2) for this character.

100. Mode of occlusion:

(0) bilateral

(1) unilateral

101. Rotation of the mandible during occlusion, as inferred from the angles of wear facets to the vertical plane:

(0) absent

(1) moderate

(2) strong

Ji *et al.* (1999) coded *Ornithorhynchus* as (1) for this character. This coding is retained for the modern platypus in the modern versus fossil analysis (see Figure 4.12). However, upon consideration of *Obdurodon* and *Steropodon*, Hu *et al.* (1997) considered rotation of the mandible to be strong in ornithorhynchids ancestrally. Hence I have coded Ornithorhynchidae as (2) for this character.

As well as the alterations noted above, coding in the data matrix (below) differs from Ji *et al.*

(1999) in line with the updated coding of Lou *et al.* (2001a and 2001b) and Wang *et al.* (2001)\* of the following characters for:

*Gobiconodon* for characters 16 (?→1); 19 (?→1); 24 (?→1); 27 (?→0); 31(?→0); 58 (?→0); 59 (?→1); 60 (?→0); 61\* (?→0); 62 (?→0); 63 (?→0); 64 (?→0); 65 (?→0)

*Jeholodens* for characters 65 (?→0); 97 (?→1)

*Henkelotherium* for character 54 (?→1)

*Vincelestes* for character 27 (?→1)

As explained in Section 3.2.1, characters 61-64 were coded ? by Ji *et al.* (1999), but are here coded as for other Triconodontidae (Rougier *et al.* 1996).

Character matrix for Chapters 3 and 4, with numbering as for Ji et al. (1999)

	1	1
0000000011 111111222 222223333 333334444 444445555 555556666 666677777 888888888 999999990 0		
1234567902 3456789123 4567890123 4567890123 4567890124 5678901234 5789123456 0123456789 0123467890 1		
Cynodont (outgroup)		
0000000001 0000000000 0000000000 0000000000 0000000000 00?0000000 ?000000000 00000?0000 0000?00000 0		
Morganucodontids		
?001000101 ?010?00?00 0000000000 0100000100 0000000000 0100100000 0000000010 1111000000 0000011121 1		
<i>Jeholodens</i> (Triconodontidae)		
100?000001 111111011 10??011010 0100000100 0000001010 0100100000 0111120111 1111110000 0000011121 1		
<i>Gobiconodon</i>		
???????0?? ??11??1?11 1000011000 ?100000100 0????0???1 0100100000 0111120111 1111002000 0000021121 1		
Multituberculates		
10?1001100 1111011011 0100B00011 0111110211 0100121111 010010A0A1 011211A111 1102??1??0 1??0?32310 0		
<i>Zhangheotherium</i> (Spalacotheriidae)		
?11000?00 1121111011 1111111010 1101110200 0100110??1 ?111101111 1111110120 1111102100 2110022221 2		
<i>Henkelotherium</i>		
????????1? 1121111?11 ?11?101011 ?101110211 ?????1????1 1????????? ?111010110 1112112110 2221122221 2		
<i>Vincelestes</i>		
1011?1111 1?21011011 1101001010 0111100211 0201110200 1111211111 1112010111 1112112111 2111122221 2		
Marsupials		
1111A11111 1121011121 1111202111 1111111211 1211110200 1111221111 1112021111 1112112111 2222122221 2		
Placentals		
1111111111 1121011121 1111202111 A111111211 1211110200 1111221111 111B00A111 1112112101 2222122221 2		
Tachyglossidae		
1111A1100 0000100000 0000000000 1010000210 000?021101 1111012001 01120?1121 ?????????? ?????????? ?		
Ornithorhynchidae		
1111011100 0100100000 0000010000 1110000210 000?021111 01AA110000 0112021121 111211???? 22221?22C1 2		
Ornithorhynchidae (platypus)		
1111011100 0100100000 0000010000 1110000210 000?021111 0100110000 0112121121 1212?1???? ??????????B1 1		
Ornithorhynchidae (fossils)		
1111011100 0100100000 0000010000 1110000210 000?021111 0111110000 0112021121 111211???? 22221?22C1 2		

? is uncertainty; letters indicate polymorphism: A=(0/1), B=(1/2), C=(0/2)

## Appendix E: Determining expected character-map incompatibility values

### a. expected character-map incompatibility ( $CMI_E$ ) across the whole background phylogeny

The  $CMI_E$  (across the whole tree) for each of the character-map pairs can be determined by simple equations that depend on the character-map categories of each member of character pairs (see Section 4.2.4 and Figure 4.3). Equations 1 and 4 are for trees generally, while equations 2, 3, 5 and 6 are only guaranteed for pectinate trees such as the background phylogeny used in Chapters 3 and 4. The four parameters used in the equations are:

- The number of nodes in the unrooted background phylogeny ( $r$ ), which equals 5 for the background phylogeny of generalized insectivores.
- The number of taxa (or leaves) on the background phylogeny ( $t$ ), which equals 7 for the background phylogeny of generalized insectivores. Some of the equations below will simplify by recognizing that  $r = t - 2$ .
- The number of nodes included in the character map for each member of the character pairs being compared ( $n_1$  and  $n_2$ ).
- The number of terminal nodes (nodes to which two external branches are attached) in the background phylogeny ( $e$ ), which equals 2 for the background phylogeny of generalized insectivores.

In Table 4.1 the  $CMI_E$  values are expressed as fractions. The **denominator** is the total number of different combinations of positions on the background phylogeny that the two character-maps can be placed. The **numerator** is the number of these combinations for which the character-maps are incompatible.

#### 1. Where the pairwise character-map comparison involves two leaves

$$\text{denominator} = t^2$$

$$\text{numerator} = t(t-1)$$

#### 2. Where the pairwise character-map comparison involves a leaf and a node-group (or connected node-group)

$$\text{denominator} = t(r+1-n)$$

$$\text{numerator} = (t-n)(r+1-n)-e$$

3. Where the pairwise character-map comparison involves a leaf and a terminal node-group (or terminal connected node-group)

$$\text{denominator} = t \times e$$

$$\text{numerator} = e(r+1-n) \quad (\text{where } n < r; \text{ if } n = r, \text{ then } \text{CMI}_E = 0)$$

4. Where the pairwise character-map comparison involves two node-groups (or connected node-groups) ( $n_1$  and  $n_2$ ).

$$\text{denominator} = (r+1-n_1)(r+1-n_2)$$

$$\text{numerator} = \sum_{c=1}^{r-(n_1+n_2)} 2^c \quad (\text{where } n_1 + n_2 \leq r; \text{ if } n_1 + n_2 > r, \text{ then } \text{CMI}_E = 0)$$

5. Where the pairwise character-map comparison involves two terminal node-groups (or terminal connected node-groups) ( $n_1$  and  $n_2$ ).

$$\text{denominator} = e^2$$

$$\text{numerator} = (e-1)e$$

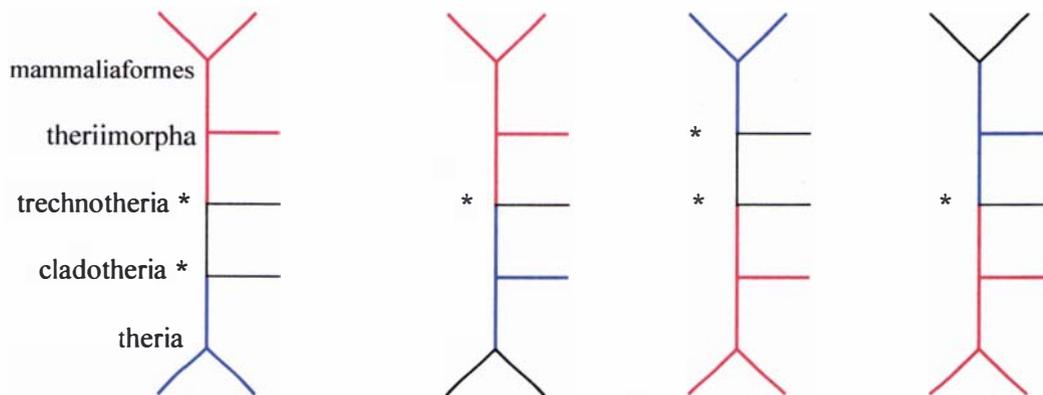
6. Where the pairwise character-map comparison involves a node-group (or connected node-group) ( $n_1$ ) and a terminal node-group (or terminal connected node-group) ( $n_2$ ).

$$\text{denominator} = (r+1-n_1)e$$

$$\text{numerator} = e(r-n_1-n_2) \quad (\text{where } n_1 + n_2 \leq r; \text{ if } n_1 + n_2 > r, \text{ then } \text{CMI}_E = 0)$$

**b. expected character-map incompatibility ( $CMI_E$ ) across specific nodes on the background phylogeny**

Determining  $CMI_E$  across specific nodes is more complex than determining  $CMI_E$  across the whole background phylogeny. So  $CMI_E$  for specific nodes was determined by summing the numbers of incompatible pair combinations (given the character-map categories for each member of the pair) and dividing by the total number of combinations. These combinatorial probabilities were determined graphically. The example that follows is for a character pair with one member that is a 2-node terminal node-group, and one member that is a single node-group. On the generalized insectivore background phylogeny there are 4 possibilities for a 2-node terminal node-group to be incompatible with a single node-group (From 10 possible combinations of the two character-map categories: using equation 6 above).



Character-map 1 (single node-group) is shown in blue and character-map 2 (2-node terminal node-group) is shown in red. As shown above, for each of the four paired combinations in which the two character-maps are incompatible (across the phylogeny overall), the specific nodes that the pairs are incompatible across are denoted with an asterisk (\*). By summing across the 4 trees for the incompatibilities at each node and dividing by the overall number of pairwise combinations (10), the  $CMI_E$  values at specific nodes are: 0.4 for the trechnotheria node, 0.1 for the theriimorpha and cladotheria nodes and 0 for the mammaliaformes and theria nodes.

**Appendix F: Character-map incompatibility values within and between anatomical regions**

(a) Relative CMI ( $CMI_R = (CMI_O - CMI_E) / CMI_E$ ) among characters within the six anatomical regions and between anatomical regions that are drawn from the same meta-region (LB and MVD). The CMI values are for incompatibility across the entire background phylogeny. Note that n = the number of pairwise character comparisons. p-values indicate the level of significance for the difference between  $CMI_O$  (observed CMI) and  $CMI_E$  (expected CMI) according to the z-ratio binomial probability test (for n pairwise comparisons).

Regions	Characters	Pairs	$CMI_O$	$CMI_E$	Resolvability ( $CMI_E$ /pairs)	$CMI_R$ (overall)	p-value
<b>within region CMI (pairs from the same region)</b>							
U	22	231	12	55.990	0.242	- 0.786	<0.0001
L	20	190	16	17.571	0.092	- 0.089	0.7872
B	11	55	4	4.150	0.075	- 0.036	0.5970
M	9	36	2	3.171	0.088	- 0.369	0.3743
V	8	28	0	1.750	0.063	- 1.000	0.1641
D	21	210	0	4.305	0.021	- 1.000	0.0135
<b>between region CMI with pairs from different regions within LB</b>							
L*B	20*11	220	19	20.521	0.093	- 0.074	0.8103
<b>between region CMI with pairs from different regions within MVD</b>							
M*V	9*8	72	3	4.732	0.066	- 0.366	0.2960
M*D	9*21	189	5	9.260	0.049	- 0.460	0.2041
V*D	8*21	168	0	7.500	0.045	- 1.000	0.0091

(b) Relative CMI ( $CMI_R = (CMI_O - CMI_E) / CMI_E$ ) among characters between anatomical regions that are drawn from different meta-regions.

Regions	Characters	Pairs	$CMI_O$	$CMI_E$	Resolvability ( $CMI_E$ /pairs)	$CMI_R$ (overall)	p-value
<b>between region CMI for pairs with one character from U and the other from LB</b>							
U*L	22*20	440	72	73.495	0.167	- 0.020	0.8966
U*B	22*11	242	37	43.765	0.181	- 0.155	0.2937
<b>between region CMI for pairs with one character from U and the other from MVD</b>							
U*M	22*9	198	45	28.427	0.144	+0.583	0.0011
U*V	22*8	176	51	25.048	0.142	+1.036	<0.0001
U*D	22*21	462	110	49.987	0.108	+1.201	<0.0001
<b>between region CMI for pairs with one character from LB and the other from MVD</b>							
L*M	20*9	180	18	17.164	0.095	+0.049	0.9283
L*V	20*8	160	18	12.464	0.078	+0.444	0.1362
L*D	20*21	420	33	21.400	0.051	+0.542	0.0139
B*M	11*9	99	14	8.141	0.082	+0.720	0.0500
B*V	11*8	88	13	7.125	0.081	+0.825	0.0357
B*D	11*21	231	22	9.860	0.043	+1.231	<0.0001

## Appendix G: Phylogenetic relationships among placentals

Relationships between 43 placental mammals and five outgroup taxa (marsupials and monotremes) were inferred from their mtDNA sequences. In this preliminary analysis, selecting the tree that is most consistent with the data is emphasized, rather than exploring statistical support for the relationships recovered. The GenBank accession numbers for each sequence are given in Table 1. The mtDNA sequences were RY-coded. Relative to standard NT-coding, this is expected (in most cases) to increase the ratio of phylogenetic signal to signals from biases (see Chapter 2). Furthermore, only the PTN12 and RNAsperms data were used. Chi-square testing showed that composition stationarity among the 48 taxa is rejected at a high significance level ( $p < 0.0001$ ) if the third codon positions and RNAloops data are included. In contrast, the corresponding p-value for the RY-coded PTN12+RNAsperms dataset is 0.8079.

**Table 1** Taxon names (including abbreviations from Figure 5.2.1) and GenBank accession numbers for complete mt genome DNA sequences. Asterisk (\*) denotes NADH2 and partial NADH4 and COIII sequences from the revised hedgehog sequence (see Lin *et al.* 2002). The Hector's dolphin mt sequence is unpublished and was kindly made available by Patricia McLenachan, Alicia Gore and David Penny.

echidna	<i>Tachyglossus aculeatus</i>	AJ303116
platypus	<i>Ornithorhynchus anatinus</i>	X83427
common brushtail possum	<i>Trichosurus vulpecula</i>	AF357238
northern brown bandicoot	<i>Isodon macrourus</i>	AF358864
Virginia opossum	<i>Didelphis virginiana</i>	Z29573
armadillo	<i>Dasyus novemcinctus</i>	Y11832
elephant	<i>Loxodonta africana</i>	AJ224821
dugong	<i>Dugong dugon</i>	AY075116
aardvark	<i>Orycteropus afer</i>	Y18475
tenrec	<i>Echinops telfairi</i>	AJ245806
hedgehog	<i>Erinaceus europaeus</i>	X88898, (AF513818, AF513819, AF513817)*
gymnure	<i>Echinosorex gymnurus</i>	NC_002808
mole	<i>Talpa europaea</i>	Y19192
formosan shrew (Shrew)	<i>Soriculus fumidus</i>	NC_003040
long-clawed shrew (LClawShrew)	<i>Sorex unguiculatus</i>	AB061527
little Red Flying-Fox (FlyingFox)	<i>Pteropus scapulatus</i>	AF321050
Taiwanese horseshoe bat (Rhinolophus)	<i>Rhinolophus monoceros</i>	AF406806
Jamaican fruit-eating bat (JFEBat)	<i>Artibeus jamaicensis</i>	AF061340
long-tailed bat (LtailBat)	<i>Chalinolobus tuberculatus</i>	AF321051
Japaneses pipistrelle (PipBat)	<i>Pipistrellus abramus</i>	AB061528
cat	<i>Felis catus</i>	U20753
dog	<i>Canis familiaris</i>	U96639
brown bear	<i>Ursus arctos</i>	NC_003427
New Zealand fur seal (FurSeal)	<i>Arctocephalus forsteri</i>	NC_004023
harbor seal (Harb Seal)	<i>Phoca vitulina</i>	X63726
gray seal	<i>Halichoerus grypus</i>	X72004
horse	<i>Equus caballus</i>	X79547
Indian Rhinoceros (Ind Rhino)	<i>Rhinoceros unicornis</i>	X97336

**Table 1** continued

alpaca	<i>Lama pacos</i>	Y19184
pig	<i>Sus scrofa</i>	AJ002189
cow	<i>Bos taurus</i>	V00654
hippopotamus (hippo)	<i>Hippopotamus amphibius</i>	AJ010957
blue whale	<i>Balaenoptera musculus</i>	X72204
sperm whale	<i>Physeter catodon</i>	AJ277029
Hector's dolphin (Hect dolphin)	<i>Cephalorhynchus hectori</i>	
rabbit	<i>Oryctolagus cuniculus</i>	AJ001588
pika	<i>Ochotona collaris</i>	AF348080
red squirrel (Squirrel)	<i>Sciurus vulgaris</i>	AJ238588
fat dormouse (Dormouse)	<i>Glis glis</i>	AJ001562
Guinea Pig	<i>Cavia procellus</i>	AJ222767
cane rat	<i>Thyromys swinderianus</i>	NC_002658
mouse	<i>Mus musculus</i>	V00711
vole	<i>Volemys kikuchii</i>	NC_003041
tree shrew	<i>Tupaia belangeri</i>	AF217811
slow loris (Loris)	<i>Nycticebus coucang</i>	AJ309867
tarsier	<i>Tarsius bancanus</i>	AF348159
babbon	<i>Papio hamadryas</i>	Y18001
gibbon	<i>Hylobates lar</i>	X99256

The rooting of the placental tree has been identified as a particularly difficult problem, both by studies of nuclear (e.g. Madsen *et al.* 2001) and mt (e.g. Waddell *et al.* 1999) sequences. Further, the erinaceids have a high rate of mt evolution and have not only proved difficult to place on the tree, but also affect other placental relationships (see Lin *et al.* 2002). Hence the initial ME analyses only examine relationships among the ingroup (Placentalia) and do not include erinaceids (hedgehog and gymnure). The results provide an unrooted placental background phylogeny on to which the placement of the root and the erinaceids are tested in the following section.

The ME analyses used ML(CF87+I+ $\Gamma_8$ ) distances and 500 heuristic-search bootstrap replicates. The bootstrap support values are shown in Table 2, with each of the clades referable to Figure 5.2.1 as nodes a-f. It is interesting to note how the bootstrap support values change as the extent of among-site heterogeneity incorporated in the CF87 model is increased. Initially, a conservative (80%) proportion of the observed constant sites were excluded and the remainder of sites assumed to be equally variable (gamma shape parameter set at infinity). In order to examine how bootstrap support for relationships changes with increased among-site rate heterogeneity, the analysis was repeated with the value of the gamma shape parameter being decreased (1.2, 0.8, 0.5).

In Chapter 2 it was suggested that assuming a single model for among-site rate heterogeneity may not be reliable for phylogeny reconstruction across the whole tree. This is because the relative variability of sites (including probability of being constant) is likely to increase as sequences and their protein and/or RNA products diverge (see Penny *et al.* 2001). A partial solution to the problem of testing the reliability of a clade when rate heterogeneity (among sites) differs across the

tree, might be to examine trends in support for clades, as different levels of among-site heterogeneity are incorporated into models.

Support for all relationships among the placentals varied (to some degree) with changes in the gamma shape parameter ( $\alpha$ ) used in the CF87+I+ $\Gamma$  model. However, most relationships were recovered in more than 60% of bootstrap replicates in each analysis and on this basis were incorporated into the placental background phylogeny (Figure 5.2.1). The six clades identified as nodes a-f in Figure 5.2.1 were the exceptions. Nevertheless, four of these groupings (which have not been favoured in previous mtDNA studies) appear quite promising with the PTN12+RNAstems dataset being RY-coded. These are: a. dolphin+sperm whale, b. tarsier+anthropoid primates, c. non-camelid cetartiodactyls, and d. tree shrew+primates.

As examination of Table 2 shows, groupings b-d are favoured in each of the analyses, and are attributed increasing bootstrap support as  $\alpha$  decreases from infinity to 0.5. With only 80% of constant sites assumed to be invariable and rate homogeneity among the sites assumed to be variable (i.e.  $\alpha$ =infinity) the ME model is expected to be underestimating among-site rate heterogeneity. Indeed, with 80% of constant sites assumed to be invariable, the PAUP\* ML estimate (CF87+I+ $\Gamma_8$ ) for  $\alpha$  is approximately 0.5. Hence as  $\alpha$  decreases over the range shown in Table 2, the ME analyses are likely to be providing better branch-length estimates and so more reliable phylogeny reconstruction. It is on this basis that I prefer the odontocete (dolphin+sperm whale) grouping (a) over a sperm whale plus mysticete grouping, despite the latter grouping being favoured in the analyses that employed the higher values of  $\alpha$ .

**Table 2** Effect of the value of the gamma shape parameter ( $\alpha$ ) on bootstrap support for seven clades (a-f on Figure 5.2.1) in ME analyses of the RY-coded PTN12+RNAstems dataset, using ML(CF87+I+ $\Gamma_8$ ) distances. Asterisk (\*) denotes that a murid + lagomorph grouping is preferred and (\*\*) denotes that a sperm whale + blue whale grouping is preferred.

Clade	Node	Gamma shape parameter ( $\alpha$ ) for ML(CF87+I+ $\Gamma_8$ )			
		$\alpha$ =infinity	$\alpha$ =1.2	$\alpha$ =0.8	$\alpha$ =0.5
dolphin+sperm whale	a	4**	17**	23**	51
tarsier+anthropoids	b	49	48	53	55
alpaca as basal cetartiodactyl	c	48	49	50	53
Euarchonta	d	40	48	52	53
Rodentia	e	64	44	30*	18*
Perissodactyla+Carnivora	f	93	83	74	58

In contrast to the situation for nodes a-d, support for the clades at nodes e and f falls with decreases in  $\alpha$ . (the alternative relationships are noted with broken arrows in Figure 5.2.1). In the case of the Perissodactyla+Carnivora grouping (f), the decrease in bootstrap support is from 93% with  $\alpha=\infty$  to 58% with  $\alpha=0.5$ . Much of the signal supporting this grouping when  $\alpha$  is high may be a branch-length effect, because the perissodactyls and carnivorans have the slowest rates of evolution among the PTN12+RNAstem dataset (inferred from branch-lengths: not shown). With the higher values of  $\alpha$ , among-site heterogeneity is being underestimated and this results in the amount of parallel change between long branches being proportionately underestimated relative to that among short branches (see Waddell 1995). This would tend to lead to attraction between the longer branches of the outgroups of Fereuungulata and Artiodactyla, effectively providing signal for grouping together the slower evolving perissodactyls and carnivorans. Hence the relationships among the fereuungulates (perissodactyls, artiodactyls and carnivorans) in Figure 5.2.1 should be treated with some suspicion.

Rodent monophyly (node e) is the only grouping included in the placental background phylogeny that is not favoured by the ME analysis for which the incorporated among-site rate heterogeneity was greatest (and optimum, according to ML estimation). Instead the tendency in this case is for the murids (mouse and vole) to group with the lagomorphs (rabbit and pika). Being the most divergent taxa (in terms of ME distances from marsupials and monotremes) among the placental background phylogeny, it is the rodents, particularly the murids, for which the ML estimates for rate heterogeneity are most likely to be overestimates. If this is the case for the analyses incorporating higher  $\alpha$  values, then the amount of parallel change between the murids and the other rodents may be overestimated, causing these long branches to be repelled (see Swofford *et al.* 2001). This possibility may be worth further investigation. Moreover, as rodent monophyly is favoured in the analyses for which  $\alpha=\infty$  and 1.2, there is clearly signal among the data for rodent monophyly. Hence, while the RY-coded PTN12+RNAstems dataset does not clearly favour rodent monophyly, it is not inconsistent with this hypothesis. Furthermore, Pesole (2002) have recently shown that support for rodent monophyly from analysis of complete mt protein-coding data is boosted by the inclusion of the basal murid, *Spalax*.

The placements on the placental background phylogeny of the erinaceids and the root were examined with ML(CF87) analyses. Table 3 shows the results of these analyses. Notably, when rate homogeneity among sites is assumed, both the erinaceids and the marsupial/monotreme outgroup join with the murid stem, the longest branch on the placental background tree. However, for the CF87+I+ $\Gamma_4$  models, preference for the placements of both erinaceids and the root switch to

positions that are more consistent with recent nuclear studies (e.g. Madsen *et al.* 2001; Murphy *et al.* 2001a, 2001b).

**Table 3** ML Shimimodaira-Hasegawa (S-H) testing for the placement of erinaceids and the root on the placental background phylogeny. The CF87 and with CF87+I+ $\Gamma_4$  ML models were used to estimate  $-\ln L$  scores for each tree representing the alternative placement of erinaceids and the root. For the I+ $\Gamma_4$  treatments, 80% of the constant sites were assumed to be invariable, and PAUP\* ML estimates of the gamma shape parameter were used.

Placement with the	Shimimodaira-Hasegawa test probabilities			
	for erinaceid placement		for the placement of the root	
	CF87	CF87+I+ $\Gamma_4$	CF87	CF87+I+ $\Gamma_4$
shrew stem	0.357	0.920	<0.001	0.001
mole	0.521	<b>ML tree</b>	0.001	0.001
eulipotyphlan stem	0.135	0.784	<0.001	0.002
laurasiantherian stem	0.041	0.552	0.018	0.372
supraprimates stem	0.039	0.144	0.031	0.384
euarchontan stem	0.053	0.086	0.043	0.230
Glires stem	0.123	0.085	0.069	0.318
lagomorph stem	0.129	0.112	0.074	0.319
rodent stem	0.167	0.078	0.080	0.319
murid stem	<b>ML tree</b>	0.080	<b>ML tree</b>	0.329
boreoeutherian stem	0.008	0.107	0.394	<b>ML tree</b>
armadillo	0.003	0.002	0.853	0.618
afrotherian stem	<0.001	0.002	0.424	0.574

An association with the mole is favoured for erinaceids, though alternative placements with the shrew stem, the eulipotyphlan (core insectivores) stem, or with the Laurasiatherian stem cannot be rejected even at  $p < 0.50$ . Indeed, only a small change in the gamma shape parameter is required for preference for erinaceid placement to swap over to the shrew stem.

Rooting the placental tree on the Boreoeutherian stem under the CF87+I+ $\Gamma_4$  model (see Table 3) implies that placentals can be divided into two clades, one containing the afrotherians and xenarthrans (Atlantogenata; Waddell *et al.* 1999a) and the other containing the supraprimates and laurasiatherians (Boreoeutheria; Murphy *et al.* 2001b). However, as shown in Table 3, rooting the placentals on either the xenarthran branch, or the afrotherian stem cannot be rejected at  $p < 0.50$ . In fact, the root can be placed in ten positions on the tree without being rejected at  $p < 0.20$ .

**Appendix H: "Long-range" PCR primers.**

At least three of the following primer combinations were used for amplifying mtDNA from the northern brown bandicoot, common brushtail possum and hedgehog liver samples.

Long 16S-For (AATTAGGGTTTACGACCTCGATGTTGGATCAGG) to H11685-Rev (CCTAAGACCAATGGATTACTTCTATCCT)

L11012-For (AGCTCTATCTGCTTTCGTCAAACAG) to Long16S-Rev (TGATTATGCTACCTTTCACGGTCAGGATACC)

L23-For (GCAAGGCACTGAAAATGCCTAGAT) to H5100-Rev (AGGCTTTTGAAGGCCTTGGTCT)

L2050-For (CCGTGCAAAGGTAGCATAATCAC) to H7580-Rev (CGCCTGGAATAGCATCTGCTTTT)

L7371-For (GGYCATCAATGATAYTGAAGCTA) to H13734-Rev (AGGCCAAATTGRGCTGATTTTCC)

L12175-For (TGRGAAGGAGTRGGMATTATRTC) to H29-Rev (AAACCCATCTARGCATTTCAGTG)