SEQUENCES AND SIGNALS: EVOLUTIONARY HISTORIES OF NEW ZEALAND SKINKS.

A thesis presented in partial fulfilment of
the requirements for the degree of
Doctor of Philosophy in Genetics
at Massey University
New Zealand

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May 1993

Abstract

The application of DNA sequencing to studies of the New Zealand biota is illustrated by investigations into the evolutionary relationships of skinks in the genus *Leiolopisma*. DNA sequences from a region of the mitochondrial 12S rRNA gene were determined for 20 taxa by use of the polymerase chain reaction. A vertebrate secondary structure model for this part of the gene was developed using comparative sequence analysis and calculations of RNA folding energies. Approximately one third of the molecule does not vary between the vertebrates examined, and there are similar patterns of sequence variability among the vertebrates. The secondary structure model was subsequently used to assist phylogenetic analyses of the skink sequence data set.

Analyses of the mitochondrial DNA sequence information, using newly developed and more sophisticated algorithms, did not produce a fully resolved phylogenetic tree for all the skinks, though relationships between some taxa are less ambiguous. The lack of resolution does not appear to be due to limitations in the analytical methods, nor to the patterns of nucleotide substitutions in the skink 12S rRNA sequences. The skink sequence data set is unusual in that most of the taxa have similar numbers of nucleotide substitutions when compared to each other. These results are interpreted as reflecting a rapid divergence of the *Leiolopisma* group of skinks. Simulation studies support this interpretation.

Three hypotheses are presented to account for the patterns of sequence differences between the skinks. One proposes a Gondwanan divergence of the *Leiolopisma* group, about 80 million years ago. Under this hypothesis the distribution of the skinks on islands in the Pacific and Indian oceans can be explained, in part, by continental drift. A second hypothesis suggests that New Zealand *Leiolopisma* are derived from a Miocene (15-25 million years ago) evolutionary radiation in New Zealand. This hypothesis however is inconsistent with observations of the sequence similarities between Mauritian, Australian, and New Zealand skinks. A third hypothesis, proposing several independent colonizations of New Zealand by *Leiolopisma*, is also not as well supported by the available sequence data. However, a close relationship between the 12S rRNA sequences of one New Zealand species *L. infrapunctatum* and the Australian *Lampropholis guichenoti* suggests that at least two skink immigrations to, or emigrations from, New Zealand may have occurred. Predictions of the three hypotheses and strategies to test them are discussed.

Some of the conclusions derived from analyses of the mitochondrial DNA sequences conflict with those obtained from allozyme information, though there are points of agreement. Comparison of the allozyme and sequence data do also revealed a case of hybridization between two sympatric species, *L. n. polychroma* and *L. maccanni*, at a site in Southland. Analyses of both data sets indicate that the morphological similarity of *Leiolopisma* species obscures a large amount of genetic diversity, and the evolutionary histories of New Zealand *Leiolopisma* are older and more complex than previously considered. Further genetical and ecological studies of *Leiolopisma* are required, but this thesis emphasizes both the suitability and necessity of molecular genetic approaches for evolutionary investigations in New Zealand.

Acknowledgments

Barry Scott and Garry Latch are penultimately to blame for this thesis. None of us knew what we were really in for.

Financial support was provided by the University Grants Committee, the Nga Manu Trust, Massey University, the Molecular Genetics Unit and Department of Microbiology and Genetics. Additional support came from the Dean of Science, the Royal Society of New Zealand, the Alfred P. Sloan Foundation (not once but twice!), and my parents. To all I am very grateful.

Iain Lamont and Liz Poole of Otago University provided hospitality and Liz showed me how to double-strand sequence. Brad Shaffer, Centre for Population Biology, University of California Davis, permitted me time, space and consumables in his lab. Ross Sadlier of the Australian Museum, Sydney, provided tissue samples of New Caledonian skinks.

With Robert Fisher, Centre for Population Biology, University of California Davis, I enjoyed many hours of entertaining and interesting discussions, and he let me share a room with his lizards. Thanks Robert, but get on with your thesis! Mike Hendy, Mike Steel, Mike Charleston, Peter Waddell, and Chris Simon are also thanked for stimulating discussions and ideas.

Charlie Daugherty, Victoria University, provided the initial inspiration for this study. He gave me access to the skink tissues and was ever willing to fill me in on the details of New Zealand skinks.

Barry Scott, Tim Brown, and Geoff Malcolm provided a stimulating and friendly atmosphere in which to work, freedom to do as I pleased and to go where I wanted, and money to make it possible. Mister Tucker, Paul, Laura and Terri covered administration and clerical things.

Thanks also to my travel agent Sue. But absolutely no thanks to Guido and the Linguine Gang, Milano Station, Italy, even though they too relieved me of a burden.

Lots of people have been and gone, or are still around. Thanks to them for all sorts. Especially Carolyn and Miranda.

To the following, words are no recompense:

David and Eric, both much more than superb and challenging supervisors and friends.

Alan, Gina, and particularly Mary of "Capital City Campus".

Stan, Pete, Trish and Liz of the Farside (and other places).

But most of all I thank my parents, with much love.

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Chapter One: ***Introduction***

New Zealand's biota is a largely untapped resource for evolutionary investigations. Despite the fact that our flora and fauna rank alongside life forms from the islands of Hawaii, Madagascar, New Guinea and the Galápagos' in furnishing vivid examples of evolution in action (Hooker 1853, Hutton 1872, Wallace 1880, Fleming 1958, Carlquist 1974), this potential has not yet been fully exploited. Some reasons for this are discussed below. This thesis illustrates the importance of New Zealand and its biota for addressing evolutionary questions. It does this by examining mitochondrial DNA sequences and from this data inferring aspects of the evolutionary history and relationships of skinks (Reptilia: Lacertilia: Scincidae) in the genus *Leiolopisma*. It also investigates what phylogenetic information can be obtained from DNA sequences by consideration of the sequences' secondary structure and by the use of new sequence analysis algorithms.

The major conclusions of this thesis are that firstly, phylogenetic trees are hypotheses only, and close examination of DNA sequences is necessary to have confidence in the reliability of phylogenetic relationships. New DNA sequence algorithms are used which allow exploration of information in sequences. Secondly, the use of molecular genetic techniques are valuable for investigations of evolutionary problems in New Zealand, particularly when applied in conjunction with ecological and population genetic studies. Thirdly, that the evolutionary history of New Zealand skinks is more complex and fascinating than previously suspected. Use of model groups, such as New Zealand skinks, is essential for an understanding of the patterns and processes of evolution.

New Zealand in an Evolutionary Context.

New Zealand contains great geological variety and complexity, diverse topography and habitats, and has been, and is being, subjected to extensive geological, climatic and ecological disturbances (Gage 1961, Suggate *et al.* 1978, Fleming 1980, Burrows & Greenland 1979, Mildenhall 1980, Stevens 1980, Pocknall 1989, 1992). In addition to the origin and separation of New Zealand from Gondwanaland, four periods have been suggested to have had a significant effect on the biota.

The Oligocene

During the Oligocene (25-35 million years ago [MYA]), New Zealand was a series of low lying islands, and fluctuating sea levels changed their size and number (Suggate *et al.* 1978; Cooper, A., Chambers, G.K., Cooper, R.A. *in prep.*). Extinction of species may have been high at this time because of the due to rising sea levels (Cooper *et al. in prep.*). Subsequent tectonic uplift, and increases in land area and relief, temperature, and habitat diversity during and after the Miocene (15-25 MYA) may have promoted conditions suitable for diversification of species (see Suggate *et al.* 1978, Cooper *et al. in prep.*).

The Pliocene

The Pliocene (1-15 MYA), marked the start of the rise of the southern alps in New Zealand (Suggate *et al.* 1978, Fleming 1980). With no previous alpine environment, the origins of the alpine flora has been considered problematical. Raven (1972) suggested the alpine flora came from Australia, but adaptation of indigenous New Zealand plants is more likely (Fisher 1965, Wardle 1978, Fleming 1980). During the Pliocene the north of the North Island was a series of archipelagoes, which changed in size and shape. The diversity of beetles and land snails now found there may be due to such geological changes (Powell 1949, Holloway 1963, Climo 1978; see also Fleming 1980).

The Pleistocene

Pleistocene glaciations are considered to have had an important effect upon the distribution and speciation of many groups both here (Powell 1949, Willett 1950, Dell 1955, Wardle 1963, 1988, Burrows 1965, Bigelow 1967, Irving 1967, Petersen 1968, Bull & Whitaker 1975, Fleming 1980, Solem *et al.* 1981), and elsewhere (for example, Sylvester-Bradley 1963, Haffer 1969, Mayr & O'Hara 1986). In New Zealand, the existence of several "refugia" for cold intolerant species during glacial periods has been suggested (Burrows 1965, Wardle 1963, Fleming 1980). McGlone (1985) argues however that plant distributions in New Zealand may have been influenced more by pre-Pleistocene tectonic activity than by glacial advances. Evaluating these hypotheses requires accurate assessments of both taxonomic relationships and times of divergence. Genetic investigations of Australian and European fauna indicate that the effects of Pleistocene glaciation may have had less influence on the speciation of some groups than previously assumed (Roberts & Maxson 1985, Wallis & Arntzen 1989). Similar evaluations for the New Zealand biota are lacking, and the phylogenetic relationships of many New Zealand groups are still poorly known (Solem *et al.* 1981, Fife 1985, Wardle 1988).

The Holocene

Human settlement of New Zealand over the last millennium has also had a major influence on the biota, through direct action and indirectly via introduced plants and animals (Molloy *et al.* 1963, Anderson 1983, McGlone 1983, Holdaway 1990). Evolutionary studies have relevance in this context. They can identify taxonomically important populations in urgent need of conservation (for example, Daugherty *et al.* 1990a). Investigations of genetic diversity within and between populations is also essential to develop rational conservation strategies (Vrijenhoek *et al.* 1985, Cohn 1990, May 1990, Vane-Wright *et al.* 1991).

Evolutionary Studies in New Zealand

How and why species may change have been illustrated by studies in the Galápagos and Hawaiian islands (see for example Berry 1984, Simon 1987, Grant & Grant 1989), and also by detailed investigations of *Drosophila* species (see Lewontin *et al.* 1981, DeSalle & Hunt 1987). Despite recognition that New Zealand is an important place to investigate evolutionary patterns and processes (Hooker 1853, Hutton 1872, Wallace 1889, Cockayne 1911, Godley 1949, Hair 1966, Fleming 1958), the biota have not yet been examined in sufficient detail to complement Hawaiian and Galápagan studies.

New Zealand's antiquity, continental origins, and the large size of some of its islands make it different from the islands of Hawaii and the Galápagos'. Studies of the origins of New Zealand's biota and how they have evolved may therefore offer different perspectives on patterns and processes of evolution.

Studies of evolutionary relevance have been conducted here but have been primarily descriptive and species-based, rather than being of a more theoretical and experimental nature. Few of the studies have directly examined the evolutionary concepts established by Lyell (1830), Darwin (1859), and Wallace (1889), and subsequently elaborated upon by Dobzhansky (1941), Stebbins (1950), Mayr (1963, 1982), Lewontin (1974), Kimura (1983) and Nei (1987). Exceptions are studies of alpine plants (Fisher 1965, Raven & Raven 1976, Ornduff 1964), morphological and genetic variation among introduced birds (Baker 1975, 1992, Ross 1983, Baker & Moeed 1987), floral biology (Lloyd & Yates 1982, Lloyd & Webb 1986), and behavioural ecology (for example, Craig 1984). Recent advances in molecular genetic techniques are however leading to renewed interest in evolutionary investigations here.

Biogeographic Studies in New Zealand

One of the major problems facing evolutionary studies in New Zealand is establishing from where, and when, many elements of our biota came. Biogeographic studies in New Zealand (see Fleming 1980, McGlone 1985, Craw 1989) laid the groundwork for examination of the evolution of species. As a crude distillation two, often competing, views of the history of New Zealand's biota have been presented. "Traditional" biogeographers (Fleming 1980) have assumed that much of the flora and fauna arrived here after the separation of the New Zealand land mass from Gondwanaland, approximately 80 MYA (Lawver *et al.* 1991), and various means and routes of dispersal have been proposed to account for their arrival (Fleming 1980, McGlone 1985, see also Kuschel 1975). Panbiogeographers (see Craw 1989) took the alternate view that present distributions may reflect more about past geographical connections than dispersal. Both perspectives are limited by uncertainty about the taxonomic relationships of some of the biota that they discuss. Inaccurate assessments of the degree of genetic separation between groups results in speculative accounts of dispersal routes (for example Hardy 1977), or biological scenarios in conflict with geological evidence (see Cooper 1989).

The tendency to create "narratives" (Craw 1988) rather than testable hypotheses is also evident in other discussions about the evolution of New Zealand's biota . ~~The role of hybridization~~ For example, ~~A~~ a notable feature of many New Zealand plant groups is their readiness to hybridize (see Allan 1961, Fisher 1965, Carlquist 1974). Rattenbury (1962) and Raven & Raven (1976) suggested that hybridization has played a major role in the evolution of New Zealand plants, but the true extent of hybridization has often been poorly investigated (Connor 1985). Failure to recognize that many of the hybrids occur in disturbed habitats may also have overemphasized the role of hybridization in New Zealand (Hair 1966). Wardle (1988, and Wardle *et al.* 1988) attempted to explain some disjunct plant distributions in terms of long distance pollen dispersal, interspecific hybridization and subsequent environmental selection to reconstitute a parental genotype. Genetic investigations to determine the taxonomic status of such putative hybrids have not yet been conducted, so proposals involving ill defined selection processes are not warranted at this stage. Similar uncertainty over taxonomy is also evident in discussions of the evolution of some animal groups, such as land snails (Solem *et al.* 1981, Climo 1989).

Assessment of hypotheses about distributions and speciation requires information on genealogical relationships. Taxonomic investigations in New Zealand have relied primarily upon morphological comparisons, and as already noted, have not always adequately resolved relationships. The first priority for evolutionary studies then, is to reliably determine phylogenies.

Assessing Taxonomic Relationships

Morphological variation is at the core of the theory of descent with modification by means of natural selection (Wallace 1858, Darwin 1859, 1875, Mayr 1942, 1963, Stebbins 1950). Morphological characters however, being only indirect manifestations of the genotype, can be uninformative or misleading about the closeness of evolutionary relationships (for example, King & Wilson 1975, Systma & Gottlieb 1986, Fitch & Atchley 1987, Wayne *et al.* 1989, Daugherty *et al.* 1990a, 1990b). Biochemical and cytological investigations of taxonomy are an essential complement to morphological studies (Hillis 1987, Patterson 1987, Hillis & Moritz 1990). These techniques are described below, and examples of their applications in New Zealand noted.

Secondary Metabolites

Analysis of secondary metabolites have been used for taxonomic purposes and provide an interesting adjunct to other phylogenetic methods (Cronquist 1980, Wright 1980). Chemotaxonomy has been used to study several New Zealand plant groups (for example, Taylor 1964, Markham & Godley 1972, Wilson 1984, see also Connor 1985), but genetic methods are required to resolve their relationships.

Immunological Techniques

Immunological affinities (Champion *et al.* 1974, Maxson *et al.* 1990), and DNA-DNA hybridization (Sibley & Ahlquist 1987), are useful for establishing taxonomic affinities, particularly between more distantly related groups. They provide little insight however into the genetical processes at work within populations. The relationships of New Zealand *Leiopelma* frogs (Daugherty *et al.* 1981) and the short-tailed bat (*Mystacina tuberculata*; Pierson *et al.* 1986) have been investigated immunologically. DNA-DNA hybridization studies have been conducted for ratites and a few New Zealand passerines (Sibley & Ahlquist 1981, 1987, Sibley *et al.* 1982).

Chromosomal Studies

Examination of chromosomal organisation can be useful both for systematics and for inferences about processes (Dobzhansky 1941, White 1978, Grant 1981, Carson 1983, Moritz 1986, Systma 1990), but it is difficult to prove that such changes are causally related to the formation of new species (Bush *et al.* 1977, White 1978) rather than being subsequent effects of isolation (see Endler 1986). Chromosomal atlases for many New Zealand plant species have been compiled (see Frankel & Hair 1937, Hair 1966, 1977, Rendle & Murray 1989), but the range and patterns of chromosomal changes have not been extensively examined. Hair (1966) noted that chromosomal instability was associated with advancing fronts of podocarp dispersal but this has not been investigated closely. Chromosomal studies of blackflies (McLea & Lambert 1985) and *Leiopelma* frogs (Green 1988, Green & Sharbel 1988), and preliminary descriptions of skink chromosomes (Hardy 1977) have been published, while karyological investigations of weta and other invertebrates are underway (M. Richards pers. comm.).

Allozyme Studies

Enzyme electrophoresis is also applicable to studies of evolutionary patterns and processes. The potential resolving power of a well conducted allozyme survey can exceed both restriction mapping and nucleotide sequencing studies if 30 or so loci are examined (Nei 1987). The relative simplicity, speed, cost effectiveness and the number and range of nuclear loci which can be surveyed by protein electrophoresis give it great practical value (Powell 1975, Buth 1984, Murphy *et al.* 1990). The extent of genetic variability revealed by this technique however depends upon assay conditions, such as temperature, pH and buffer solutions (see Nei 1987). Allozyme surveys can be used for both phylogeny reconstructions and for analyses of population structuring and dynamics (Powell 1975, Spieth 1975, Gottlieb 1984, Murphy *et al.* 1990). Analysis of electrophoretic data for phylogeny reconstruction can present problems though because of the uncertainty over whether to use similarity or distance information, and whether to code the data in terms of presence or absence of alleles or in terms of allele frequencies (Buth 1984, Swofford & Olsen 1990). Assumptions used in calculations of genetic distance estimates from allozyme data can also be unrealistic and lead to inaccurate views of relationships (Hillis 1984, Swofford & Olsen 1990).

In New Zealand the taxonomic status of molluscs (Phillips & Lambert 1990), freshwater fish (Allibone 1990), *Leiopelma* frogs (Daugherty *et al.* 1981, Green *et al.* 1989), skinks (Vos 1988, Daugherty *et al.* 1990b, C.H. Daugherty & G.B. Patterson pers. comm.), tuatara populations (Daugherty *et al.* 1990a), and ducks (Hitchmough *et al.* 1990), have been investigated with allozymes. Relationships of New Zealand weta (M. Richards pers. comm.) and geckos are also being studied (R. Hitchmough pers. comm.). The techniques have also been used to assess the genetic diversity of molluscs (Freeth & Sin 1986, Smith *et al.* 1989), marine fish stocks (e.g., Smith & Johnston 1985), introduced birds (Ross 1983, Baker & Mooed 1987, Baker 1992), the kakapo (Triggs *et al.* 1989), possum (Triggs & Green 1989), rimu (*Dacrydium cupressinum*; Hawkins & Sweet 1989) and beech (*Nothofagus* species; Wilcox & Ledgard 1983, Haase 1992).

DNA Fragments

Restriction mapping of nuclear or organelle DNA had a central role in the development of molecular systematics (Wilson *et al.* 1985, 1989, Nei 1987). Specific pieces of nuclear DNA can be investigated (for example, Hillis & Davis 1987, Crowhurst *et al.* 1990), but it is often easier and more informative to isolate and use cytoplasmic genomes. Phylogenetic inferences from cytoplasmic DNA can be made by simply noting the occurrence and sizes of fragments cut by a range of enzymes, but more accurate reconstructions are obtained by mapping the restriction endonuclease sites (Wilson *et al.* 1985, Crozier 1990). Restriction analyses require relatively large amounts of DNA, and can have limited resolving power, particularly for more distant divergences (Wilson *et al.* 1989, Hillis & Moritz 1990). Recent developments for rapidly obtaining and sequencing specific DNA sequences (see below) are resulting in a decline in use of restriction analyses for phylogenetic studies.

DNA Sequences

Information from protein or nucleotide sequences has been extensively utilized to study taxonomic relationships over both vast tracts of evolutionary time (for example, Goodman *et al.* 1987, Gray 1989, Lake 1990, Martin *et al.* 1993) and much briefer periods (for example, Hahn *et al.* 1986, Koop *et al.* 1989, Henderson *et al.* 1989, Vigilant *et al.* 1990, Miyamoto & Goodman 1990). The availability of sequence data permits direct examination of the the basis of genetic variation, and the widespread application of sequencing has led to a renaissance in systematics (Patterson 1987 & 1990).

The effort and expense involved in 'conventional' approaches to isolating and sequencing genes (Sambrook *et al.* 1989) prohibited their application to population studies, although some attempts have been made (e.g., Hahn *et al.* 1986). The development of the polymerase chain reaction ("PCR", Saiki *et al.* 1988, Arnheim & Erlich 1992) however now provides a system which can bring nucleotide sequencing to a level matching or exceeding the utility of allozymes in terms of ease, speed, and sample handling (Gyllensten & Erlich 1988, Kocher & White 1989, White *et al.* 1989, Arnheim *et al.* 1990). The procedure (see Chapter Two) is elegantly simple (in theory), allowing specific regions of DNA to be isolated and sequenced within hours, and from organisms for which no detailed genetic information is available. Consequently it is being rapidly employed for both taxonomic investigations and assessment of sequence diversity (for example, Kocher & White 1989, Kocher *et al.* 1989, White *et al.* 1989, Arnheim *et al.* 1990, Simon *et al.* 1991), and it is the method used in this study.

Mitochondrial DNA and Evolution

The mitochondrial DNA (mtDNA) of animals in particular has several features which facilitate analysis of evolutionary divergence over periods of time ranging from one generation to about 100 million years (Moritz *et al.* 1987, Harrison 1989, Kocher *et al.* 1989). It is, firstly, the epitome of economy - small and simple. Only 14 to 39 thousand nucleotide pairs ("kbp") in size, it contains information for 22 transfer RNAs, two ribosomal RNAs and about 13 open reading frames. In addition it lacks intervening sequences, and recombination appears to be infrequent (Brown 1983, Moritz *et al.* 1987). Insertion and deletion events tend to occur in the control region (Moritz *et al.* 1987). Gene order can vary among invertebrates (Cantatore *et al.* 1987, Thomas *et al.* 1989), and among vertebrate groups (Desjardins & Morais 1990, Moritz 1991, Pääbo *et al.* 1991). The only region of non-coding DNA is the control region or D-loop (D for displacement), around the site of initiation of mtDNA replication. Parts of this region are the most rapidly changing in the mitochondrial genome, though conserved domains occur within it, and consequently it is used for examination of intra- and inter-population diversity (Cann *et al.* 1984, Vigilant *et al.* 1991, Wilkinson & Chapman 1991, Ward *et al.* 1991). The protein-encoding and ribosomal RNA mitochondrial genes are being used to study older divergences (for example, Irwin *et al.* 1991, Simon *et al.* 1991, Smith & Patton 1991, Ballard *et al.* 1992, Cooper *et al.* 1992, Hickson *et al.* 1992, Pashley & Ke 1992).

The predominance of maternal mitochondrial inheritance, absence of recombination and the apparent rarity of different types of mtDNA within an organism (but see Bermingham *et al.* 1986, Gyllensten *et al.* 1991, Hoeh *et al.* 1991) make analyses simpler than for nuclear loci (Brown 1983, Wilson *et al.* 1985, Moritz *et al.* 1987). The relatively rapid rate of sequence evolution of animal mtDNA in comparison to nuclear rates is also advantageous when comparing closely related groups. In some vertebrates the rate of mitochondrial genome evolution can exceed that of single-copy nuclear DNA by up to ten-fold (Brown *et al.* 1979, 1982, Wilson *et al.* 1985), but this is not universal (Britten 1986, Vawter & Brown 1986, Thomas & Beckenbach 1989, Goddard *et al.* 1990, Palmer 1990).

Rates of Evolution in Cytoplasmic Organelles

Reasons for variation in the rate and types of changes in mtDNA are unknown, but such variability implies that the small mitochondrial genome size in itself is insufficient to explain rapid rates of change. Chloroplast genomes are also relatively small but change at a slower rate than animal mtDNA (Palmer 1990). Other possible causes for a fast rate of evolution, such as reduced replicative fidelity and translational constraints, and the absence of recombination (Cann *et al.* 1984, Wilson *et al.* 1985, Clayton 1982), have not been investigated in detail.

Cytoplasmic genomes of plants and fungi are larger and more complex than animal mtDNA and do not exhibit such rapid sequence changes (although fungi are poorly studied; Gray 1989). Nor do they appear to have the large bias (10:1) in transitions (changes between nucleotides of the same class, for example adenine to guanine) over transversions (for example adenine to cytosine) found in some animal mtDNA (Brown 1983, Palmer 1990). Chloroplast DNA and fungal mtDNA can however be used for population comparisons (Taylor 1986, Soltis *et al.* 1989, Clegg & Durbin 1990, Palmer 1990). Plant mitochondrial genomes can be both very large (sizes up to 25 000 kbp have been reported) and can undergo rapid and extensive organisational rearrangements without accompanying sequence alteration (Palmer & Herbon 1988). One reason for the latter may be due to the creation of a panmictic mtDNA population by fusion of mitochondria within cells with the consequent reduced probability of fixation of mutations (Lonsdale *et al.* 1988). Paternal transmittance of mitochondria and chloroplasts can occur (for example, Harrison & Doyle 1990, Kondo *et al.* 1990, Gyllensten *et al.* 1991), but maternal inheritance predominates (Wilson *et al.* 1985).

Maternal inheritance and rapid rates of change in animal mtDNA make it possible to investigate gene evolution over relatively short periods of time, as well as permit examination of geographic structuring and gene flow in populations (reviewed by Wilson *et al.* 1985, Avise *et al.* 1987, Avise 1989a, 1991, Harrison 1989, Slatkin 1989). The haploid state and uniparental acquisition of mtDNA also make it more sensitive than nuclear DNA to changes in population size, so low mtDNA variability can be indicative of past reductions in population size (Wilson *et al.* 1985). Examination of more distant relationships using mtDNA sequences include studies by Ballard *et al.* (1992), Meyer & Dolven (1992), Irwin *et al.* (1991), and Allard *et al.* (1992). Several molecular systematic studies are now underway in New Zealand. Published reports are available for ratites (Cooper *et al.* 1992) and skinks (Hickson *et al.* 1992). DNA fingerprinting of sea bird populations have also been initiated (Millar *et al.* 1992) and satellite DNA has been examined in the native frog *Leiopelma hochstetteri* (Zeyl & Green 1992).

Limitations of Sequence Data

Analysis of mtDNA sequences is a convenient starting point to investigate relationships among animals, but it should be borne in mind that the uniparental inheritance of mtDNA means that only the evolutionary history of the maternal line (and only a small part of that) can be examined. Reliance on single genes can be misleading (Penny *et al.* 1982, Wilson *et al.* 1987, Wyss *et al.* 1987). Examination of short regions of DNA also reflects the evolutionary history of that gene rather than the organism (Nei 1987, Pamilo & Nei 1988, Martin *et al.* 1990). Confidence in a phylogeny requires congruence from other data sets, such as morphology and allozymes (Hillis *et al.* 1987, Patterson 1987, Hillis & Moritz 1990). Sequence information from several thousand base pairs and from unlinked loci is also preferred (Saitou & Nei 1986, Nei 1987, Martin *et al.* 1990), but sequencing studies employing the PCR usually concentrate on sequences under one kilobase pairs in length (Kocher *et al.* 1989).

Study of nuclear sequences with PCR may require separation of allelic variants by denaturing gel electrophoresis (Sheffield *et al.* 1990), use of allele specific primers (Gyllensten & Erlich 1988), or "single molecule PCR" (Jeffreys *et al.* 1990) but are technically more demanding. Use of nuclear ribosomal sequences (Hillis & Dixon 1991) and intron sequences (Palumbi & Baker submitted) are proving useful however for examination of older and more recent diveregences, respectively.

Generating sequence data is now becoming routine. The primary difficulties lie in data analysis. Most phylogenetic trees derived from sequence data are probably incorrect (Penny *et al.* 1990, Rohlf *et al.* 1990), since no current algorithm meets all the necessary criteria of being fast, efficient, consistent, robust, and falsifiable (Henderson *et al.* 1989, Penny *et al.* 1990). Reviews of phylogeny reconstruction generally recognise the limitations, though users of the programs may not (Felsenstein 1988, Swofford & Olsen 1990, Cracraft & Helm-Bychowski 1991, Nei 1991, Penny *et al.* 1992, Stewart 1993). Phylogenetic trees should be regarded as hypotheses and subject to error. Statistical analyses are being developed to assess the reliability of phylogenies (Li & Gouy 1991). The emphasis of this thesis is on what information can be extracted from DNA sequences, and confidence in relationships is obtained by examination of conflicting associations of taxa using newly developed sequence analysis algortithms (Penny *et al.* 1992, 1993). This is discussed in more detail in Chapter Five.

Rates of Sequence Evolution

While patterns of evolution may be intrinsically interesting, understanding evolutionary processes also requires a temporal framework. The apparent regularity in the accumulation of amino acid and nucleotide substitutions led to the suggestion that evolutionary time can be measured from sequences (Zuckerkandl & Pauling 1965, Wilson *et al.* 1977 & 1987, see also Ingram 1961). There is a growing awareness however that single genes or proteins may not be reliable as markers of time (see Wilson *et al.* 1987, Easteal 1990), that rates are lineage dependent (Thorpe 1982, Goddard *et al.* 1990, Palmer 1990), and that models used to describe the process of substitution rates require more investigation (Gillespie 1986, Wilson *et al.* 1987). Caution must be used if rate estimates are extrapolated from one group, such as mammalian mtDNA, to another (see Wilson *et al.* 1985, 1987).

Skinks as a Model Group for Evolutionary Studies

Integrated approaches are essential to an understanding of evolutionary patterns and processes. One emphasis of this thesis is on developing a "model" system with which to investigate in detail the workings of evolution, as has been done for *Drosophila* (Lewontin *et al.* 1981, DeSalle & Hunt 1987), and Galapágos' finches (Grant & Grant 1989).

Regrettably few studies have taken a comprehensive view of evolution by utilizing ecological, morphological, biochemical and genetic data for a specific group (but see Larson 1984, DeSalle & Hunt 1987, Sytsma 1990). As well as enhancing our understanding of how organisms and genes evolve, this approach also has the advantage that congruence of diverse data sets encourages confidence in phylogenetic conclusions, while inconsistencies can identify false assumptions and limitations in models or data (Hillis 1987, Patterson 1987, Hillis & Moritz 1990, Sytsma 1990). As discussed above, no comprehensive evolutionary investigations of New Zealand taxa have been undertaken. New Zealand skinks are one of many groups which offer promise however (see also Fisher 1965, Raven 1988). Extensive morphological (McCann 1955, Hardy 1977) and allozyme studies of skink populations (Vos 1988, Daugherty *et al.* 1990b, C.H. Daugherty & G.B. Patterson pers. comm.) have provided a framework upon which to develop detailed evolutionary investigations.

Information on the basic biology (Barwick 1959, Towns 1975a,b) and ecology (Barwick 1959, Whitaker 1968, Towns 1975a, Gill 1976, Patterson 1985, Porter 1987), as well as some physiological data (Morris 1971, Pollock & MacAvoy 1978, Werner & Whitaker 1978, Evetts & Grimmond 1982) is also available for various species of skinks.

Views on the Origin of New Zealand Lizards

New Zealand lizards (and the tuatara) trace their ancestry, in Maori mythology, back to Tangaroa, God of the Oceans (Andersen 1969). Seeking to escape the avenging wrath of Tawhiri-matea (Father of winds & storms) for the separation of Rangi (Earth) and Papa (Sky), Tu-te-wehiwehi and his descendants fled from the sea to the land adopting lizard-like forms.

Three terrestrial groups of reptiles now survive here. The ancient reptilian order Sphenodontidae has two surviving *Sphenodon* species, both endemic to New Zealand (*S. punctatus* and *S. guntheri*, Daugherty *et al.* 1990a). Tuatara ecology and reproductive biology has been extensively studied (Crook 1975, Bell *et al.* 1985, Cree *et al.* 1992). Geckos and skinks are cosmopolitan families, each comprising about 600 species, in approximately 82 and 40 genera respectively (Cogger & Heatwole 1981). On the basis of morphology, 18 species of gecko, in three endemic genera, are currently recognised in New Zealand (Towns 1985), but the validity of one genus is uncertain (Thomas 1982a). The New Zealand skinks pose a more difficult taxonomic problem because of their limited morphological diversification. Two species were originally described by J. E. Gray but more were recognised (see Dieffenbach 1843), and numbers have been continually revised upwards (McCann 1955, Hardy 1977). Two genera are currently described here, *Leiolopisma* Dumeril & Bibron and *Cyclodina* Girard, which currently contain 20 and six described species respectively (Towns 1985, Daugherty & Patterson 1990), but allozyme studies indicate that these are still underestimates (C.H. Daugherty pers. comm.). *Leiolopisma* skinks are generally diurnal insectivores found in grassland or coastal habitats. *Cyclodina* skinks tend to be slightly larger, nocturnal, forest dwellers, and are now found primarily on offshore islands in the Northern parts of New Zealand (Bull & Whitaker 1975, Pickard & Towns 1988). Rats, cats and collectors have considerably reduced lizard numbers in New Zealand (Whitaker 1978, Thomas 1982b).

Our terrestrial reptilian fauna lacks a fossil record (Bull & Whitaker 1975) so there is uncertainty about the origins of New Zealand lizards. Tuatara seem to be long term residents, dating back about 80 million years when Gondwanaland was unfragmented (Crook 1975). Geckos and skinks have been suggested to be more recent colonists with affinities to New Caledonian and Australian groups (Bull & Whitaker 1975, Hardy 1977, Towns *et al.* 1985). Morphological assessments suggest that New Zealand geckos have closest affinities to species from New Caledonia, and could have arrived here during the Miocene, up to 30 MYA (Kluge 1967). Members of the genus *Cyclodina* are known only from New Zealand but some morphological and karyotypic features suggest closer relationships to New Caledonian and Australian species than to New Zealand *Leiolopisma* (Hardy 1977, 1979), and Hardy (1977) proposed that *Cyclodina* may have arrived here independently, possibly during the early Pleistocene (up to 2.5 MYA). *Cyclodina* has morphological similarities to the Lord Howe skink, *L. lichenigerum* (Bull & Whitaker 1975), and to *L. alazon* from Fiji (Zug 1985). Interpretation of allozyme data (Vos 1988, C.H. Daugherty & G.B. Patterson pers. comm.) indicates that *Cyclodina* form a separate group, distinct from the New Zealand *Leiolopisma*.

Ecological Aspects of Some *Leiolopisma*

The widespread distribution and ecological flexibility of some New Zealand *Leiolopisma* is an interesting and important feature. Allozyme studies (Towns *et al.* 1985, Patterson & Daugherty 1990) corroborated ecological information (Patterson 1985) which suggested that *L. nigriplantare* is a species complex which cannot be adequately resolved by anatomical characters. One subspecies, *L. n. polychroma*, is found in the lower half of the North Island and over many areas of the South Island, encompassing approximately six degrees of latitude and an altitudinal range of 1700 metres (Pickard &

Towns 1988). A morphologically distinct subspecies, *L. n. nigriplantare*, occurs on some of the Chatham Islands, 900 km east of the South Island, but it's allozyme profile is very similar to the mainland subspecies (Daugherty *et al.* 1990b).

L. n. polychroma is apparently closely related to *L. notosaurus* from Stewart Island and to two other southern South Island species (*L. maccanni*, *L. inconspicuum*; Patterson & Daugherty 1990). It occurs sympatrically at several sites with these latter two but all are ecologically distinct (Patterson 1985) and apparently reproductively isolated from each other (Daugherty *et al.* 1990b). The sympatric species can be distinguished on the basis of colour patterns ("striped", "spotted", and "speckled"), though this is not always an infallible guide since the colour patterns are associated more with habitat type (for example "striped" is common in tussock habitats) than species (as identified on the basis of allozyme profiles; Patterson 1985, Daugherty *et al.* 1990b).

Genetical Investigations of *Leiolopisma*

The genus *Leiolopisma* is a particularly complex group however, and taxonomic relationships of members of the genus are uncertain. Preliminary immunological studies of albumin (Baverstock & Donnellan 1990, Hutchinson *et al.* 1990) support the view (Greer 1974 & 1982) that the genus is polyphyletic, some members being more closely related to other genera. Hutchinson *et al.* (1990) recommended restricting *Leiolopisma* to the type species *L. telfairi* from Mauritius, and they resurrected or created several generic epithets to accommodate the Australian species. Insufficient work has been done to clarify the affinities of New Zealand and New Caledonian species, but initial comparisons (Hutchinson *et al.* 1990) indicate that New Zealand and New Caledonian species are related to, but immunologically quite distinct from, the Australian taxa. A new genus for the New Zealand *Leiolopisma* is currently under review (C.H. Daugherty pers. comm.), but the term *Leiolopisma* is retained in this thesis for the New Zealand species as well as for other taxa which are or have been included in the genus.

Members of the *Leiolopisma* group occur in New Zealand, Australia. Lord Howe Island, New Caledonia, Fiji, and also Mauritius in the Indian ocean. Morphologically, New Zealand *Leiolopisma* have affinities to some New Caledonian and Australian species, and the degree of similarity between them was used to suggest a 'recent' arrival here, within the last 5 million years (Towns 1975a, Hardy 1977). This would have necessitated crossing hundreds of kilometers of open ocean. The Australian *L. delicata*, utilizing modern forms of transportation, became established here during the 1960's (Robb 1980).

The biochemical data (Baverstock & Donnellan 1990, Hutchinson *et al.* 1990, Daugherty *et al.* 1990b, C.H. Daugherty & G.B. Patterson pers. comm.) make it unlikely that the initial *Leiolopisma* colonisation(s) of New Zealand occurred as recently as the Pliocene (as suggested by Towns 1975a and Hardy 1977), but the data is either insufficient or inadequate (see Avise & Aquadro 1982, Nei 1987 and Chapter Five) to determine precisely how long skinks have been here. A more detailed view of

Leiolopisma evolution based on DNA sequences is presented in this thesis. Similar analyses of *Cyclodina* are being conducted by others.

A region of the mitochondrial 12S (small subunit) ribosomal RNA gene is examined using PCR and direct DNA sequencing (described in Chapter Two). No DNA sequence information was available for skinks before this study commenced so the 12S rRNA gene was selected for initial investigations since it is well conserved among vertebrates (Kocher *et al.* 1989, Palumbi *et al.* 1991). The 12S rRNA gene has been used for evolutionary investigations of other animal groups (Hixson & Brown 1986, Hedges *et al.* 1991, Simon *et al.* 1991, Allard *et al.* 1992, Ballard *et al.* 1992, Cooper *et al.* 1992, Meyer & Dolven 1992). The skink sequences are analyzed with respect to a secondary structure model developed in Chapter Three. However, the relationships of the *Leiolopisma* skinks based on the sequence information are unclear, contrasting with the results from allozyme analyses (Chapters Five and Six). The patterns of DNA sequence differences are interpreted as suggesting a period of rapid *Leiolopisma* diversification and hypotheses to account for this are presented in Chapter Five.

An important aspect of this study is that individuals of known allozyme profile were used, so DNA sequence and allozyme information from the same individuals was available. An advantage of this is illustrated in Chapter Six where comparison of the allozyme and sequence information leads to the suggestion that sexual hybridization has occurred at one site between two sympatric species, *L. n. polychroma* and *L. maccanni.*

The evolutionary histories of the New Zealand skinks are not resolved and the DNA sequence information is suggestive of a complex set of relationships. Continued molecular investigations of this group are advocated, not only to aid in resolution of their phylogenetic relationships, but also to build upon and extend existing studies of *Leiolopisma* to explore broader ecological and evolutionary questions. Avenues for such research are discussed in the final chapter.

Chapter Two: Methods

This thesis uses the polymerase chain reaction (PCR) to obtain specific regions of the mitochondrial genome from frozen skink tail muscle. The sequences of these pieces of DNA are then determined and analyzed to make inferences about the evolutionary relationships between the skinks. While the principle of PCR is elegantly simple (Saiki *et al.* 1988, Arnheim *et al.* 1990), there can be several difficulties in it's implementation. The sensitivity of PCR and consequent possibility of amplifying the wrong DNA make it necessary to check the accuracy and identity of the DNA sequences. The procedures used to extract amplify and sequence skink mitochondrial 12S rRNA sequences are described in this chapter, along with steps taken to insure the correct identity and accuracy of the sequences. A preliminary account of the methods was published in Hickson *et al.* (1992).

The Polymerase Chain Reaction

The polymerase chain reaction (PCR) is a simple and rapid means of amplifying specific regions of DNA from tissues, cells or other sources. Its versatility, the detailed information which can be obtained, as well as the relatively low cost make it not only suitable, but essential, for the investigation of a variety of problems in population and evolutionary biology (Arnheim *et al.* 1990). The essence of the procedure is to have two short pieces (about 20 to 30 nucleotides long) of single-stranded DNA - the **primers**. These need sufficient sequence specificity to bind to the DNA bracketing the region of interest; one primer for each of the complementary DNA strands. The first step in the reaction is a high temperature (usually between 90 and 94°C) denaturation to separate the DNA strands, thus allowing the primers access to their complementary region(s). Rapid lowering of the temperature (to between 37 and 60°C) in the second stage permits the primers to bind with the complementary sequence. In the presence of the four nucleotide triphosphates the region of DNA between the pair of primers can be copied using a heat stable DNA polymerase, such as *Taq* polymerase (Saiki *et al.* 1988). These three steps are repeated sequentially 20-40 times. The amount of primers in the reaction is in sufficient excess so that the process is exponential and able to produce a million or more copies of the desired region in a few hours.

Having appropriate DNA primers is critical to the success of the PCR. This information is obtained either by conventionally cloning and sequencing the region of interest, or by using information held in DNA sequence databases such as Genbank and EMBL. Relatively highly conserved regions of DNA - such as the 12S ribosomal RNA encoding gene in animal mitochondria - can be isolated from a wide range of organisms using "universal primers" (e.g. Kocher *et al.* 1989), but faster evolving regions of DNA require more taxon-specific sequence data. This can pose difficulties for groups that have been less well characterized at the molecular level; for instance reptiles, invertebrates, and many plant groups.

A difficulty of PCR is that its very power means that even slight contamination from other tissue or DNA can lead to the amplification of the wrong sample. Extreme care must therefore be taken to ensure that the correct sample of interest is amplified. A further difficulty was that no other reptilian sequences were available to compare our gene sequences with. Considerable effort therefore was made in this study to ensure that we were obtaining skink gene sequences.

Mitochondrial DNA Isolation

To ensure that the primers amplified the correct mitochondrial region intact mitochondria were isolated and then the mtDNA purified. Laboratory rats were used as a test of the methods, then mitochondria were prepared from individual *L. n. polychroma*. Mitochondria were prepared following the procedure of Fleischer *et al.* (1979). Livers were removed from freshly killed animals, washed in ice cold 0.3 M sucrose, cut into small pieces, washed again, and ground in a glass tissue grinder in ice cold 0.3 M sucrose. Nuclear debris was removed by a low speed spin of 755 x **g** for 5 minutes at 0°C. The supernatant was respun at 6800 x **g** for 15 min., 0°C, and the brown mitochondrial pellet removed with a sterile glass rod, leaving behind the black nuclear debris. Mitochondria were resuspended in cold 0.3 M sucrose, and the purification repeated. A final spin at 7700 x **g** gave a pellet which subsequently proved to be largely free of nuclear DNA. This pellet was resuspended in cold TES buffer (10 mM Tris, pH 8, 1 mM EDTA, 100 mM NaCl). Confirmation that mitochondria had been isolated was obtained by measuring respiratory activity using an oxygen electrode with sodium glutamate as the substrate (Lessler & Brierly 1969). The remaining suspensions were frozen (-20°C) as 250 μl aliquots.

Mitochondrial DNA was prepared from these aliquots by addition of sodium dodecyl sulphate (SDS) and proteinase K to final concentrations of 1% and 50 μg/ml, respectively, and incubating at 37°C for 1 hour. RNA was removed by a further 10 minute incubation with 4 μg/ml of RNase A. The solution was then sequentially extracted with, respectively, one volume each of phenol, phenol and chloroform, and finally chloroform, to remove proteinaceous material. The supernatant was precipitated with 95% ethanol and 0.3 M sodium acetate and resuspended in 25 μl sterile water.

Total DNA Isolation

If the PCR primers are specific enough for their target and do not cross react with other regions of the genome then highly purified DNA solutions however are not required for the PCR, it is simpler to obtain preparations of total DNA (containing both nuclear and mitochondrial DNA). For the main part of the study total DNA was extracted from frozen tail muscle tissue, obtained from the reptile collection of the National Museum in Wellington (Table 2.1). These were some of the same specimens used by Daugherty *et al.* (1990b; C.H. Daugherty & G.B. Patterson pers. comm.) for their allozyme studies.

In addition, isolation of DNA from tissues preserved in other ways was also investigated. Extractions of DNA were performed on fresh tail muscle and tails preserved by desiccation with silica gel, storage in 95% ethanol, or fixing in formalin.

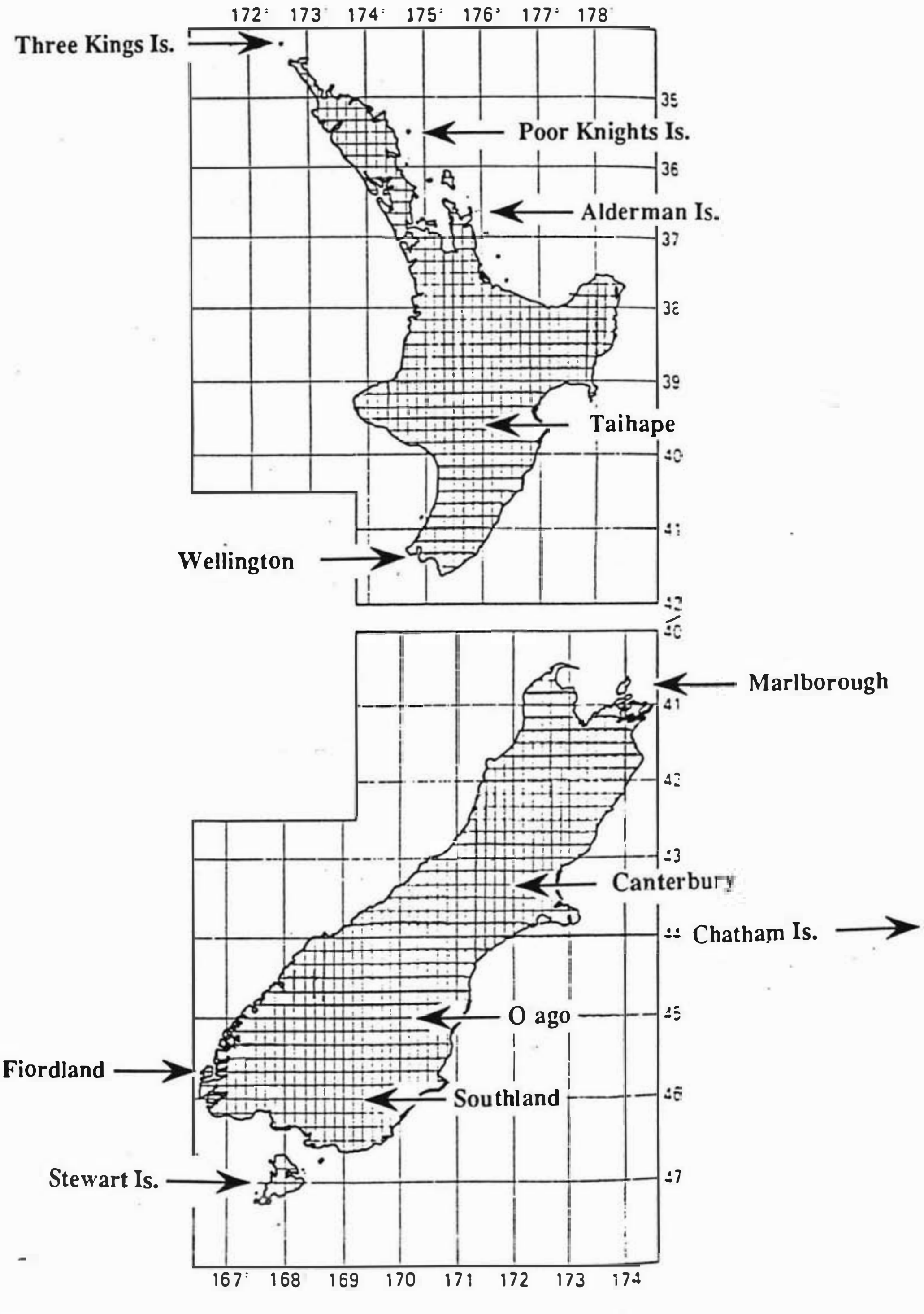
Table 2.1. Skink taxa from which DNA sequence information was obtained. Species, locality, and National Museum of New Zealand reptile collection catalogue numbers are given. The three letter abbreviation for each taxon, which are used in later tables, are shown in parentheses. Figure 2.1 shows the New Zealand localities.

Taxon	Locality[a]	Cat. No.
"Stewart Island Green"[1] (SIG)	Table Hill, St. Is.	FT6
Leiolopisma grande (Lgr)	Central Otago	CD1055
L. notosaurus (Lno)	Masons Bay, St. Is.	CD1089
L. lineoocellatum/chloronotum[2] (Llc)	Tekapo, Otago	CD1217, CD1218
L. suteri (Lsu)	Aorangi, Poor Knights Is.	CD1027
L. nigriplantare nigriplantare(Lnn)	Chatham Islands	CD1058, CD1060
L. n. polychroma (Lnp)	Gorge Burn, Southland	CD1110-1112
	Twizel, Canterbury	CD2126
L. microlepis (Lmi)	Taihape	CD2123
Cyclodina aenea (Cae)	Somes Is., Wellington	CD1962
L. acrinasum (Lac)	Fiordland	CD826
L. inconspicuum (Lin)	Gorge Burn, Southland	CD1100, CD1101
L. smithi (Lsm)	Ruamahua-iti, Alderman Is.	FT569
L. zelandicum (Lze)	Outer Chetwode Is., Marlb.	CD1952
L. fallai (Lfa)	Great Island, Three Kings	FT598
L. maccanni (Lma)	Gorge Burn, Southland	CD1106-CD1108
L. infrapunctatum (Lfr)	Stephens Is., Marlb.	CD535
L. otagense (Lot)	Central Otago	CD1053
L. moco (Lmo)	Poor Knights Is.	CD848, CD1031
L. telfairi (Lte)	Round Is., MAURITIUS	CD2021
Lampropholis guichenoti (Lag)	AUSTRALIA	CD536

[a] Locality abbreviations: St. Is. = Stewart Island, Marlb. = Marlborough Sounds, Three Kings = Three Kings Island.

[1] Stewart Island Green has not yet been formally described.
[2] *L. lineoocellatum/chloronotum*; these individuals could not be identified definitively on the basis of morphology and allozymes as either *L. lineoocellatum* or *L. chloronotum* (C.H. Daugherty pers. comm.).

Fig. 2.1. Map of New Zealand showing locations of the skinks examined in this thesis. Latitude and longitude are indicated along the edges of the map.



Small samples (about 0.05 g) of muscle were removed from near the base of the tail, cut into small pieces and incubated in 300 µl of extraction buffer (10 mM Tris pH 7.5, 100 mM EDTA, 50 µg/ml proteinase K, and 1% SDS) at 65°C for two to four hours (Sambrook *et al.* 1989). The solution was then extracted with phenol and chloroform, precipitated, and resuspended in 25 µl of sterile milliQ water.

Amplification of DNA by the Polymerase Chain Reaction.
The primers found to work best with skink DNA were:

12SAR `5'-AAACTGGGAT TAGATACCCC ACTAT-3' (L1091)` and
12SBR `5'-GAGGGTGACG GGCGGTGTGT-3' (H1478),`

where L and H signify the light and heavy strands of the mitochondrial DNA (mtDNA) and the numbers refer to the 3' ends of the primers according to the complete human mtDNA sequence (Anderson *et al.* 1981). These primers isolate an approximately 400 nucleotide long region in the second half of the gene and have been used to amplify 12S rRNA from a variety of vertebrate and invertebrate groups (Palumbi *et al.* 1991)

DNA amplifications were performed in 0.5 ml reaction tubes using a DNA thermal cycler (Perkin Elmer Cetus, Connecticut, USA). Initial trials were carried out in 20 µl volumes, while 80 µl was used for the production of DNA templates for sequencing. Reactions contained 200 µM of each deoxynucleotide, reaction buffer (Promega Corporation, Madison, USA; 50 mM KCl, 10 mM Tris-HCl, pH 8.8, 1.5 mM $MgCl_2$, 0.1% Triton X-100), 0.1 µM of each primer, and 2 units of *Taq* polymerase (Promega). About 100 ng of pure mitochondrial DNA or 100 to 200 ng of total DNA were used as the template for polymerase chain reactions. Samples were overlain with a drop of mineral oil to prevent evaporation.

Several factors influence the success of the PCR (including the quality and quantity of the DNA template, primer sequence and concentration, magnesium concentration, the quality of the DNA polymerase, and the type of thermal cycler used). Various temperature and time settings need to be tried to obtain optimal specificity and amplification of the desired product (Innis & Gelfand 1990). We tried a variety of conditions and found that good amplifications for the 12S primers and skink DNA were obtained using the following parameters:

> 94°C for 60 sec to separate DNA strands,
> 54°C for 60 sec to allow primer annealing, and
> 72°C for 60 sec for DNA copying.

Successful DNA sequencing requires good quality templates. Direct precipitation of the PCR reaction, either with or without phenol extraction, did not give clear sequencing gels. Initially, good sequences were obtained by purifying PCR products through a 1% Seaplaque agarose gel (FMC BioProducts, USA) prior to sequencing. Isolation of the product from the agarose used the hot phenol method of Thuring *et al.* (1975). After precipitation, DNA fragments were resuspended in 11 µl of sterile milliQ water. The availability of inexpensive centrifugal dialysis filters to remove excess primer DNA, unincorporated dNTPs, and other inhibitors from the PCR reaction proved more efficient and cost effective however. Most of the samples were processed using the Promega Magic PCR Preps^{TM} DNA purification system (Promega Corporation, Madison, Wisconsin, USA).

Mineral oil from the PCR reaction was removed by addition of approximately 30 µl chloroform and brief centrifugation. The PCR sample (approximately 70 µl) was mixed with 100 µl of direct purification buffer. This was mixed for one minute with 1 ml of DNA purification resin and then passed through the Magic PCR Preps^{TM} mini-column using a syringe. The column was washed with 2 ml 80% isopropanol. Excess alcohol was removed by centrifugation for 20 seconds. Complete evaporation of the isopropanol was ensured by leaving the column at room temperature for a further two minutes. Thirty microlitres of sterile milliQ water was then added to the mini-column and the DNA allowed to resuspend for one minute. The sample was then collected by a 20 second centrifugation step. Adding the solution back to the mini-column and respinning increased the DNA recovery. Two microlitres of the purified PCR product was checked on a mini-gel prior to sequencing. Recovery of the product using the mini-columns was consistent and high.

Direct Sequencing of PCR Products

Attempts to directly produce single-stranded DNA templates for sequencing by limiting one primer in the amplification reaction (Gyllensten & Erlich 1988) were neither consistent nor reliable. Smearing of DNA above the PCR product was usually seen when the single-stranded amplification reaction was checked on a gel. Increasing annealing temperature and decreasing the template DNA concentration in the PCR reaction had very little effect. Consequently, a double-stranded sequencing procedure was tried based on the method of Casanova *et al.* (1990), using the modified T7 DNA polymerase (USB, Ohio, USA) and ^{35}S dATP as the radiolabel source. Two microlitres of gel purified template (about 100 ng) was denatured at 96°C for 5 minutes in a 10 µl volume, containing 10 ng of one primer, and reaction buffer. This was then immediately snap frozen in liquid nitrogen to reduce reassociation of the DNA strands and then 5.5 µl of the sequencing cocktail added (as described in the Sequenase kit). Termination reactions with the dideoxynucleotides were incubated at 37°C for two minutes. Sequencing reactions were run on 6% acrylamide gels, 8 M urea, then fixed, dried, and exposed to Kodak X-omat AR film (Eastman Kodak Co., Rochester, New York, USA).

Four primers were used for sequencing. Two were the PCR amplification primers while two more were constructed after obtaining preliminary skink sequence data. This set of four primers allowed the complete sequence of both strands to be determined, though use of primer 12SBR for sequencing consistently gave faint sequencing lanes. The skink-based primers, designated SK12SL and SK12SR, were situated approximately half-way along the PCR product and were complementary, designed to read

off opposite strands of the product (as shown in Appendix 1). Their sequences and corresponding location relative to the human mtDNA sequence are, respectively,
SK12SL `5'-CTTCTTTCAT AAGGTAGGC-3'`(L1408) and
SK12SR `5'-GCCTACCTTA TGAAAGAAG-3'`(H1390).

Attempts to amplify and sequence another mitochondrial gene were less successful. Conserved primers to the cytochrome *b* gene (Kocher *et al.* 1989) did not consistently amplify the equivalent region in the skinks. Altering reaction temperatures and times (to both higher and lower levels), Mg^{2+} concentration, primer concentrations, *Taq* polymerase and buffer, did not result in improved yields. This suggests that either the cytochrome *b* primers of Kocher *et al.* (1989) may not match the skink cytochrome *b* gene target sites very well, or that there may be a secondary structure interaction inhibiting amplification. The same cytochrome *b* primers have been successfully used in other studies of vertebrates (e.g. Meyer *et al.* 1990, Hedges *et al.* 1991, Irwin *et al.* 1991, Moritz *et al.* 1993). Information was however obtained for three taxa and will be discussed in Chapter Five. The cytochrome *b* primers and their location relative to the human mtDNA sequence are:
CB5 `5'-GCTTCCATCC AACATCTCAG CATGATG-3'` (L14813) and
CB3 `5'-GCAGCCCCTC AGAATGATAT TTGTCCTC-3'` (H15171)

Both strands of the PCR product were sequenced and where possible sequences from several individuals from each population were obtained. Sequences were compared and aligned using the University of Wisconsin GCG sequence analysis software package, Version 6.2 (Devereux *et al.* 1984) and phylogenetic inferences made using the Hadamard (discrete Fourier) conjugation (Hendy & Penny 1989, 1993, Penny *et al.* 1992). The Hadamard conjugation is described in more detail in Chapter Five, ~~and a reduced version of the HADTREE program is included in Appendix 2 on the diskette at the back of the thesis~~.

RESULTS

Oxygen electrode assays confirmed the presence of mitochondria from the rat liver preparations, though respiratory activity could not be detected in any mitochondrial preparations from skinks. Digestion of the DNA with the restriction endonuclease *Bam*HI gave three distinct fragments with a total size of approximately 16 000 base pairs, the expected size of the rat mitochondrial genome (Gadaleta *et al.* 1989). The absence of background smearing in these preparations confirmed that there was little contamination with nuclear DNA. Digestion of the skink DNA with both *Bam*HI and *Eco*RI also confirmed that the preparations were free of nuclear DNA. Enzymatic digestion also indicated that the skink mitochondrial genome was at least 15 000 base pairs in size, but an accurate estimate of its size was not obtained.

High molecular weight DNA was obtained from fresh, frozen, dried, and ethanol preserved tissues and all proved suitable substrates for PCR amplification of the mitochondrial DNA fragment. DNA could not be obtained from tissues stored in formalin. Formalin rapidly cross-links DNA making it difficult to obtain DNA suitable for the PCR, even from samples stored for short periods in formalin (Greer *et al.* 1991). Other sources of DNA, such as subfossil bones (eg. Cooper *et al.* 1992), preserved museum skins (e.g. Thomas *et al.* 1990), and herbarium samples (Rogers & Bendich 1985) can be suitable for such studies.

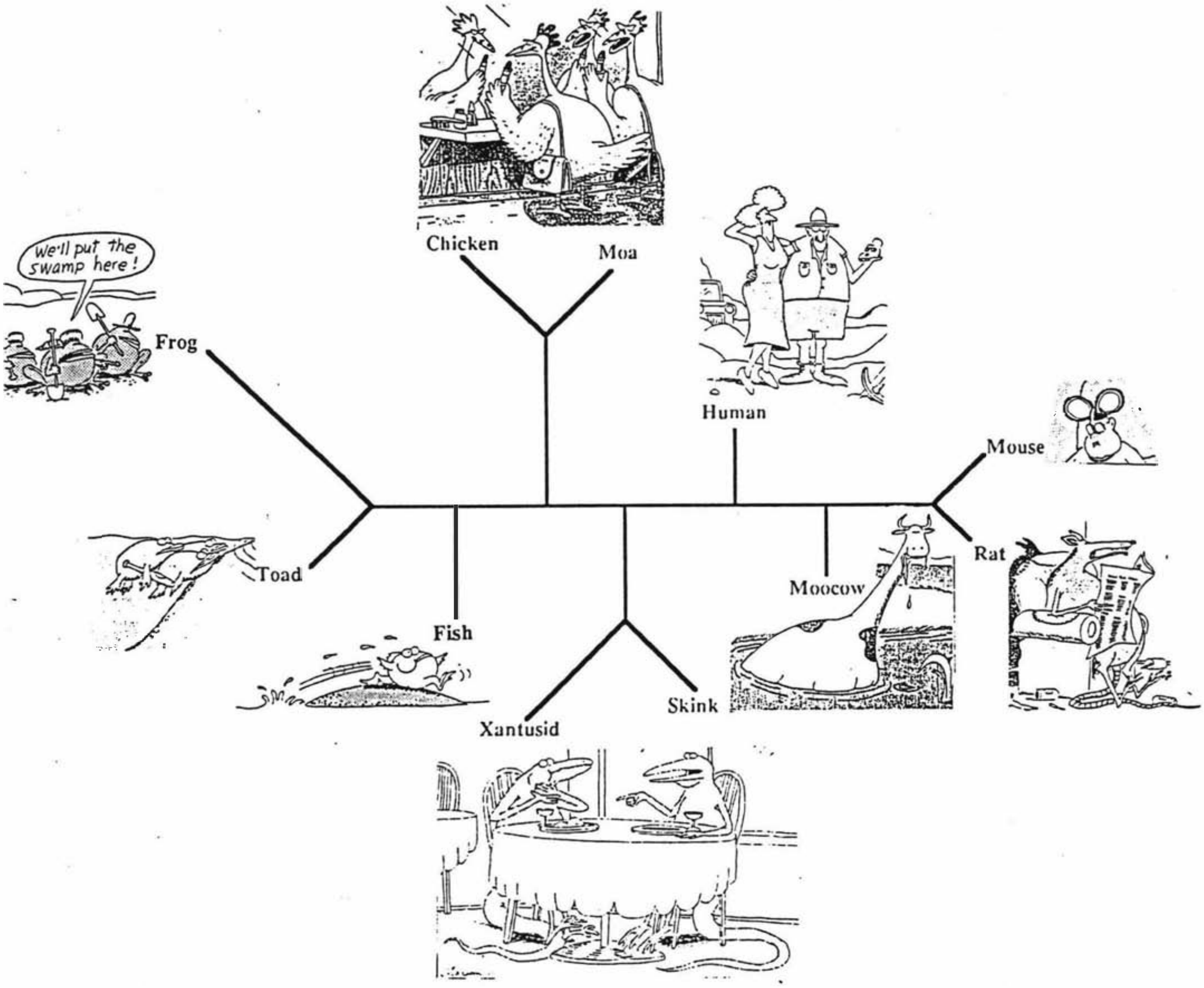
The same single PCR product was obtained from preparations of both total DNA and purified mtDNA. Sequence analysis of the product showed it to be most similar to other vertebrate 12S rRNAs from the region of the gene to which the PCR primers anneal. The very sensitivity of the PCR however makes the procedure vulnerable to the amplification of contaminating DNA, such as that from the hands or skin of the investigator (Kitchin *et al.* 1990). When this study was begun no reptilian mtDNA sequences were available on the GenBank or EMBL sequence databases, so confirmation that skink sequences had been amplified was established indirectly in several ways.

Amplification of a contaminating, non-skink, DNA template was discounted by the consistency of the skink DNA sequences. The same sequence was obtained from separate DNA preparations of the same individual, from separate amplifications of the same DNA sample, and from different individuals from the same population (see Table 2.1). The latter observation is to be expected for a relatively highly conserved molecule such as 12S rRNA (Mindell & Honeycutt 1990). This consistency also indicates the fidelity of the *Taq* polymerase. Errors can be introduced however. In one instance (out of several hundred amplifications) a single nucleotide difference was found for separate amplifications of the same skink DNA. Such misincorporation errors by the *Taq* polymerase are known to occur at relatively low frequencies (Bloch 1991). As these results show, repeating amplification and sequencing reactions safeguards against these errors. Inadvertent amplification of rat 12S rRNA rather than skink sequence was also detected on one occasion, but in general contamination was not a problem.

A final verification that the PCR products were not derived from other sources was performed by aligning a skink sequence against the same region of the 12S rRNA gene from other vertebrates and establishing their phylogenetic relationships. The alignment is presented in the next chapter in the context of secondary structure features (Table 3.3). The phylogenetic relationships of the skink and ten other vertebrate sequences are shown in figure 2.2. The Hadamard conjugation using the two character state option (see Chapter Five) was used. Omitting one taxon to permit four character state analysis gave the same relationships for the remaining taxa. Parsimony analysis using PAUP 3.0s (Swofford 1990) also produced the same result (not shown). The skink sequence is most similar to the other reptile used, a xantusiid lizard from the Caribbean (Hedges *et al.* 1991), confirming that the correct target sequence had been obtained by PCR.

In this analysis (Fig. 2.2), the birds are slighlty closer to the reptiles than to the mammals. The phylogenetic relationship of reptiles, birds and mammals to each other are still uncertain. Benton (1990) places birds closer to the reptiles than mammals on the basis of morphological characters. Sequence analyses of 18S rRNA, beta-haemoglobin and myoglobin group birds with mammals, but relationships

Fig. 2.2. Inferred phylogenetic relationships of 11 vertebrate 12S rRNA sequences. Alignment was done with reference to sequence's secondary structure and analysis performed using the Hadamard conjugation. The skink sequence is most similar to that of the xantusiid lizard, *Xantusia riversiana*, confirming that reptilian DNA had been amplified by the polymerase chain reaction. Branch lengths are proportional to the probability of change along that branch. The tree is unrooted.



inferred from insulin, alpha-crystallin and alpha-haemoglobin sequences place birds with reptiles (Hedges *et al.* 1990). Inferences about the order of deep branches in vertebrate evolutionary relationships are however, unlikely to be robust using the short sequence of 12S rRNA analysed here.

Two different 12S rRNA sequences were found for *L. n. polychroma* in this study. The sequence of *L. n. polychroma* from Gorge Burn was identical to that of *L. maccanni* at the same site, while the 12S rRNA sequence of the *L. n. polychroma* individual from Twizel was quite different (see Table 3.1). Partial sequence information from other *L. n. polychroma* populations in the North and South Islands (data not presented) were the same as the sequence from Twizel, while partial sequence data from other populations of *L. maccanni* were the same as the sequence from Gorge Burn. DNA was extracted independently from several tissues (muscle and liver) and from several individuals for these taxa, and amplified and sequenced several times. The same sequences were obtained from each individual every time indicating that the identical sequences were not the result of contamination of tissue samples or PCR amplification reactions. This point will be discussed again in Chapter Six.

The complete 12S rRNA sequence information for 20 skink taxa are given in Appendix 1, in a format suitable for analysis using the Hadamard conjugation. The sequences are also presented in the next chapter (Table 3.1). The nucleotide frequencies for the 20 skink taxa which are considered in most detail in this thesis are very similar, with a slight predominance of adenine (Table 2.2). The results of the analyses presented in the subsequent chapters are therefore not affected by differing nucleotide compositions between taxa (see Lockhart *et al.* 1992, Hasegawa & Hashimoto 1993).

Table 2.2. Base composition of the skink 12S rRNA sequences.

	Frequency of					
Taxon	**A**	**C**	**G**	**U**	**A+U**	**C+G**
"St. Is. Green"	0.36	0.27	0.19	0.19	0.54	0.46
L. grande	0.35	0.26	0.19	0.20	0.55	0.45
L. notosaurus	0.35	0.27	0.19	0.19	0.54	0.46
L. lin/chl	0.35	0.26	0.19	0.20	0.55	0.45
L. suteri	0.35	0.26	0.19	0.20	0.55	0.45
L. n. nigriplantare	0.35	0.26	0.20	0.20	0.54	0.46
L. microlepis	0.34	0.27	0.20	0.19	0.53	0.47
La. guichenoti	0.35	0.26	0.19	0.20	0.55	0.46
C. aenea	0.34	0.28	0.20	0.18	0.52	0.48
L. acrinasum	0.35	0.27	0.19	0.19	0.54	0.46
L. inconspicuum	0.34	0.28	0.20	0.19	0.53	0.47
L. smithi	0.34	0.28	0.20	0.19	0.52	0.48
L. zelandicum	0.34	0.28	0.19	0.19	0.53	0.47
L. fallai	0.35	0.27	0.19	0.19	0.54	0.46
L. maccanni	0.34	0.27	0.19	0.20	0.54	0.46
L. telfairi	0.34	0.28	0.19	0.19	0.53	0.47
L. n. polychroma	0.34	0.26	0.20	0.20	0.54	0.46
L. infrapunctatum	0.35	0.26	0.19	0.20	0.55	0.45
L. otagense	0.34	0.27	0.20	0.19	0.53	0.47
L. moco	0.34	0.28	0.20	0.19	0.52	0.48
Mean	0.35	0.27	0.19	0.19	0.54	0.46
Standard Deviation	0.01	0.01	0.01	0.01	0.01	0.01

Chapter Three: A Refined Secondary Structure Model for Domain III of Vertebrate 12S rRNA

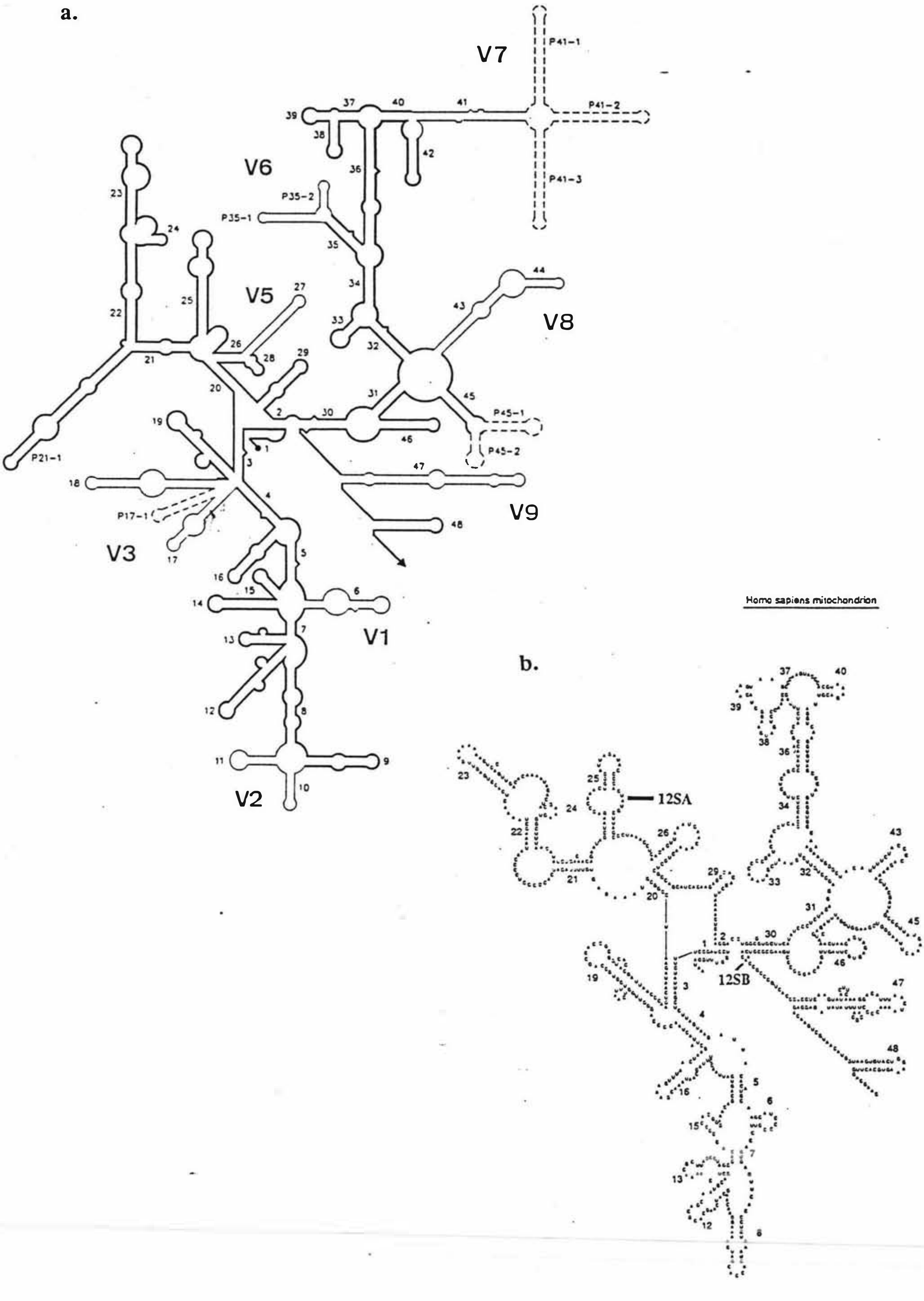
A DNA sequence is not a simple string of abstract letters. Appreciation of the higher order structure of DNA has several advantages for evolutionary analyses. Different positions and regions in the molecule have different functional and structural constraints, and so different rates and patterns of change (see for example Kimura 1983, Noller *et al.* 1990) which should be taken account of when attempting to reconstruct phylogenies. Knowledge of how and where the molecule varies, and where it is constrained, is also essential for understanding the patterns and processes of molecular evolution. The purpose of this chapter is to firstly refine a vertebrate secondary structure model for domain III of mitochondrial 12S rRNA and then to determine patterns of sequence variability among the vertebrate groups. This information will then be used in analyses in subsequent chapters.

Refining a Secondary Structure Model

Comparative sequence analyses, free energy predictions, and experimental investigations (such as chemical modification, nuclease digestion and intra-RNA crosslinking) have been used to determine secondary structure models for ribosomal RNA (reviewed in Woese *et al.* 1983, Noller 1984). Three different secondary structure models exist for the prokaryotic small subunit (16S) rRNA, to which the mitochondrial 12S rRNA is related (Stiegler *et al.* 1981, Zwieb *et al.* 1981, Gutell *et al.* 1985). The three models are generally similar, though they differ in the size and or placement of some base-paired regions (Noller 1984, Gutell *et al.* 1985).

Structurally, the small subunit rRNA is divided into four domains (labelled I, II, III and IV), each separated by a highly conserved single-stranded region (Fig. 3.1a). Domains III and IV are the most highly conserved regions of the molecule (Noller 1984, Neefs *et al.* 1990). The mitochondrial form of the small subunit rRNA is a reduced structure, lacking some helices (base-paired regions) but still having all four domains (Fig. 3.1b). Structural and sequence conservation among rRNAs is high (even from different kingdoms), with major changes in size involving insertion or deletion of blocks of sequence (Noller 1984). Comparative sequence analysis is therefore an effective way of estimating secondary structures of rRNA, the validity of which can be subsequently examined experimentally (Noller 1984). Several compilations of rRNA sequences in a structural context are available for a wide range of organisims (Dams *et al.* 1988, Gutell & Fox 1988, Gutell *et al.* 1990, 1992, Neefs *et al.* 1990, 1991, Specht *et al.* 1991, de Rijk *et al.* 1992). The small subunit rRNA compilation of Neefs *et al.* (1990) formed the basis for refining a secondary structure model for part of animal mitochondrial 12S rRNA. Neefs *et al.* (1991) now also provide the compilation in computer format. Models for the 12S rRNA structure are limited by the fact that only a few mitochondrial sequences have been used in previous compilations (Zwieb *et al.* 1981, Clary & Wolstenholme 1985, Gutell *et al.* 1985, Neefs *et al.* 1990). The PCR primers 12SAR and 12SBR (Chapter Two) amplify the last part of domain II and all of domain III (Fig. 3.1b), though for simplicity in this thesis, the whole PCR fragment is usually referred to as domain III. In this chapter I present alignments for domain III of the 12S rRNA from all five classes

Fig. 3.1. ***a.*** Secondary structure model for the prokaryote small subunit (16S) rRNA (from Neefs *et al.* 1990). The mitochondrial small subunit (12S) rRNA gene is derived from this. Helices are numbered, and regions which may be absent in some prokaryotes are drawn in thin lines and labelled V1-V9. ***b.*** Secondary structure model for human mitochondrial small subunit rRNA (from Gutell *et al.* 1985). Helices are numbered as in 3.1a. The location of the PCR primers 12SAR and 12SBR are indicated.



of vertebrates. Complete 12S rRNA sequences are available for only a few vertebrates, but the use of the polymerase chain reaction and universal 12S rRNA primers (Kocher *et al.* 1989, Palumbi *et al.* 1991) are leading to an increasing availability of domain III sequences from a wide range of taxa.

The Skink and Other Vertebrate 12S rRNA Sequences

The sequences from 20 skink taxa are presented in Table 3.1, overlain with Neefs *et al.* suggested helical (stem) regions. Other sources of vertebrate sequences used in this chapter are listed in table 3.2. The 12S rRNA sequences for several snakes (Knight & Mindell 1993), and a complete seal mitochondrial genome (Arnason & Johnsson 1992) were also recently published , but were obtained too late to be included in the analyses presented in this thesis. A more general structural model of domain III for both vertebrate and invertebrate 12S rRNA is being developed (A.J. Cooper, R.E. Hickson, G.M. Lento, C. Simon & D. Penny *in prep.*).

Approaches for Refining a Vertebrate Domain III Secondary Structure Model

Three strategies were used to determine how the region of the 12S rRNA between PCR primers 12SAR and 12SBR could fold. Comparative sequence analysis established which helices had the potential to form (Table 3.3). Identification of fixed compensatory mutations (changes preserving base-pairing in helical regions) was especially useful for confirming helices and locating those whose sequences varied between the different groups. The vertebrate sequences in table 3.3 were relatively easy to align when done so in the context of secondary structure. Gaps to maintain alignments were introduced at the beginning or end of unpaired regions.

Energetics of the Folding of the rRNA Sequences

The third approach was to compare minimal free energies of some of these suggested helix structures using the MFOLD algorithm (Zuker 1989, Zuker *et al.* 1991) in the University of Wisconsin Genetics Computer Group (GCG) Package, version 7.2. The program takes account of the energetic costs and benefits of potential base pairings, and the occurrence, locations and interactions among unpaired nucleotides (Zuker 1989, Zuker *et al.* 1991). The MFOLD program can show both optimal and suboptimal folded structures. Other folding programs are also available (e.g., Abrahams *et al.* 1990) but were not used in this study.

On their own, and in the absence of knowledge about tertiary or quaternary structure, such energy calculations may not indicate what can actually form, but when used in conjunction with the other two lines of inquiry provide a well-founded basis for suggesting secondary structure models. Structures determined on the basis of comparative sequence analysis are usually found to have a minimal free energy within 10% of the value of the optimal structure determined by MFOLD (Zuker *et al.* 1991).

Table 3.1. Alignment of 20 skink taxa for 384 bases of 12S rRNA. The PCR primers 12SAR and 12SBR are not included but start immediately before and after, respectively, the sequences shown. Conserved sites are in uppercase and variable positions are in lowercase, with transversions in **bold**. The suggested helical (stem) regions of Neefs *et al.* (1990) are shown above the sequence alignment (a ′ denotes the distal arm of a helix). Taxa are arranged in order of increasing numbers of differences (see Table 4.1a), and the three letter abbreviation for each taxon is shown on the right. A dot indicates no difference from the consensus, while deletions are shown as dashes. The internal sequencing primers SK12SL and SK12SR anneal between bases 185 and 203. *L. telfairi* has two single base deletions (positions 25 & 60) and one single base insertion (between 202 & 203). *L. maccanni* also has a deletion at position 25. When these two taxa are included in phylogenetic analyses (Chapter Five), the sites of deletion and insertion are not included.

```
                  _25′__        __26___              _26′_ __20′___     _29__    29′_        _2′__ ______30______
                           1         2         3         4         5         6         7         8         9        10
                  1234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890
                  GCucAGCCGUcAACAAAgAcAGuauaaaauACAauaCUgUUCGCCAGAGAAcUAcAAGcuAAaaCUcaAAACuCcAAGGACUUGGCGGUGCUCCAcAuCa
StIsGreen         .............................c...................................................................c.. Sig
L.grande          ........................c........................................................................c.. Lgr
L.notosaurus      ......................cc..............................u..........................................c.. Lno
L.lin/chl         ...u......a..................c........a....................c..gg.................................... L1c
L.suteri          ........................c........................................................................c.. Lsu
L.n.nigripl.      ...................u.........c...g....a.......................g....g................................ Lnn
L.microlepis      ........................c..........g.......................a........................................ Lmi
La.guichenoti     ..c..........................c....c.......................u...c..................................... Lgu
C.aenea           ..c...............................c............................g..a.....c........................... Cae
L.acrinasum       ..c.......a.............a....c....c...........................gg.................................... Lac
L.inconspicuum    .......................c..........c..............................................................c.g Lin
L.smithi          ........................c..................................a........................................ Lsm
L.zelandicum      ...u......u......a.......g..cc....c........................a...g.................................... Lze
L.fallai          ..................................c................u...........g..a.....c........................... Lfa
L.maccanni        ..........a......a......-........g...............................................................c.g Lma
L.telfairi        ..........a......a......-....c.............................-.......g............................u.... Lte
L.n.polychroma    ...................u.............g.g..a....................a..g...........u......................... Lnp
L.infrapunctatum  ..c.......a..................c....c.......................u...c..................................... Lfr
L.otagense        ..c.......a...........c.c.........cg...............................................................g Lot
L.moco            ..........u...........c......c....cg...........................g..g................................. Lmo
```

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                     31       32        33          33'          34                    36              37  38     38'
                  ------ ----------    -----       -----       ------            ----------------    --------    ----
                         11        12        13        14        15        16        17        18        19        20
                 1234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890
                 aCCUAGAGGAGCCUGUCCUAUAAUCGAUACCCCcCGAUCuACCuGAGGacUUUUUGAAacUCAGcCUAUAUACCGCCGUCGuCAGCcUACCUUaUGAaAG
StIsGreen        .................................................................................................... Sig
L.grande         .................................................................................................... Lgr
L.notosaurus     ...........................................................u.....................c.................. Lno
L.lin/chl        .................................................................................................... Llc
L.suteri         .................................a.................................................................. Lsu
L.n.nigripl.     ...........................................................u........................................ Lnn
L.microlepis     c................................................................................................... Lmi
L.guichenoti     c................................................u.................................................. Lgu
C.aenea          c......................................c...................u.................................g...... Cae
L.acrinasum      u................................................................................................... Lac
L.inconspicuum   ...........................................................u.....................c.................. Lin
L.smithi         c................................................................................................... Lsm
L.zelandicum     c................................................................................................... Lze
L.fallai         c......................................c.........u.................................................. Lfa
L.maccanni       .......................................c.........u.........u.....................c...........g...... Lma
L.telfairi       g...............................................g............................................g...... Lte
L.n.polychroma   .................................a..............g.........gu..........................u............. Lnp
L.infrapunctatum c................................................u.................................................. Lfr
L.otagense       u..........................................c...............u.....................................g.. Lot
L.moco           c................................................u........gu....u................................... Lmo
```

```
                           37'      40       40'                36'          34'       32'         43       43'
                         _____    ____     _____         _____________     ____  ___________     ____     ____
                          21        22        23        24        25        26        27        28        29
                 12 3456789012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789
                 aa-guauAGuAaGcaAAAuAguCaccaAcUAaAACGuCAGGUCAAGGUGUAGCAcAUaaaguGGaAGAGAUGGGCUACACUCUCUcCCcCAGAGaAcACg
StIsGreen        .. .a.c..............................c..................................................a..u........... Sig
L.grande         .. ...c.......g.....................c...................................................u........... Lgr
L.notosaurus     .. a..a....g........................................................................................ Lno
L.lin/chl        .. .c.a.................u.............................u.....a...........................u........... Llc
L.suteri         .. ..........u..........u......................................................................g..... Lsu
L.n.nigripl.     .. ac......g................................................c...........................u........... Lnn
L.microlepis     .. ...c..................................................gu.............................u........... Lmi
L.guichenoti     .. .a...................u......g............................a........................a..u........... Lgu
C.aenea          .. .cg.......................................................c...................................... Cae
L.acrinasum      .. .................a.......u...............................a........................a.............. Lac
L.inconspicuum   .. a..a....................................................g....c.............................g..... Lin
L.smithi         .. .c.c..................................................gu.............................u........... Lsm
L.zelandicum     .g a..c..........................................................................................u... Lze
L.fallai         .. ac.c...............................................u.....a......................................u Lfa
L.maccanni       g. .c.a.................u.............................u.....a....................................... Lma
L.telfairi       ..ac..a..............c.c.uu..................................c...................................... Lte
L.n.polychroma   .. .c.a....g............................................................................u........... Lnp
L.infrapunctatum .. aa...................u......g............................a...........................u........... Lfr
L.otagense       .. ......................u...........................................................g.............. Lot
L.moco           .g cc....c........c.........................................a....................................... Lmo
```

```
                       45                45'                31'          46                46'                  30'
                  3
                  0          31          32           33          34           35          36          37           38
                  012345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234
                  AAcaGCAucaAUGAAAcaCuGCucaAAGGuGgAUUUAGuAGUAAGAuaaaCaAGAgACuuaucUuAAAcCAGCcCUGGAGCGCGC
StIsGreen         .............................................................c...........u........... Sig
L.grande          .......................u......................................a..........u........... Lgr
L.notosaurus      ..................................................................................... Lno
L.lin/chl         .................c................................................................... Llc
L.suteri          .................c....................c................a............................. Lsu
L.n.nigripl.      .................c................................................................... Lnn
L.microlepis      .......c..............................c..........g........c.........u................ Lmi
L.guichenoti      .................c...............................g.......................u........... Lgu
C.aenea           .......c.....................c....................................................... Cae
L.acrinasum       .................c.c.............................g.......................u........... Lac
L.inconspicuum    .......................u.....c..................g.........c.......................... Lin
L.smithi          .......c..............................c........g.g........ccg.......u................ Lsm
L.zelandicum      .......c........................................g.........c.......................... Lze
L.fallai          ..............................................c.............g...a.................... Lfa
L.maccanni        .......................ug.......................g.................................... Lma
L.telfairi        .......c.....................c....................................................... Lte
L.n.polychroma    .................c....................c.............................................. Lnp
L.infrapunctatum  ..u.....ag......uu..............................g..........a......................... Lfr
L.otagense        ...g..................cu.....c.a..................................................... Lot
L.moco            ........................g....c.....................g................................. Lmo
```

Table 3.2. Species and sources of 12S rRNA sequences used for Table 3.3. Whether the sequence was obtained by PCR and direct sequencing or by cloning is also indicated. An asterisk indicates a group for which several sequences are available (see Appendix 2).

Taxon	Genbank Acc. No.	Ref.	Note
Coelocanth	S111210	1.	PCR product
Lungfish	S111206-S111209	1.	PCR
Crossostoma lacustre {fish}	M91245	2.	cloned
Rana castesbeiana {frog}	X12841	3.	cloned
Xenopus laevis {toad}	M10217	4.	cloned
Xantusiid lizards*	M65110-M65116	5.	PCR
Chicken	X52392	6.	cloned
Ratites*	X67626-X67638	7.	PCR
Whale	S79330	8.	cloned
Rat	X14848	9.	cloned
Mouse	J01420	10.	cloned
Cow*	J01394	11.	cloned
Human*	J01415	12.	cloned

References:

1. Meyer & Dolven (1992).
2. Tzeng *et al.* (1992).
3. Nagae *et al.* (1988).
4. Roe *et al.* (1985).
5. Hedges *et al.* (1991). See Appendix 2.
6. Desjardins & Morais (1990).
7. Cooper *et al.* (1992). See Appendix 2.
8. Gadaleta *et al.* (1989).
9. Bibb *et al.* (1981).
10. Arnason *et al.* (1991).
11. Anderson *et al.* (1982). See Appendix 2.
12. Anderson *et al.* (1981). See Appendix 2.

Table 3.3. Alignment of the region of vertebrate 12S rRNA between the PCR primers 12SAR and 12SBR. The primer sequences are not included, but 12SAR is immediately to the left, and 12SBR immediatedly to the right of the sequences shown. Where more than one taxon is available for a group (see Table 3.2), the positions that are variable among them are shown in lower case. **Bold** regions denote helical (stem) regions following Neefs *et al.* (1990), with the exceptions that stem number 39 is omitted. The regions into which the sequences have been divided for further analyses are indicated below the human sequence (S denotes a stem &/or hairpin region, and L a loop). Nucleotides conserved in all the vertebrates listed here are also shown below the alignment. Coelocanth and lungfish are included in the alignment but not used in the subsequent analyses because they are incomplete. Helix regions marked with a prime (′) denote the distal (3′) arm of the helix. Gaps, introduced to maintain alignments, are indicated by "-".

	25′ 26 26′ 20′ 29 29′ 2′ 30 .
Coelocanth	**GG**AACAAC**AAGC**CACA--**GCUU**AAAACUCAA**AGGA**CU**UGGC**G**GUGCUUCA**UA
Lungfish	**CCAGG**AaCUAC**aAGC**cCAa--**GCUu**AAAACcCAA**AGGA**CU**UGGC**G**GUGCCUCA**cA
Fish	G**CUCAGC**UAUAAACCUA**GACGUUU**--AAUCACAAC**AAACGUCCGCCAGGG**UACUAC**GAGC**GUCA--**GCUU**AAAACCCAA**AGGA**CU**UGG**CG**GUGCCUUA**GA
Frog	G**CCUAGC**CGUAAACAA-----**UUA**--AUUUACACC**AAU**AAG-**CGCCAGGG**AAUUAC**GAGC**AAU---**GCUU**AAAACCCAA**AGGA**UU**UGAC**G**GUGUCCCA**--
Toad	G**CCUAGC**CAUAAAC------**UUUG**ACUACUUACG**CAAA**AAUC**CGCCAG**--AACUAC**GAGC**CUAA--**GCUU**AAAACCCAA**AGGA**CU**UGGCGGUGCUCCA**AA
Xantusid	G**CuuaAC**uguaAACcuA**gacagC**--caaaaacauuu**gCuGucCGCCAGgG**aAcUACA**AGc**AAAA--**gCU**caAAACuCaA**AGGA**CU**UGGC**G**GUG**cu**CuA**uA
Skink	G**CucAGC**CGUcAACAAA**gAcAGu**-auaaaauACAau**aCUgUUCGCCAGAG**AAcUAcA**AGc**uAAa--**aCU**caAAACuCcA**AGGA**CU**UGGC**G**GUGCUCCA**cA
Chicken	G**CCUAGC**CCUAAAUCUA**GAUAC**C**U**-CCCAUCACAC**A**U**GUAUCCGCCUGAG**AACUAC**GAGC**ACAAAC**GCUU**AAAACUCUA**AGGA**CU**UGGC**G**GUGCC**C**CA**AA
Moa	G**CUUaGC**CcUAAAUCca**GaUaC**U**u**--aCcccACac**a**A**GuAuCCGCCcGAG**AACUAC**GAGC**ACAAAC**GCUU**AAAACUCUA**AGGA**CU**UGGC**G**GUGCCCcA**AA
Whale	G**CUUAGU**CGUAAACCCC**AAUAGUC**--ACAAAACAA**GACUAUUCGCCAGAG**UACUACUA**GC**AACA--**GC**CUAAAACUCAA**AGGA**CU**UGGC**G**GUGCCUCA**UA
Rat	G**CUUAGC**CCUAAACCUU**AAUAAUU**-AAACCUACAA**AAUUAUUUGCCAGAG**AACUACU**AGC**UACA--**GCU**UAAAACACAA**AGGA**CU**UGGC**G**GUACUUUA**UA
Mouse	G**CUUAGC**CAUAAACCUA**AAUA**A**UU**AAAUUUAACAA**AA**C**UAUUUGCCAGAG**AACUACU**AGC**CAUA--**GCU**UAAAACUCAA**AGGA**CU**UGGC**G**GUACUUUA**UA
MooCow	G**CuuAGC**CcUAAACAcA**gaUaauu**-acauaAACAA**aAuUaUUCGCCAGAG**uACUACu**aGC**aAca--**GCu**uaAAACUCAA**aGGA**CU**UGGC**G**GUGCUUuA**uA
Human	G**CuUAGC**CCUAAACuUc**A**A**CAGUU**-aAAUuAACAA**aACUG**C**UCGCCAGAA**CACUAC**GAGC**CACA--**GCUU**AAAACUCAA**AGGA**CC**UGGC**G**GUGCUUCA**UA
Conserved	GC AA GCC A G C AAAC C A GGA UG CGGU A
	S25′ ↑ *S26* ↑ *S20′* ↑ *S29* ↑ *S2′* ↑ *S30* ↑

```
                     31       32        33          33'           34        "35'            36            37
Coelocanth    CCC-CCUAGAGGAGCCUGUUCUAGAACCGAUAAACCCCGAUCAACCUCAACCACACUU-GC-UAUUUCAGCCUAUAUACCGCCGUCGCCAGCCCAC
Lungfish      CCCACCUAGAGGAGCCUGUuCUAgAACCGAUAAUCCACGUUuuACCcaA-CCuucccUgGC--AuUuCaGcCUAUAUACCgCCGUCGCCAGCcaAC
Fish          CCCCCCUAGAGGAGCCUGUUCUAGAACCGAUAACCCCCGUUAAACCUCA-CCACUUCUaGU-CAUCCCCGCCUAUAUACCGCCGUCGUCAGCUUAC
Frog          CCCCACUAGAGGAGCCUGUUCUAUAAUCGAUGAUCCCCGAUAUACCCGA-CCAUUUCUCGC-AUUAUCAGUCUGUAUACCUCCGUCGAAAGCUUAC
Toad          CCCACCUAGAGGAGCCUGUUCUGUAAUCGAUACCCCUCGCUAAACCUCA-CCACUUCUUGC-CAAACCCGCCUAUAUACCACCGUCGCCAGCCCAC
Xantusid      uCaAaCUAGAGGAGCCUGUCCcAUAAUCGAUAcCcCACGaUAaACCCgA-CCACucUUggaAuacUcCAGCCUAUAUACCGCCGUCacCAGccuAC
Skink         uCaaCCUAGAGGAGCCUGUCCUAUAAUCGAUACCCCcCGAUCuACCuCA-CCgcUUUUUG--AAacUCAGcCUAUAUACCGCCGUCGuCAGCcUAC
Chicken       CCCACCUAGAGGAGCCUGUUCUAUAAUCGAUAAUCCACGAUUCACCCAA-CCACCCCUUGC-CAGCACAGCCUACAUACCGCCGUCGCCAGCCCAC
Moa           CCCACCUAGAGGAGCCUGUUCUAUAAUCGAUAAcCCACGuUaCACCCga-CCAuCuCUuGC-CcaugCAGCCUACAUACCGCCGUCcCCAGCcCGC
Whale         CCCAUCUAGAGGAGCCUGUUCUGUAACCGAUAAACCCCGAUCAACCUCA-CCAACCCUUGC-UACUUCAGUCUAUAUACCGCCAUCUUCAGCAAAC
Rat           UCCGUCUAGAGGAGCCUGUUCUAUAAUCGAUAAACCCCGUUCUACCUUA-CCCCUUCUCGC-UAAUUCAGCCUAUAUACCGCCAUCUUCAGCAAAC
Mouse         UCCAUCUAGAGGAGCCUGUUCUAUAAUCGAUAAACCCCGCUCUACCUCA-CCAUCUCUUGC-UAAUUCAGCCUAUAUACCGCCAUCUUCAGCAAAC
MooCow        ucCuuCUAGAGGAGCCUGUUCUaUaAUCGAUAAACCCCGAUAaACCUcA-CCAauuCUuGC-UAAUaCAGuCUAUAUACCGCCAUCUUCAGCaAAC
Human         uCCCuCUAGAGGAGCCUGUUCUGUAAUCGAUAAACCCCGAUCAACCUCA-CCaCCuCUUGC-----UCAGCCUAUAUACCGCCAUCUUCAGCAAAC
                   CUAGAGGAGCCUGU C    A CGAU    C CG U  ACC    CC     U         C G CU  AUACC CC UC   AG    C
              L30'↑ S31  ↑     S32    ↑     S33       ↑  L33'  ↑  S34 ↑      L34'   ↑      S36       ↑40↑ S37

               38      38'          37'     40         40'                 36'            34'        32'
Coelocanth    CCU--GUGAAGGAAAUACAAUGGGCAAAAAUAA--A---AAAAUUAAAAACGUCAGGUCGAGGUGUAGCAAAUGAGAUGGGAAGAAAUGGGCUACA
Lungfish      CCC--cUGA-GGcccacuAGUugGCAaAAUaga--uagcaucua---aCacgucagguCGAGGUGUAGCacAUGggagG-gAAGAaAUGGGCUACA
Fish          CCU--GUGAAGGCUCAAUAGUAAGCAAAGUGGG--CACAACCCA---AAACGUCAGGUCGAGGUGUAGCGUACGAAGUGGGAAGAGAUGGGCUACA
Frog          CAU--GUGAACGGUUGC-AGUAGGCUUAAUGACCUAACACGUCA---AUACGUCAGGUCAAGGUGCAGCUUAAGAAAUGGGAAGUAAUGGGCUACA
Toad          CUC--GUGAGAGAUUCUUAGUAGGCUUAAUGAU--UUUUCAUCA---ACACGUCAGGUCAAGGUGUAGCAUAUGAAGUGGGAAGAAAUGGGCUACA
Xantusid      cuu--augAgaGcacAaaAGUaAGCAaAAcuGc--aaacaCaauU----AcGcCAGGUcAAGGUGUAGCuuAcaggGUGG-AgaAGAuGgGCUAca
Skink         CUU--aUGAaAGaaguauAGuAaGcaAAAuAgu----CaccaAcUA-aAACGuCAGGUCAAGGUGUAGCAcAUaaaguGG-aAGAGAUGGGCUACA
Chicken       CUCUAAUGAAAGAACAACAGUGAGCUCAAUAGC---UCCCUCGCUA-AUAAGACAGGUCAAGGUAUAGCCUAUGGGGUGG-GAGAAAUGGGCUACA
Moa           CUa---UGAaAGAACAauAGCGAGCACAAcAGC----cacccGCUA-aCAAGACAGGUCAAGGUAUAGCauAuGagaUGG-aAGAAAUGGGCUACA
Whale         CCU---AAAGGG-AGAAAAGUAAGCAUAACCAU-----CCUACAUAAAAACGUUAGGUCAAGGUGUAACCCAUGGGUUGGGAAGUAAUGGGCUACA
Rat           CCU--AAAAAGGCACUAAAGUAAGCACAAGAAC------AAACAUAAAaACGUUAGGUCAAGGUGUAGCCAAUGAAGCGGAAAGAAAUGGGCUACA
Mouse         CCU--AAAAAGGUAUUAAAGUAAGCAAAAGAAU-----CAAACAUAAAAACGUUAGGUCAAGGUGUAGCCAAUGAAAUGGGAAGAAAUGGGCUACA
MooCow        CCU--aAAAAGGaaaAaaAGUAAGCauAAuuAu-----gauacaUAAAaACGUUAGGUCAAGGUGUAaCCuAUGaaauGGgAAGAAAUGGGCUACA
Human         CCU-GAUGAAGGcuACaAAGUAAGCgCAAguAC------CCACGUAAAGACGUUAGGUCAAGGUGUAGCCcAUGagGuGGCAAGAAAUGGGCUACA
                      A  G      A    G   A                              C AGGU  A C  A      G       A G GCUA
                  S37    ↑ L38' ↑ S37 ↑             SL40        ↑         S36    ↑L34'↑  S34 ↑        S32    ↑
```

```
                 43       43'                            45             45'              31'        46
Coelocanth    UUUUCUACAU--AGAAUAUU------------ACGAAAAAAACAG-CGAAACCUGUACUUU-GAAGGAGGAUUUAGCAGUAAAAGGGGAAUAGAGAG---
Lungfish      UUUUCUaC-----GAAaAc-------------ACGgAcaaCcccA-UGAAAuugggGuu--ugAAGcUggaUUUAGuAGuAAGAAAA
Fish          UUUUCUACU---AGAAUAAG------------ACGAAUAGCAUCA-UGAAAACUUAAUGCUUGAAGGAGGAUUUAGUAGUAAAAAGGAAAUAGAGUG---
Frog          AUUUCU-C----AGAACAA-------------ACGAAAGACUAUA-UGAAAUUAUAAUCAU-GAAGGUGGAUUUAGUAGUAAAAAGAAAAUAGAGUG---
Toad          UUUUCUACCUU-AGAAUAA-------------ACGAAAGAUCUCUAUGAAACCAGAUCGAGAAAAGGCGGAUUUAGCAGUAAAGAGAAACAAGAGAG---
Xantusid      uUUucUAAaac-agaauacgc-----------ACGgAaaggCuuA-UGAAAaauaAcCu--aAaaggcgGAUUUAgCAGUAaaAuAaa-caAgaauu---
Skink         CUCUCUcCC---AGAGaAc-------------ACgAAcaGCAucaAUGAAAcaCuGCuc---aAAGGuGgAUUUAGuAGUAAGAuaaa-CaAGAgA----
Chicken       UUUUCUACU---AGAACAAA------------CGAAAAAGGAUG--UGAAACCCGCCCU-UAGAAGGAGGAUUUAGCAGUAAAGUGAGAUCAUACCCCCU
Moa           UUUUCUAacaU-AGAAcAccA-----------CGaAAGAgaAGa--UGAAAcuC-UCcU-cagAAGGcGGAUUUAGCAGUAAAauaGgAcaAGAacG---
Whale         UUUUCUACUA--AGAACAUCCCCUAUACUCACACGAAAGUUUUUA-UGAAACUUAAAAACU-AAAGGAGGAUUUAGUAGUAAAUCAAGAGCAGAGUG---
Rat           UUUUCUUCCC--AGAGAACAUU----------ACGAAACCCUUA--UGAAACUAAAGGAC--AAAGGAGGAUUUAGUAGUAAAUUAAGAAUAGAGAG---
Mouse         UUUUCUUCAA--AGAACAUU------------ACUAUACCCUUUA-UGAAACUAAAGGACU--AAGGAGGAUUUAGUAGUAAAUUAAGAAUAGAGAG---
MooCow        UUcUCUacaccaAgAgaAucaagc--------ACGAAAGuuauuA-UGAAAccaauaACc--AAAGGAGGAUUUAGcAGUAAaCUaagAAUAGAGuG---
Human         UUUUCUACC---AGAAAAcU------------ACGAUAaCCCUuA-UGAAAccUAAgGGUc-gAAGGUGGAUUUAGCAGUAAACUAAGAGUAGAGUG---
               U   U                                         GAAA                    UUUA  AG A          A
              ↑    S43          ↑     L43'          ↑     S45           ↑   L45'    ↑  S31    ↑        S46


                   46'                   30'
Coelocanth    ---CCCCUCU
Lungfish
Fish          ---UCCUUUUGAACC-CGGCUCUGAG-GCGCGU
Frog          ---UUCUUUUUAACC-CGGCUCUGGG-ACGCGU
Toad          ---UUCCUCUUUAAAACGGCCCUGGA-GCGCGC
Xantusid      ---uuuacuugaAGa-uugcuCUaGa-GcacGC
Skink         ---CuuaucUuAAAc-CAGCcCUGGA-GCGCGC
Chicken       AAGCUCACUUUAAGA-CGGCUCUGAG-GCACGU
Moa           ---cCcAuUUUAAgc-uGGCcCUgGG-GCACGU
Whale         ---CUUGAUUGAAUA-AGGCCAUGAGGGCACGC
Rat           ---CUUAAUUGAAUA-GAGCAAUGAA-GUACGC
Mouse         ---CUUAAUUGAAUU-GAGCAAUGAA-GUACGC
MooCow        ---CUuAGuUGAAuu-AGGCuAUGAA-GCACGC
Human         ---CUUAGUUGAACA-gGGCCCUGAA-GCGCGU
                          A         U        G
                       ↑    L46'   ↑   S30     ↑
```

Identifying Individual Helices

Helices 30-33 and 36 are well conserved among the vertebrates (Table 3.3), while six helices (26, 29, 34, 43, 45, & 46) are quite variable in sequence but their pairings are easy to identify (Table 3.3). The helices 26 and 29 are part of domain II (Fig. 3.1). The area bounded by helix 34/34′ shows the greatest potential for alternate structures (Table 3.3; Glotz & Brimacombe 1980, Dams *et al.* 1988, Neefs *et al.* 1990, Simon 1991) and was examined in more detail. Helices 34, 36 and 37 tended to form spontaneously in most of the vertebrates and so particular attention was paid to the effects of forcing other helices to be included in the structure.

Helix 35

Examination of the base sequence between the proximal arms of helices 34 and 36 indicated that nucleotides in this region had the potential to pair in several of the vertebrates (see Table 3.3). This putative "helix 35" was not included in either Neefs *et al.*'s (1990) compilation or in Hixson & Brown's (1986) primate model. Nucleotides which could participate in the helix are well conserved amongst members of a group (for instance skinks and ratites; Table 3.3). Except for the toad however, helix 35 did not form spontaneously using the MFOLD algorithm and its forced creation increased (made more positive) local free energies by more than 20% (Table 3.4). In addition, few fixed compensatory mutations occur to support it's existence (Tables 3.1 & 3.3). The 5′ nucleotides for this helix could also be used in an extension of helix 34 (Table 3.3), so without experimental investigations (Noller 1984, Gutell *et al.* 1985) to test for its presence, helix 35 is not considered to be a general feature in vertebrate 12S rRNA.

Helix 36

This is one of the most conserved regions of domain III (Table 3.3) but it can potentially pair in several ways (Figure 3.2). Structure ***a.*** in figure 3.2 is the one presented in table 3.3, but structures ***b.*** and ***c.*** tend to form in different taxa and/or when different helices are forced into the structure. Note that all forms of the helix have several unpaired "bulges". Such unpaired bases may act as protein recognition sites (Peattie *et al.* 1981, Noller 1984, Gutell *et al.* 1985), and this point is discussed later.

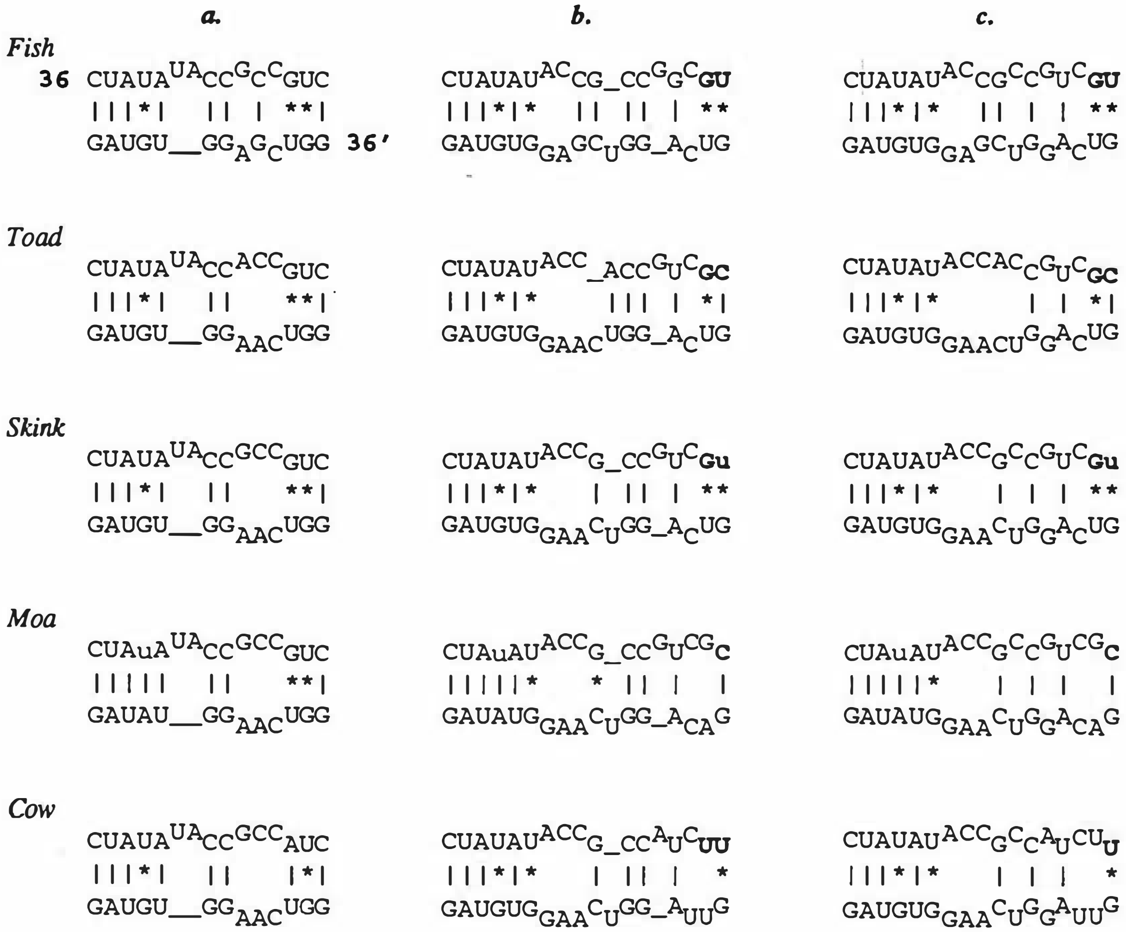
Helix 37

Helix 37 can contain from four to six nucleotide pairs, though Neefs *et al.* (1991) only show it as a two base-pair structure. This helix may contain an internal unpaired bulge (Table 3.3). Conserved nucleotides and compensatory changes strongly support helix 37, and it forms without forcing using MFOLD (Table 3.4).

Table 3.4. Local minimal free energies for folding of helices 34, 36 and 37 in vertebrate 12S rRNA domain III. The additional energy cost required (less negative energy value) to include other helices are shown, along with their percent energy differences from the minimal value. Energies were calculated using the MFOLD algorithm in the GCG package, version 7.2. The minimal energy for forming the alternate helix 40 in humans is also shown, and is lower than for placing helix 40 in the same position as other vertebrates. Energy differences more than 10% different (in kcal/mol) from the optimal value are considered less structurally feasible (Zuker *et al.* 1991 & pers. comm.). A value of 0% additional energy indicates that the helix forms without forcing.

Taxon	**Energy (kcal/mol)**	**Forcing of helix:**		
		35	**38**	**40**
Fish	-11.2	-7.3	-7.3	-11.2
		(35%)	*(35%)*	*(0%)*
Toad	- 2.5	-2.5	+0.2	-2.5
		(0%)	*(108%)*	*(0%)*
Skink	- 3.3	-2.5	-1.7	-3.3
		(25%)	*(49%)*	*(0%)*
Chicken	- 5.7	-3.3	-5.5	-5.7
		(42%)	*(3%)*	*(0%)*
Moa	- 2.1	+0.3	+0.3	-2.1
		(114%)	*(114%)*	*(0%)*
Mouse	- 6.2	+1.4	-2.8	-6.2
		(123%)	*(55%)*	*(0%)*
Cow	- 6.8	-3.3	-6.1	-2.9
		(52%)	*(10%)*	*(57%)*
Human	- 2.1	+2.9	-2.1	-2.1
		(238%)	*(0%)*	*(0%)*
(alt. S40)	- 4.5	+1.0	-4.5	-4.5
		(122%)	*(0%)*	*(0%)*

Fig. 3.2. Potential pairings for helix 36 in vertebrate 12S rRNA. Structure *a.* is the one used in Neefs *et al.*(1990) compilation and shown in Table 3.3. The additional guanine and pyrimidine nucleotides which can participate in the pairings are shown in bold in *b.* and *c.* Base pairings are indicated by "|" and "*" (for G*U), and gaps by "_". The proximal (36) and distal (36′) arms of the helix are labelled. Several sequences are available for the skink, moa, and cow groups, so variable sites in these groups are indicated by lower case letters.



Helix 38

The energetic cost required to form helix 38 is high (Table 3.4). Although there is a large unpaired bulge between the distal arms of helices 38 and 37 (Table 3.3), this bulge in itself may not be responsilbe for the increased energetic cost. A similar size bulge occurs between helices 34 and 36 (Table 3.3). Helix 38 is well conserved in sequence and size among the vertebrates however, and compensatory changes also occur (Table 3.3). Furthermore, in the skinks and many of the other vertebrates the adjacent unpaired region is one of the most variables part of the sequence (Tables 3.1 & 3.3), suggesting that the bases potentially able to form helix 38 are functionally constrained. Helix 38 is therefore included in the vertebrate secondary structure model.

The hairpin loop at the end of helix 38 is often a "tetra-loop" (i.e. it consists of four bases; Table 3.3). tetra-loops can be unusually stable and frequently exist in the form of **GNRA** or **UNCG** (where N represents any base, and R is a G or A; Woese *et al.* 1990, Antao *et al.* 1991). They have been suggested to act as nucleation sites for organizing tertiary folding of the RNA (Tuerk *et al.* 1988). Fish, amphibians and lizards have an **RTGA** motif (Table 3.3). This also occurs in the great apes and birds, but the apes and chicken have an additional one or two bases in the loop respectively. Bovids and rodents have **AAAA**. Fur seals tend to have five adenines in this loop (G. Lento pers. comm.). The stability of GNRA tetra-loops are recognised in the MFOLD algorithm and assigned low (more negative) free energy values. The RTGA and AAAA motifs are not allocated similar bonus energies, but it may be possible that they too could confer increased stability in the molecule.

Helix 39

Helix 39, a two base pair structure in the Neefs *et al.* (1990) alignment, has little support in any of the vertebrates. Its suggested position falls within the most variable region of the skink molecule (Table 3.1), and is also variable in lungfish, xantusiids, and bovids (Table 3.3).

Helix 40

Helix 40 is not shown for 12S rRNA in the Neefs *et al* compilation. In the vertebrates however a helix, usually four base pairs in size, can be formed starting four nucleotides after the end of helix 37 (and preceeded by two A's; Table 3.3). Fixed compensatory mutations (Table 3.3) and minimal energy calculations (Table 3.4) support this helix's presence and location in all but the rodents and primates. An energetically more favourable alternative helix 40 can be constructed closer to the 36′ helix for the primates (Tables 3.3 & 3.4). Additional experimental and sequence data are required to establish the existence of this alternate helix 40, but it cannot be formed in the other vertebrates (Table 3.3).

The Refined Secondary Structure Vertebrate Model

The model for domain III derived from these analyses is shown in Figure 3.3, using the skink sequence as a framework. This model differs from that of Glotz & Brimacombe (1980) and Dams *et al.* (1988) in the placement of some of the helices (Fig. 3.4). On the basis of number of bonds, free energy calculations (Fig. 3.4), and the occurrence of high sequence variability (Tables 3.1 & 3.3), neither the

Fig. 3.3. Secondary structure model for *L. n. polychroma* 12S rRNA PCR product. Sites which vary among the 20 skink taxa are shown by lowercase letters. Every tenth base is marked by ".". Dashes (-) and | signify nucleotide pairings (* for G*U), while a colon (":") deontes less certain pairings. Underscores (_) represent stylistic gaps used to simplify the diagram. For secondary structure analyses the molecule was divided into regions as indicated by the underlined numbers (S25′, S26, S20′, S2′ & S29 are part of domain II; see Table 3.3). The positions of the PCR primers 12SA and 12SB are also shown.

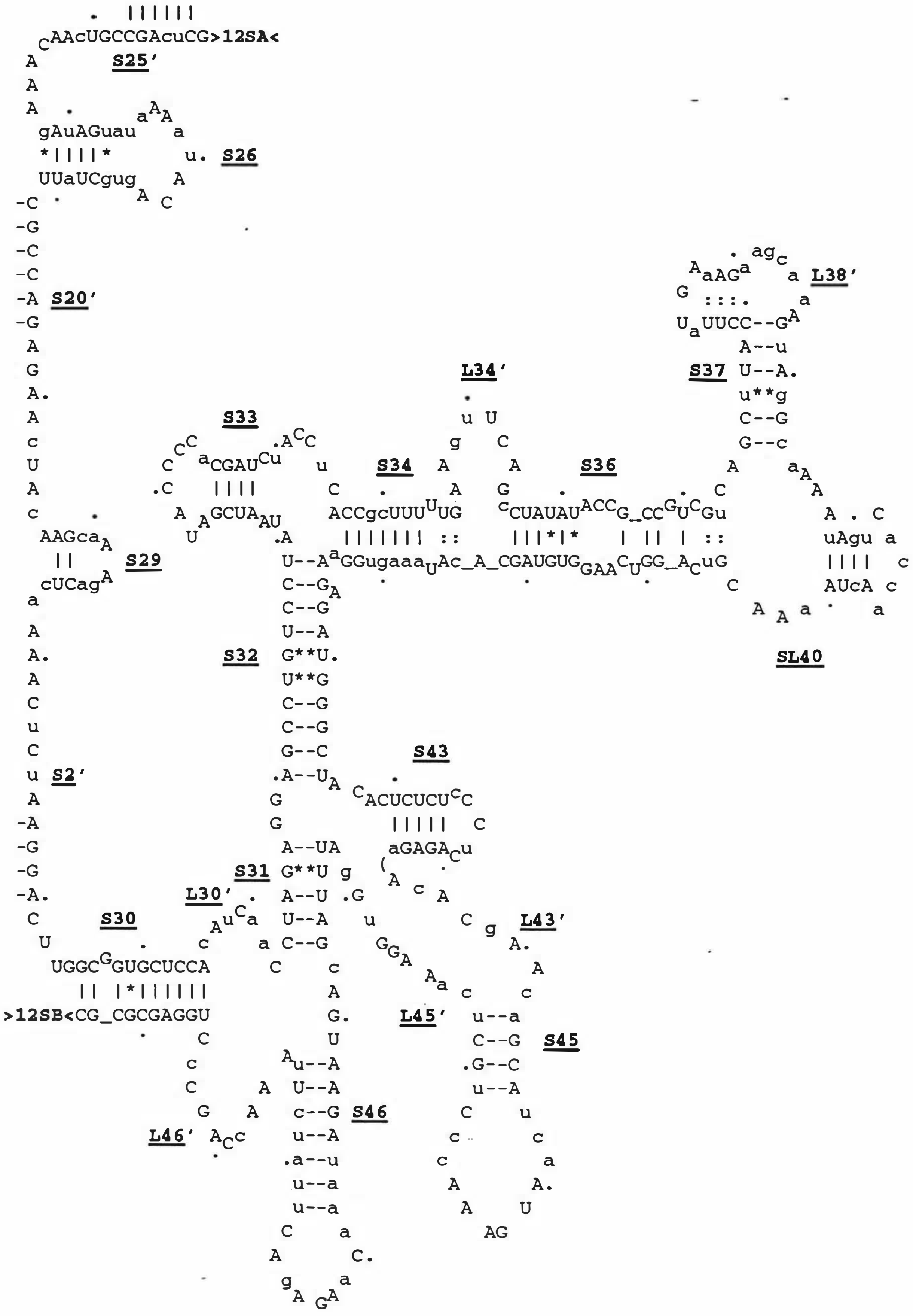


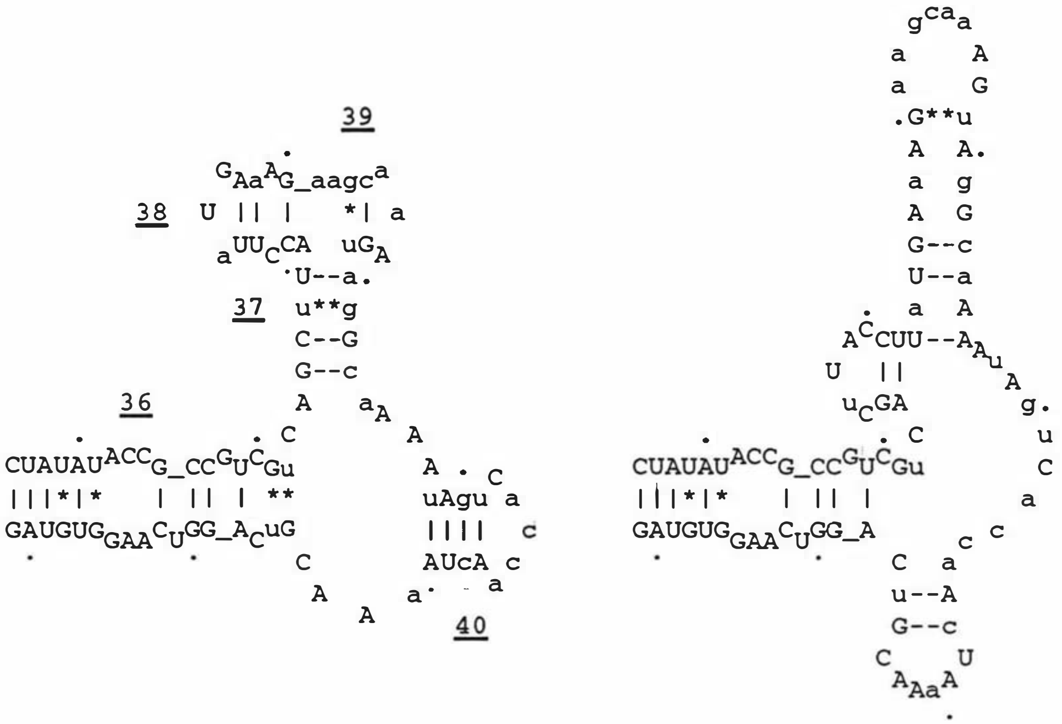
Fig. 3.4. Potential alternative secondary structures for part of skink domain III bordered by helix 36. Structure *a* is based on the Dams *et al.* (1988) model, and structure *b* on the model proposed by Glotz & Brimacombe (1980). Both these structures are energetically less favourable for the skink sequence than the model developed in this thesis (structure *c*), which has a local free energy of -1.7 Kcal/mol. Base pairs are shown by -, |, and *, while artificial gaps included to maintain alignments are indicated by underscores (_). The underlined numbers correspond to helices shown in figure 3.3, and the periods (".") mark every tenth base.

a. Dams et al. (1988) model

Free Energy = **6.8 Kcal/mol**

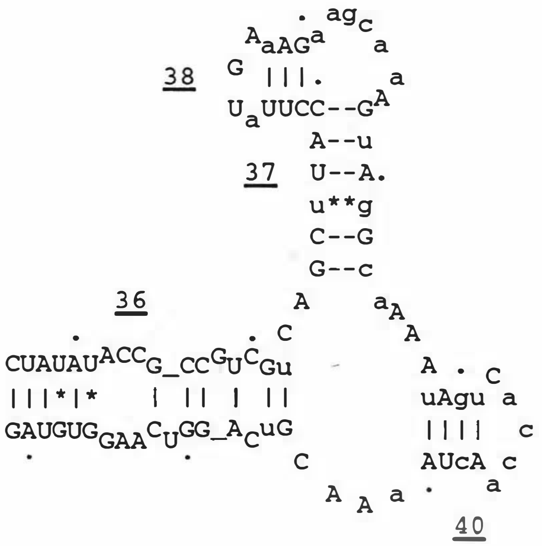
b. Glotz & Brimacombe (1980)

Free Energy = **13.1 Kcal/mol**



c. Skink Model

Free Energy = **-1.7 Kcal/mol**



Glotz & Brimacombe nor the Dams *et al.* models fits the skink or other vertebrate sequences as well as the new model presented (see also Cooper, Hickson, Lento, Simon & Penny *in prep.*).

Conserved Positions in the Secondary Structure Model

The model shown in figure 3.3 has 120 strictly conserved nucleotide positions among the vertebrates (human to fish; Fig. 3.5). Fifty (42%) of these bases are unpaired. While similar numbers of adenine, guanine, cytosine and uracil are conserved, conserved adenine residues are twice as common at unpaired sites (Table 3.5), in agreement with observations of other small subunit rRNAs (Gutell *et al.* 1985).

Variability within Domain III

To investigate how the molecule varies I have divided domain III into sections corresponding to stem+hairpin loops and other unpaired regions (see Table 3.3). The conserved vertebrate core of domain III comprises helices 31, 32, 33 and 36 (Figs. 3.3 & 3.5). Note also the generally conserved nucleotides in the unpaired region between helices 33 and 34 and between helices 45 and 31, and the conserved **UGAAA** motif in the loop of helix 45 (Table 3.3, Fig. 3.3). The single-stranded regions (S20′ & S2′) linking domains II & III are also well conserved.

The six groups of vertebrates for which several sequences are available (skinks, xantusiid lizards, ratites, bovids, apes and mammals) show similar trends in variability (Fig. 3.6). Region S26 is one of the most variable, as determined by number of positions which vary. Other commonly varying regions are S34, SL37, SL40 (Figs. 3.6a-f). Greatest variability in ratites occurs in SL40 (Fig. 3.6c), while S34 and S45 have the most variable sites in the great apes (Fig. 3.6e). Except for the xantusiids, there are relatively few insertion/deletion (indel) events within members of these families. Small, one- or two-base, indels occur in unpaired regions in S26, S29, L34′, L38′, SL40, L43′ & L46′ in the skinks, ratites, and bovids (Table 3.1, Appendix 2). Xantusiids however have indels occurring in fourteen of the 23 regions of the model (Appendix 2).

Nucleotide Substitutions in the Skink Structure

The comparisons of regions shown in figure 3.6 are a minimal estimate of variability, identifying only sites which have changed and not how frequently they may vary. When this is adjusted for in skinks (by multiplying each variable position by the number of taxa which vary at that site), regions L30′ and L38′ are the most variable (Fig. 3.7). These are both small 6-7 base internal loops (Fig. 3.3). Eight positions in the skink sequences have more than two character states, and four of this very variable sites are in either L30′ or L38′ (see Table 3.1). Whether closely related taxa tend to differ at these very variable sites will be examined in the next chapter.

In the secondary structure model for skinks (Fig. 3.3), significantly more changes ($\chi^2 = 4.79$, df = 1, $P < 0.05$) ~~0.001)~~ occur in unpaired regions than in paired regions (Table 3.6). There are 86 paired positions (excluding the bases in S25′, S20′, & S2 whose complementary pairs are not in the sequence examined). Twenty eight (33%) of these have changes in at least one taxon (Table 3.1). Nucleotide

Fig. 3.5. Conserved (█) and non-conserved (▒) sites in vertebrate 12S rRNA domain III, based on comparisons of fish, amphibian, reptile, bird and mammal sequences (see Table 3.3). Every tenth base is marked by ".". Dashes (-) and | signify nucleotide pairings (* for G*U), while a colon (":") denotes less certain pairings. Underscores (_) represent stylistic gaps used to simplify the diagram. For secondary structure analyses the molecule was divided into regions as indicated by the underlined numbers (S denotes a stem region, and L a loop region).

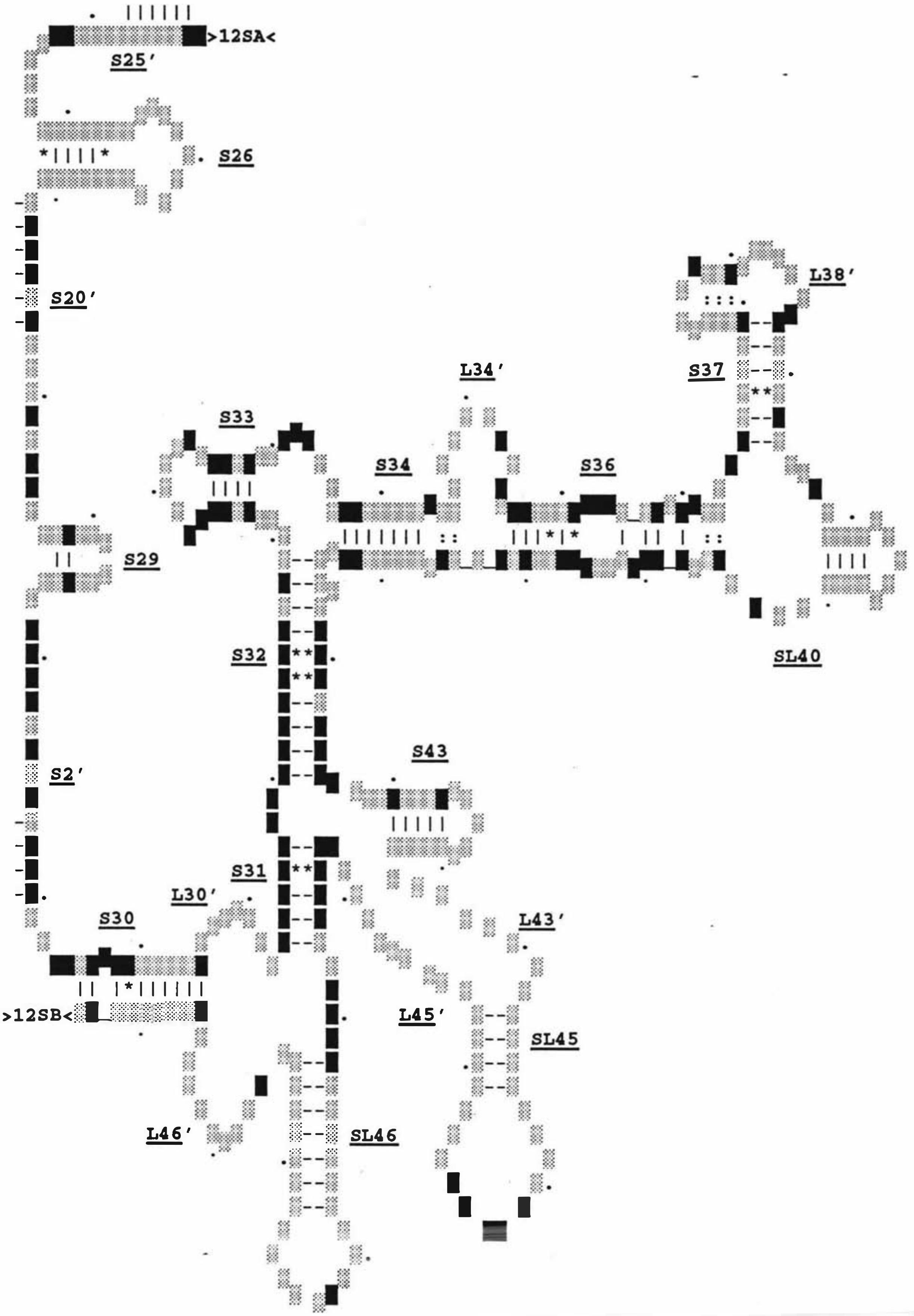


Table 3.5. Distribution of conserved nucleotides at paired and unpaired sites for the vertebrate 12S rRNA model. Since 41.7% of the conserved sites shown in figure 3.5 are unpaired, the expected numbers of conserved nucleotides at unpaired sites were calculated by multiplying the total numbers of each conserved nucleotide by 0.417. Conserved adenine residues are more common at unpaired sites, while conserved guanines tend to occur more frequently at paired sites.

		No. Conserved A	C	G	U	Total
Total		36	27	33	24	120
Paired	*Obs.*	12	17	24	17	70
Unpaired	*Obs.*	24	10	9	7	50
	Exp.	15.0	11.2	13.8	10.0	

$\chi^2 = 8.10, P < 0.05$ (3 degrees of freedom).

Table 3.6. Changes occurring in paired and unpaired regions for the skink 12S rRNA data set (see Fig. 3.3). Expected numbers of changes are calculated on the basis of numbers of paired and unpaired sites.

	No. Bases	Sites Which Vary Observed	Expected
Paired	190	39	50
Unpaired	194	62	51
Total	384	101	

$\chi^2 = 4.79$, df = 1, $P < 0.05$

Regions of variability in domain III of vertebrate 12S rRNA.

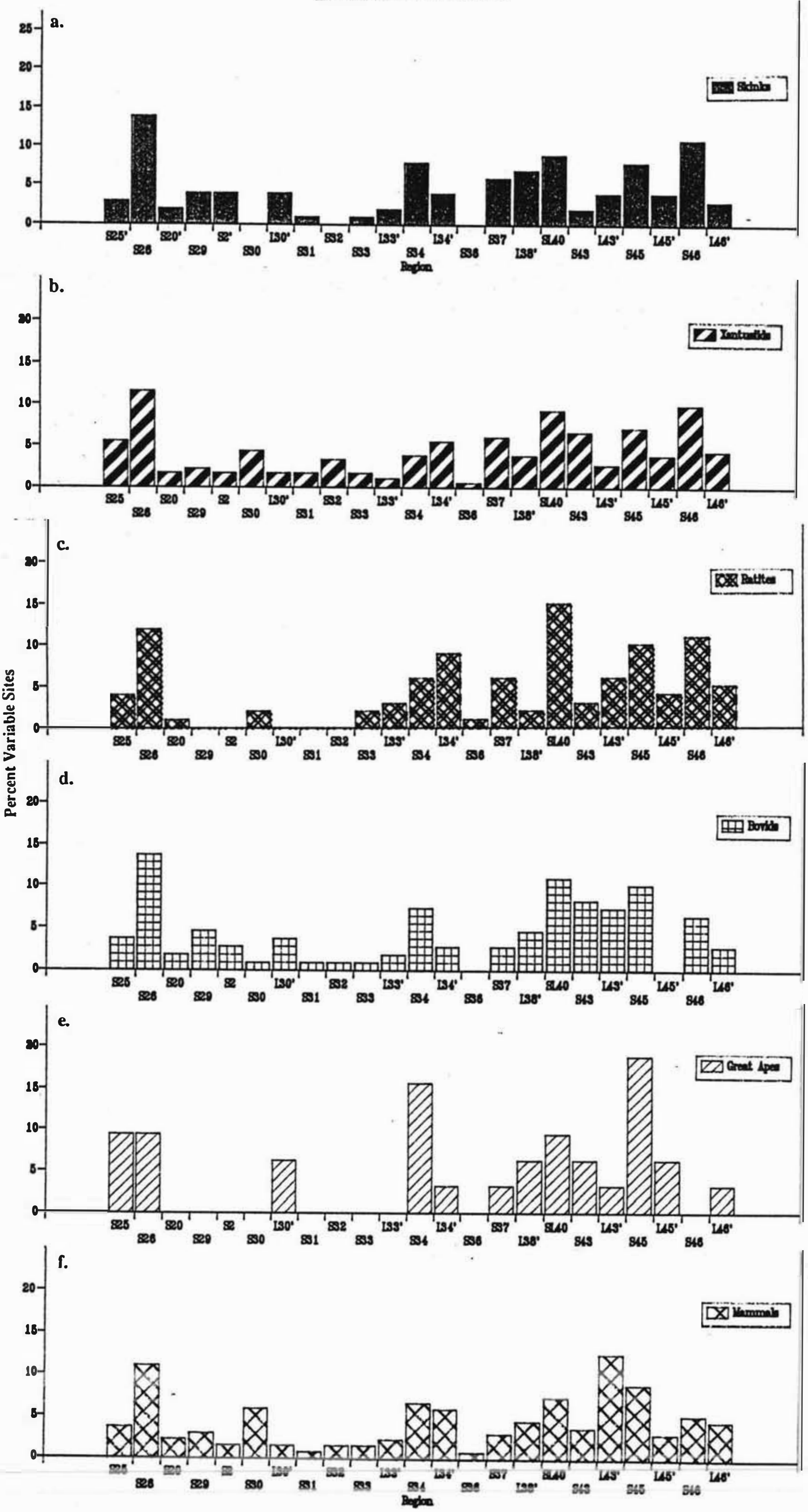
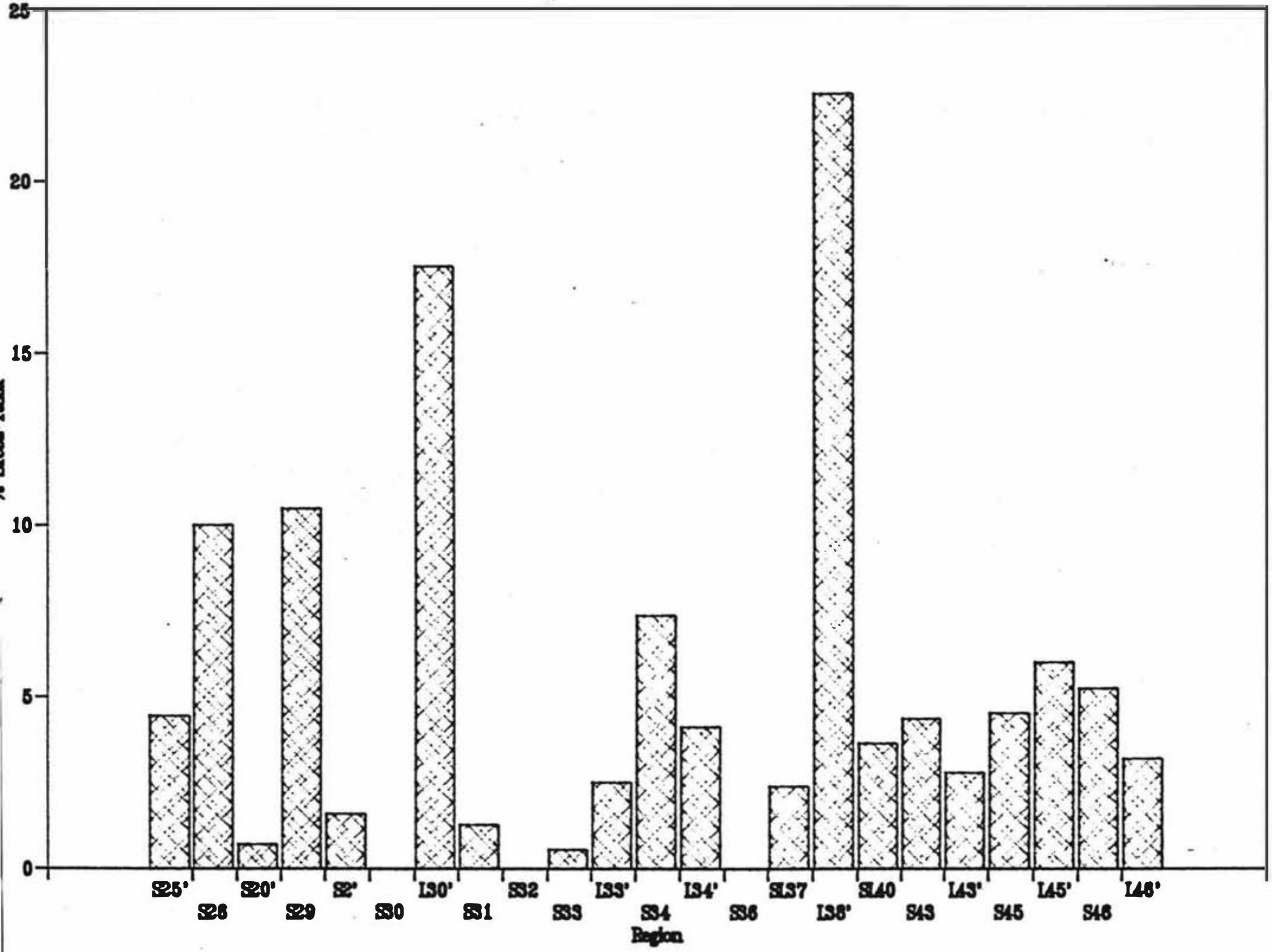


Fig. 3.7. Regions of variability in the skink 12S rRNA sequence. This figure differs from figure 3.6 in that the degree of variability of each site was accounted for using the following formula:

$$\frac{\text{(number variable sites x number taxa which vary) x 100}}{\text{(total number of sites x number of taxa)}}$$

In contrast to figure 3.6a, where S26 has the highest proportion of variable sites, the most variable positions in domain III occur in L30′ and L38′. See also figure 4.17.



pairing (including G-U) is retained in 56 (65%) cases, significantly more than would be expected to occur by chance (18%; χ^2= 84.80, degrees of freedom = 1, $P < 0.001$; see also Table 3.7). Over half (32/56) of the substitutions which maintain complementary pairing are double changes.

There are fewer than expected purine to purine (G ↔ A) transitions (χ^2= 4.11, df = 1, $0.025 < P < 0.05$; Table 3.8), and a lower number of G ↔ C transversions (Table 3.8). Although there is a tendency for more conserved areas (≤ 25% of sites vary) of the sequence to have a higher proportion of guanine residues (Table 3.9), the base composition of conserved nucleotides is similar to the proportions observed for all sites in the skink sequence (Table 3.9). This suggests that differences in the types of transitions and transversions (Table 3.8) is not due to differences in base composition.

DISCUSSION

Two major points emerge from the refinement of a vertebrate secondary structure model for domain III of 12S rRNA. The first is that one third of the sites do not change. This conservation is associated with the single-stranded region linking domains II and III, and what can be termed the structural core of domain III (helices 31, 32, 33, and 36; Fig. 3.5). The proportion of conserved sites in this model will decrease as more vertebrate sequences are added to the compilation, but the results presented here serve to indicate that many sites in the molecule may not be free to vary. Having part of the molecule effectively invariant has implications for models of sequence analysis and this will be considered in Chapter Five.

The second point is that both paired and unpaired regions can have high levels of variability. Helix 26 (and its hairpin loop), helix 34, and the unpaired region following helix 38 have many variable sites in skinks, xantusiids, ratites and bovids (Table 3.3, Fig. 3.6). The phylogenetic and functional significance of these changes are not yet clear, but complementary changes preserve the helices.

Interactions Between Domain III and Ribosomal Proteins

Adenine residues tend to be more highly conserved in unpaired than paired regions (Table 3.5). Gutell *et al.* (1985) suggested that, since adenine is the least basic nucleotide, high conservation of adenine in unpaired regions may reflect hydrophobic interactions between adenine and ribosomal proteins. This does not however account for the observation that nearly one quarter of the conserved A's are located in the single-stranded regions linking domains II and III (Figs. 3.3 & 3.5), where ribosomal proteins do not appear to specifically bind (Ehresmann *et al.* 1990, Noller *et al.* 1990). The ribosomal proteins S7, S9 and S19 bind to domain III (Stern *et al.* 1989, Ehresmann *et al.* 1990, Noller *et al.* 1990), and the conserved residues in L33′ and SL45 (Fig. 3.5) may be involved in this binding, particularly with respect to S19. Noller & Woese (1981) suggested that some phylogenetically variable regions may be protein binding sites. Helix 33 is well conserved however, and appears to interact with S19 (Stern *et al.* 1989, Ehresmann *et al.* 1990, Noller *et al.* 1990). Simon *et al.* (1990) also noted the conservation of this helix in cicadas. Helix 34 in contrast, can be very variable (Tables 3.1 & 3.3), but does not appear to be a primary site of protein binding (see Ehresmann *et al.* 1990). These observations suggest either that the

Table 3.7. Substitutions among paired nucleotides in the skink 12S rRNA sequence data (helices 25′, 20′ & 2′ are excluded). Substitutions are categorized into changes maintaining base pairing (G*U pairs allowed), and those (in either direction) between complementary and non-complementary pairs. Expected numbers of changes were calculated separately for single and double substitutions using Dixon & Hillis' (1992) probabilities for maintenance of complementary pairings (0.125 and 0.256, respectively). For example, 52 single changes were observed, of which (52 x 0.125) = 6.5 are expected to maintain base pairing by chance. Of 17 pairs where both bases changed, 16 (representing 32 substitution events) maintained complementary base pairing, whereas only (17 x 0.256) = 4.4 paired substitutions could be expected to occur. The differences between observed and expected numbers of fixed compensatory changes are highly significant ($P < 0.01$) for both single and double substitutions.

Type of Substitution	Expected	Observed	χ^2 Value
Single			
base pair to base pair	6.5	24	
base pair to non-pair	45.5	28	
			53.8
Double			
base pair to base pair	4.4	16	
base pair to non-pair	12.6	1	
			41.3
Total		86	

Table 3.8a. Expected probabilities for each type of transversion and transition in the skink data set, based on observed nucleotide frequencies (numbers in parentheses). Assuming that the base composition of the sequence determines the frequency with which, for instance, an **A** changes to a **C** rather than to a **U**, then an **A** to **C** change should occur with a probability of [0.27/(0.27 + 0.19)] = 0.59. Similarly, on the basis of the relative frequencies of **A** and **G**, a **C** is twice as likely to change to an **A** than it is to a **G**. On the basis of nucleotide frequencies, slightly more **A** to **G** transitions are expected than **C** to **U**.

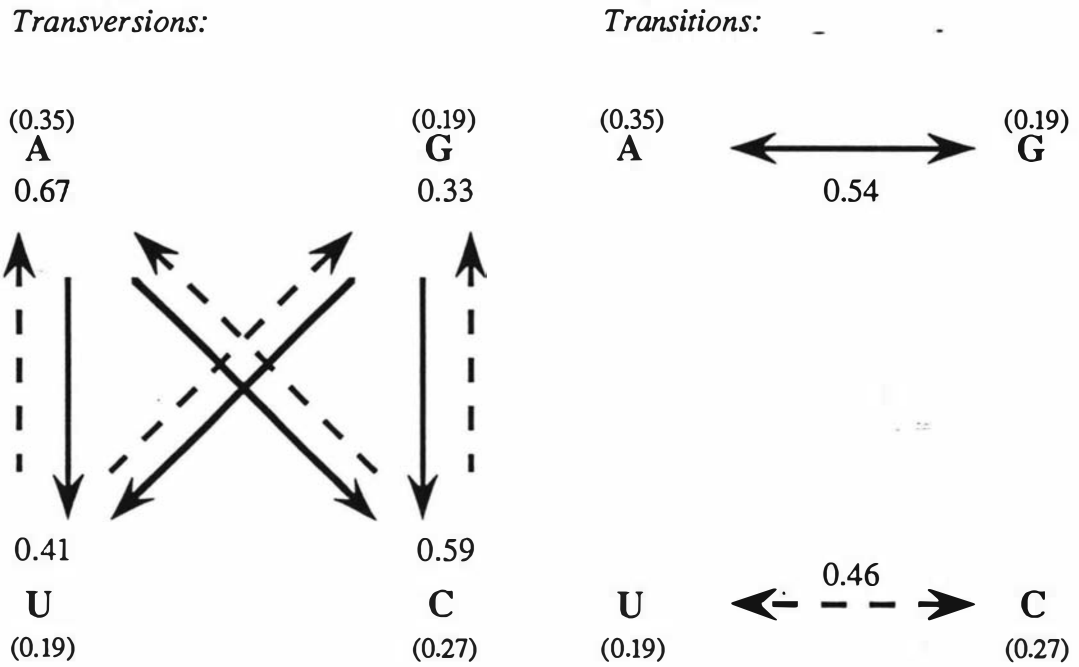


Table 3.8b. Observed and expected (based on Table 3.8a) numbers of each type of transversion and transition among the 20 skink taxa. The direction of change is not assumed.

Transversions:

	A <-> C	A <-> U	G <-> C	G <-> U	
No. Obs.	19	11	6	2	
No. Exp.	18.2	7.6	11.4	3.8	$\chi^2 = 4.97$, df = 3, $P > 0.1$

Transitions:

	A <-> G	C <-> U	
No. Obs.	40	52	
No. Exp.	49.7	42.3	$\chi^2 = 4.11$, df = 1, $0.025 < P < 0.05$.

Table 3.9. Base composition of conserved residues among regions of skink 12S rRNA domain III. Regions in bold highlight those where more than 75% of their sites are conserved. The expected proportions of each conserved nucleotide (based on nucleotide frequency; Table 2.1) is shown at the bottom of the table.

	No.	Conserved			Conserved		
Region	Bases	No.	%	% A	% C	% G	% U
S25'	17	14	82%	43	29	21	7
S26	24	12	50%	50	17	8	25
S20'	15	13	87%	46	23	23	8
S29	10	6	60%	50	17	17	17
S2'	18	14	78%	43	21	21	14
S30	21	21	100%	10	33	44	14
L30'	6	2	33%	50	50	0	0
S31	15	14	93%	29	14	29	29
S32	26	26	100%	27	23	31	19
S33	18	17	94%	29	35	12	24
L33'	8	6	75%	33	67	0	0
S34	23	14	65%	14	21	21	43
L34'	9	6	67%	50	17	17	17
S36	34	32	94%	22	25	31	22
S37&38	22	16	73%	25	19	31	25
L38'	7	1	14%	100	0	0	0
SL40	23	13	57%	69	23	0	8
S43	16	13	81%	15	46	15	23
L43'	8	5	62%	80	20	0	0
S45	20	13	65%	39	30	23	8
L45'	10	6	60%	50	0	50	0
S46	25	12	48%	50	17	17	17
L46'	9	7	78%	43	43	14	0
	384	283	*Observed:*	34%	26%	23%	17%
			Expected:	35%	27%	19%	19%

$\chi^2 = 1.11$, df = 3, not significant.

mitochondrial small subunit rRNA molecule may not have the same points of protein contact as the prokaryote molecule, or that not all protein binding regions are variable. The conserved nucleotides in the unpaired regions L33′ and SL45 (Fig. 3.5), and their probable interaction with S19 indicate that the latter point may be the more likely interpretation.

Helix 36 is the probable site of contact with the 50-S ribosome subunit in *E. coli* (Gutell *et al.* 1985). The unpaired bases in this helix, as well as the different potential alternate forms of it (Fig. 3.4), may be associated with protein recognition sites (see Peattie *et al.* 1981, Gutell *et al.* 1985, Noller 1990).

Tetra-loops

While the hairpin loop of helix 38 does not conform in either sequence (or size in some cases) to the common GNRA tetra-loop (Woese *et al.* 1990, Antao *et al.* 1991), it is interesting that the last nucleotide in the loop is generally an adenine (Table 3.3). Sea urchins (sequences not shown) do not conform to the GNRA or RTGA motif for loop 38 either, but they too have adenine as the final base. As already noted above, adenine residues have been found to be associated with protein binding (Gutell *et al.* 1985), so the helix 38 hairpin loop and the adenine residue may have a critical role in the formation of the tertiary structure. The other tetra-loop in the 12S rRNA alignment presented here is in helix 29 (Table 3.3). Its sequence is not as conserved as that in helix 38, nor does it fit the more common tetra-loop motifs (Woese *et al.* 1990), but the terminal base is also generally an adenine (Table 3.3). Insertion/deletions commonly occur in this hairpin loop however (Tables 3.1 & 3.3) so the significance of these putative tetra-loops is uncertain. The number of tetra-loops varies among vertebrate 12S rRNA sequences. There are four in human and seven in the rat (Gutell *et al.* 1985), suggesting that the tetra-loops may not have a major functional role.

Comparison of Secondary Structure Models

The model presented here is very similar to Noller & Woese's (Gutell *et al.* 1985, Noller *et al.* 1990), but it provides a more detailed view of the vertebrate 12S rRNA domain III. Previous alignments and models used only human, cow, mouse, and frog 12S rRNA sequences (Gutell *et al.* 1985, Dams *et al.* 1988, Neefs *et al.* 1990). Addition of reptile and bird sequences in the present study emphasizes differences in sites of variation between groups (Fig. 3.6). The structure in figure 3.5 is different from that proposed for the great apes (Hixson & Brown 1986). Helix 36 in the Hixson & Brown model is shifted slightly, and a five base pair helix (labelled 24 by Hixson & Brown) occupies the region encompassing helices 38 and 40 in figure 3.5. Neither of these two features is supported by the comparative approach adopted here.

Simon *et al.* (1990) found that 12S rRNA sequences from several species of cicada could fit both the Glotz & Brimacombe (1980) and Dams *et al.* (1988) models for the region between helices 37 and 40. Rather than being a conflict in structure, Simon *et al.* suggested that local switching between alternative structures may occur. Analysis of a wider range of 12S rRNA sequences indicate that this switching may not occur. On the basis of comparative sequence analysis, the presence of compensatory changes, and examination of minimal free energy structures, one structure, common to all of the vertebrates examined, as well as some invertebrates, is most favoured (Fig. 3.5 and Cooper *et al. in prep.*). As Simon *et al.* noted though, and the present analyses indicate, experimental evidence for the structure of this region is desirable. Switching may occur in other parts of the rRNA, and play a role in ribosome assembly (Glotz & Bimacombe 1980, Zwieb *et al.* 1981).

The degree of base conservation in a helix does not appear to be related to whether the nucleotides of one side of the helix are close to (short-range) or further away from (long-range) the nucleotides forming the other side of the helix, as has been suggested by Simon (1991). Helix 33 is a short range stem and well-conserved, whereas helix 34 can be classed as a long-range stem but is more variable (Figs. 3.3 & 3.5). It is interesting to note that in the skinks, most of the variability in helix 34 occurs on the distal arm of the helix, though pairing is still maintained (Table 3.1). This could imply that the five uracil residues on the proximal arm are more constrained, but both sides of this helix vary in other vertebrates (Table 3.3).

Limitations of the RNA Folding Algorithm

The energetic calculations were of some use in discounting the existence of helix 35, but implied that helix 38 was unlikely to form (Table 3.4). Zuker *et al.* (1991) noted however that optimal energy solutions for domain III of 16S rRNA were not always in agreement with the structure determined by comparative sequence analysis, though the reasons for this are unclear. Experimental evidence (Glotz & Brimacombe 1980, Gutell *et al.* 1985), or the occurrence of fixed compensatory mutations are required before helix 35 can be accepted. The potential for an alternative helix 40 to form in humans and other apes (Tables 3.3 & 3.4) also requires further sequence and experimental analyses. The secondary structure folding of the 16S rRNA appears to be primarily determined by the sequence and experimental evidence does not indicate that ribosomal proteins have a significant role in stabilizing structures (Draper 1990, Noller *et al.* 1990).

Another limitation in the folding algorithm (Zuker 1989, Zuker *et al.* 1991) is that non-standard base pairs (for instance A-C, and G-A) are not yet considered in the calculations, so minimal free energies could be underestimated. A-C pairs can be relatively common in rRNA and tRNA (Topal & Fresco 1976, Anderson *et al.* 1981, de Bruijn & Klug 1983, Kraus *et al.* 1992), though they have larger free energies (Freier *et al.* 1986). A-G pairs may also occur, but their formation may be determined by adjacent nucleotides (Cheng *et al.* 1992). Interactions between bases within loops can also occur (Heus & Pardi 1991), which may also affect the free energy level of the molecule. A-C pairings have the potential to occur in helices 36 and 37 (Table 3.3, Fig. 3.3), and could also increase the size of helix 40 in mammals (Table 3.3). Taking account of A-C pairings may therefore support the more common helix 40 over the alternative helix 40 in the great apes (see Tables 3.3 & 3.4, and Fig. 3.5). While non-standard pairing may be a possibility in helix 36, the unpaired nucleotides may be important for protein binding, and experimental investigations suggest that unpaired bases occur in helix 36 (Gutell *et al.* 1985, Noller *et al.* 1990).

Variability between Regions

Regions of relatively high or low variability may be more informative for examining more closely or distantly related taxa respectively (Kocher *et al.* 1989, Thomas & Beckenbach 1989, Simon 1991, Simon *et al.* 1991). Different regions in the 12S rRNA sequence do have different levels of variability (Figs. 3.6 & 3.7). Comparisons between closely and more distantly related skink taxa in relation to the more variable regions and sites will be considered in Chapters Four and Five.

Knowledge of which regions or sites are more conservative can be also be useful for determining the closeness of a relationship. A change shared by two taxa at a more conservative site in the molecule may be a more reliable indicator of true phylogenetic relationships than when taxa share a common substitution at more variable positions. *L. fallai* and *C. aenea* for example have the same change at position 73 (Table 3.1), a conservative region in vertebrates (Table 3.3). *C. aenea*, *L. fallai* and *L. maccanni* also differ from other taxa by a change in the otherwise conserved loop of helix 33 (Table 3.1), suggesting that *C. aenea* and *L. fallai* have a relatively close relationship. However, a change in the tetra-loop of helix 38 could also imply a strong relationship between *C.aenea*, *L. maccanni* and *L. telfairi* (position 194; Table 3.1). These conflicts will be considered in Chapter Five. In contrast, *L. fallai* shares two substitutions with a New Caledonian skink, *Tropidoscincus rohssii* (see Chapter Five), but these are both at quite variable positions in helix 46, implying that these two taxa are not that closely related.

Secondary Structure Models and Sequencing Precision

Placing nucleotide sequences in the context of a secondary structure model has the additional advantage of identifying possible errors in sequence determination. There are several cases in Table 3.3 where one taxon differs from the rest at positions which are otherwise constant. These may be real differences or the results of cloning, PCR, and/or sequencing artifacts. Seven positions in the skink sequences are variable, while they are constant among the other vertebrates. At least three primers were used to sequence the skink PCR fragment and several sequencing reactions run. Rechecking the sequencing gels did not reveal ambiguities at these seven sites suggesting that errors during sequencing are probably not the cause. At five of these positions several skink taxa vary (Table 3.1) supporting the reliabilty of the amplification and sequencing reactions. At another site (position 233) however a gel reading error was found (and corrected) when the sequence was compared to the secondary structure model.

During the compilation of table 3.3 it was noticeable that sequences obtained by cloning had more inconsistencies that sequences obtained by PCR. Changes in otherwise well conserved positions were particularly frequent in the frog and whale sequences. The frog sequence differs uniquely at seven locations, including a deletion in L30' (Table 3.3) and additional sequencing of this region is required to confirm the sequence. In the whale sequence there is a large, approximately 10 base, insertion in the unpaired region following helix 43 (Table 3.3). This unusual insertion is not remarked upon by the authors (Arnason *et al.* 1991). In addition, the whale sequence has only three nucleotides in the loop of helix 38, whereas other mammals have at least four (Table 3.3). The whale also has an apparent insertion of a guanine residue into the 30′ helix (Table 3.3).

There are also problems with some of the other sequences. Three 12S rRNA sequences for *Rattus norvegicus* can be found in GenBank (accession numbers X14848, J01438 & V00680). The latter two differ at several sites though from X14848, the one used in table 3.3. The quail 12S rRNA sequence (Desjardins & Morais 1989) was excluded from these analyses altogether because a block of about 50 nucleotides was missing. Sequencing errors are a recognized problem in the databases (see for example Clark & Whittam 1992, States 1992). Corrections to the cloned toad 12S rRNA sequence have already been published (Dunon-Bluteau & Brun 1986). It is prudent to confirm the nucleotide sequences discussed here by resequencing the relevant species.

Bias in Types of Substitutions

Bias in types of nucleotide substitutions has been observed in many sequencing studies (for example, Aquadro *et al.* 1984, Thomas & Beckenbach 1989, Marshall 1992, Knight & Mindell 1993; see also Moritz *et al.* 1987). Thomas & Beckenbach (1989) attributed a C→A transversion bias to preferential loss of guanine residues by depurination (Lindahl & Nyberg 1972). The direction of transversions cannot be determined for the skinks since an appropriate outgroup is not available, A↔C transversions are the most common however (Table 3.8), and this bias does not appear to be related to base composition (Table 3.9).

*Chapter Four: **Transitions and Transversions***

Two major features of the skink sequence data set are that, firstly, many taxa are about equally divergent from each other, but secondly, there is considerable variation in the numbers of transitions and transversions between them. These two features are examined in this chapter and compared with other vertebrate data sets and with simulated data. The patterns of differences are examined with respect to the secondary structure model developed in Chapter Three to understand both how the molecule changes and to identify the most variable regions. This information is subsequently used in Chapter Five.

Transitions and Transversions

There are 101 variable positions in the skink data set (Table 3.1), of which 58 are phylogenetically informative (parsimony sites). Fifty of the nucleotide substitutions are singletons, that is one taxon differs from all the rest. Two single base deletions occur - at positions 25 (in both *L. telfairi* and *L. maccanni*) and 6[illegible] (*L. telfairi* only). *L. telfairi* also has an extra base inserted between nucleotides 202 and 203 (Table 3.1).

Most pairs of taxa have between 20 and 25 observed differences (Table 4.1a, Fig. 4.1a), and four to five transversions in pairwise comparisons (Table 4.1b, Fig. 4.1c). There is considerable variability however. Some taxa, for instance "Stewart Island Green", *L. grande* and *La. guichenoti* have more variation associated with the number of transitions, while others, such as *L. notosaurus*, *C. aenea* and *L. inconspicuum*, show greater variation with respect to transversions (Table 4.2). *L. infrapunctatum* has the highest mean number of transversions and also the lowest transition/transversion (Ts/Tv) ratio (Tables 4.1 & 4.2).

While there is a substitution bias in favour of transitions (Table 4.1b), a very weak correlation exists between the number of transversions and the number of transitions (r^2= 0.03; Table 4.3, Fig. 4.2). Consequently, transversions are a poor predictor of the total number of differences between taxa (r^2= 0.26, Fig. 4.3), particularly when there are fewer than eight transversions (Table 4.3). As a comparison, stronger relationships exist between the number of transitions and transversions in both ratite (r^2= 0.40, Fig. 4.4a) and bovid (r^2= 0.55, Fig. 4.4b) data sets.

Saturation of Transitions?

There is no evidence to suggest that the skink sequences have become saturated with nucleotide substitutions. The skink sequences are less than 10% divergent and the proportion of transitions does not decrease as sequence divergence increases (Fig. 4.5), which is usually an indication of saturation (Wilson *et al.* 1985, DeSalle *et al.* 1987, Miyamoto & Boyle 1989, Irwin *et al.* 1991).

Table 4.1a. Distance matrices for skink 12S rRNA PCR fragment (384 base pairs). Total observed nucleotide differences are given below the diagonal and percentage transitions are above. Numbers in bold denote pairwise comparisons with 20 or fewer total differences. Underlined numbers identify where transitions comprised less than 75% of the differences. The mean number of nucleotide differences for each taxon is also given. Insertions and deletions when they occurred in pairwise comparisons were not counted. Taxon abbreviations as in Table 3.1.

Number of Differences (below diagonal)\ Percent Transitions (above)

	Sig	**Lgr**	**Lno**	**Llc**	**Lsu**	**Lnn**	**Lmi**	**Lag**	**Cae**	**Lac**	**Lin**	**Lsm**	**Lze**	**Lfa**	**Lma**	**Lte**	**Lnp**	**Lfr**	**Lot**	**Lmo**	
Sig	-	63%	73%	71%	75%	77%	72%	80%	82%	74%	76%	75%	77%	74%	74%	83%	70%	7%	88%	82%	Sig
Lgr	**8**	-	80%	80	79	85	75	71	87	73	79	79	83	79	87	75	79	71	83	87	Lgr
Lno	**15**	**15**	-	86%	75	75	76	72	81	72	92	79	78	74	90	78	81	68	78	80	Lno
Llc	**17**	**20**	21	-	84%	79	74	67	79	78	85	75	73	68	95	83	84	73	87	77	Llc
Lsu	**16**	**14**	**16**	**19**	-	90%	72	76	83	77	75	76	81	77	83	72	91	74	80	83	Lsu
Lnn	**17**	**20**	**16**	**14**	**20**	-	79%	75	83	77	79	80	81	76	84	72	69	71	83	81	Lnn
Lmi	**18**	**16**	21	23	**18**	24	-	74%	87	76	76	100	90	81	83	71	77	73	85	87	Lmi
Lag	**15**	21	25	**18**	21	**20**	23	-	78%	71	72	76	77	71	75	67	75	67	86	77	Lag
Cae	22	23	21	24	23	23	23	23	-	78%	78	88	87	88	84	68	79	75	87	96	Cae
Lac	**19**	22	25	**18**	22	22	25	**14**	23	-	72%	79	76	73	87	74	77	75	92	78	Lac
Lin	21	**19**	**12**	27	**20**	24	25	29	23	29	-	79%	74	74	85	78	83	67	74	79	Lin
Lsm	**20**	**19**	24	24	21	25	**5**	25	24	28	28	-	91%	80	84	74	80	74	87	88	Lsm
Lze	22	24	23	22	26	26	**20**	27	23	25	23	23	-	79%	83	70	84	70	87	84	Lze
Lfa	23	24	23	22	26	25	26	24	**17**	26	27	25	24	-	76%	71	75	67	84	88	Lfa
Lma	24	22	**20**	21	23	25	30	28	25	30	**20**	31	30	25	-	89%	86	76	89	83	Lma
Lte	23	24	23	24	25	25	24	30	22	27	27	27	23	31	28	-	80%	69	78	80	Lte
Lnp	23	24	21	**19**	21	**13**	22	28	28	30	29	25	32	32	29	30	-	74%	78	77	Lnp
Lfr	24	28	28	22	27	24	30	**12**	28	24	30	31	27	27	29	32	34	-	81%	72	Lfr
Lot	26	23	23	30	25	29	26	28	23	24	23	31	31	32	28	27	32	32	-	85%	Lot
Lmo	27	30	25	26	30	26	30	26	22	27	29	33	25	24	29	30	31	29	27	-	Lmo
	Sig	**Lgr**	**Lno**	**Llc**	**Lsu**	**Lnn**	**Lmi**	**Lag**	**Cae**	**Lac**	**Lin**	**Lsm**	**Lze**	**Lfa**	**Lma**	**Lte**	**Lnp**	**Lfr**	**Lot**	**Lmo**	
Means	*20.0*	*20.8*	*20.9*	*21.6*	*21.7*	*22.0*	*22.6*	*23.0*	*23.2*	*24.2*	*24.5*	*24.7*	*25.1*	*25.4*	*26.2*	*26.4*	*26.5*	*27.3*	*27.4*	*27.7*	

Table 4.1b. Pairwise comparisons of transitions (above diagonal) and transversions (below). **Bold** denotes comparisons with 20 or fewer total differences. The mean numbers of transitions and transversions for each taxon are shown on the right and below the table, respectively.

Transversions (below diagonal) \ Transitions (above)

	Sig	Lgr	Lno	Llc	Lsu	Lnn	Lmi	Lag	Cae	Lac	Lin	Lsm	Lze	Lfa	Lma	Lte	Lnp	Lfr	Lot	Lmo		Mean Ts
Sig	-	**5**	**11**	**12**	**12**	**13**	**13**	**12**	18	**14**	16	**15**	17	17	20	16	17	17	23	22	Sig	*14.3*
Lgr	**3**	-	**12**	**16**	**11**	**17**	**12**	15	20	16	**15**	**15**	20	19	19	18	19	20	19	26	Lgr	*16.5*
Lno	**4**	**3**	-	18	**12**	**12**	16	18	17	18	11	19	18	17	**18**	18	17	19	18	20	Lno	*16.3*
Llc	**5**	4	3	-	**16**	**11**	17	**12**	19	**14**	23	18	16	15	20	20	**16**	16	26	20	Llc	*17.1*
Lsu	**4**	**3**	**4**	**3**	-	**18**	**13**	16	19	17	**15**	16	21	20	19	18	19	20	20	25	Lsu	*17.2*
Lnn	**4**	**3**	**4**	**3**	**2**	-	19	15	19	17	19	20	21	19	21	18	**9**	17	24	21	Lnn	*17.4*
Lmi	**5**	**4**	5	6	**5**	5	-	17	20	19	19	**5**	**18**	21	25	17	17	22	22	26	Lmi	*17.8*
Lag	**3**	6	7	**6**	5	**5**	6	-	18	**10**	21	19	21	17	21	20	21	**8**	24	20	Lag	*17.1*
Cae	4	3	4	5	4	4	3	5	-	18	18	21	20	**15**	21	15	22	21	20	21	Cae	*19.1*
Lac	**5**	6	7	**4**	5	5	6	**4**	5	-	21	22	19	19	26	20	23	18	22	21	Lac	*18.6*
Lin	5	**4**	1	4	**5**	5	6	8	5	8	-	22	17	20	**17**	21	24	20	17	23	Lin	*18.9*
Lsm	**5**	**4**	5	6	5	5	**0**	6	3	6	6	-	21	20	26	20	20	23	27	29	Lsm	*19.9*
Lze	5	4	5	6	5	5	**2**	6	3	6	6	2	-	19	25	16	27	19	27	21	Lze	*20.2*
Lfa	6	5	6	7	6	6	5	7	**2**	7	7	5	5	-	19	22	24	18	27	21	Lfa	*19.4*
Lma	4	3	**2**	1	4	4	5	7	4	4	**3**	5	5	6	-	25	25	22	25	24	Lma	*22.0*
Lte	7	6	5	4	7	7	7	10	7	7	6	7	7	9	3	-	24	22	21	24	Lte	*19.7*
Lnp	6	5	4	**3**	2	**4**	5	7	6	7	5	5	5	8	4	6	-	25	25	24	Lnp	*21.0*
Lfr	7	4	9	6	7	7	8	**4**	7	6	10	8	8	9	7	10	9	-	26	21	Lfr	*19.7*
Lot	3	4	5	4	5	5	4	4	3	2	6	4	4	5	3	6	7	6	-	23	Lot	*23.0*
Lmo	5	4	5	6	5	5	4	6	1	6	6	4	4	3	5	6	7	8	4	-	Lmo	*22.7*

	Sig	Lgr	Lno	Llc	Lsu	Lnn	Lmi	Lag	Cae	Lac	Lin	Lsm	Lze	Lfa	Lma	Lte	Lnp	Lfr	Lot	Lmo
MeanTv	*4.7*	*4.3*	*4.6*	*4.5*	*4.5*	*4.6*	*4.8*	*5.9*	*4.1*	*5.6*	*5.6*	*4.8*	*4.9*	*6.0*	*4.2*	*6.7*	*5.5*	*7.6*	*4.4*	*5.0*

Fig. 4.1. Frequency distributions for pairwise comparisons of nucleotide substitutions in the 20 skink 12S rRNA sequences. *a.* Total numbers of differences between taxa. *b.* Numbers of transitions. *c.* Numbers of transversions.

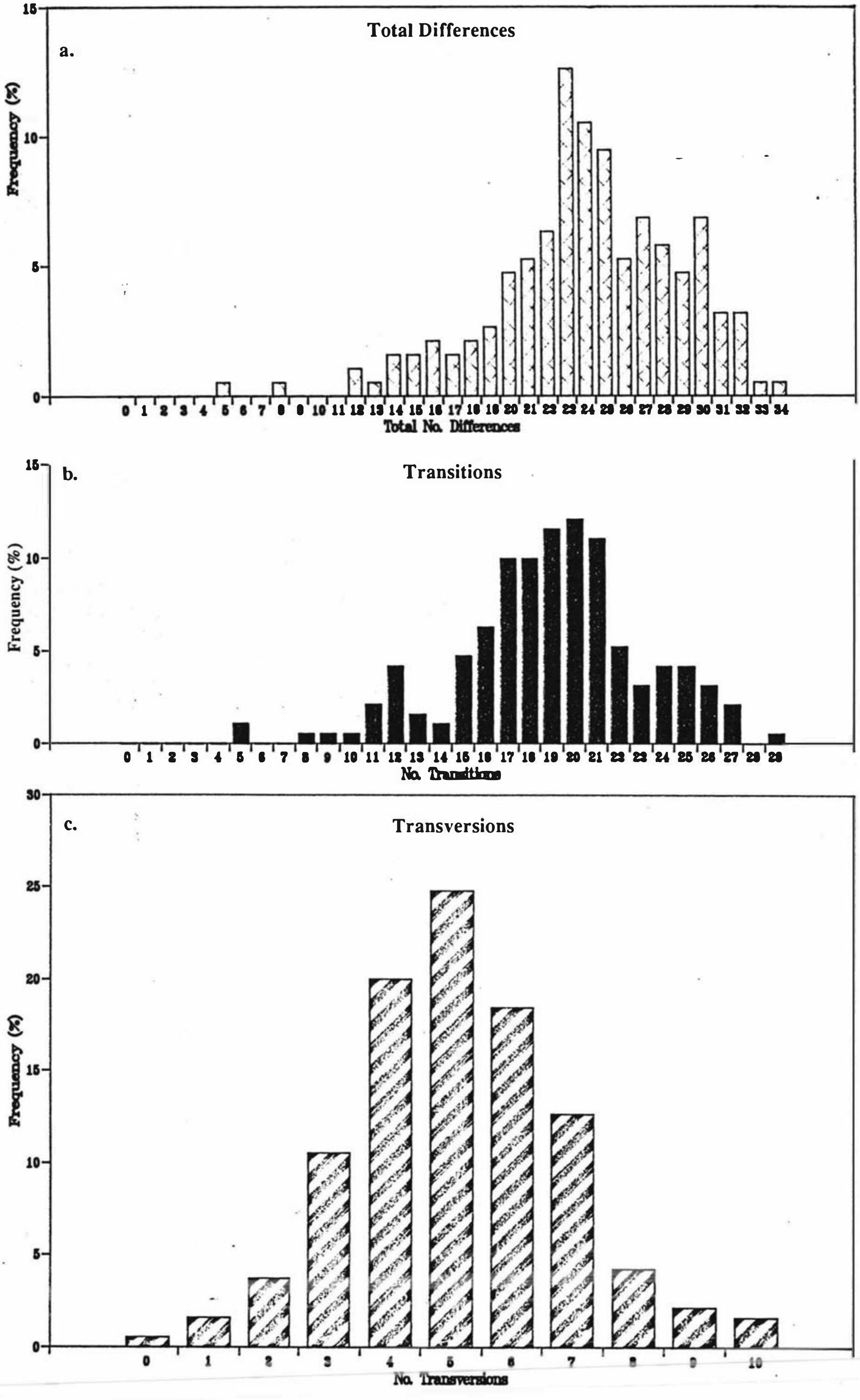


Table 4.2. Mean numbers (± standard deviation) of total observed differences, transitions, transversions, and transition/transversion ratios for each skink taxon. The five most variable taxa for each column (assessed as the largest standard deviations relative to their means) are shown in bold.

Taxon	Total	Transitions	Transversions	Ts/Tv
St.Is.Green	**20.0 ± 4.6**	**15.3 ± 4.4**	4.7 ± 1.2	3.4 ± 1.3
L.grande	**20.8 ± 5.1**	**16.5 ± 4.5**	4.3 ± 1.4	4.1 ± 1.4
L.notosaurus	20.9 ± 4.3	16.3 ± 3.0	**4.6 ± 1.8**	4.2 ± 2.2
L.lin/chl	21.6 ± 3.8	17.1 ± 3.8	4.5 ± 1.5	**4.7 ± 4.0**
L.suteri	21.7 ± 4.2	17.2 ± 3.6	4.5 ± 1.4	4.2 ± 2.0
L.n.nigriplantare	22.0 ± 4.4	17.4 ± 3.9	4.6 ± 1.3	4.0 ± 1.5
L.microlepis	**22.6 ± 5.8**	**17.8 ± 4.9**	**4.8 ± 1.8**	4.0 ± 1.8
La.guichenoti	**23.0 ± 5.3**	**17.1 ± 4.3**	5.9 ± 1.6	3.0 ± 0.9
C.aenea	23.2 ± 2.3	19.1 ± 2.0	**4.1 ± 1.6**	**5.7 ± 4.0**
L.acrinasum	24.2 ± 4.2	18.6 ± 3.7	5.6 ± 1.4	**3.7 ± 2.0**
L.inconspicuum	24.5 ± 4.7	18.9 ± 3.3	**5.6 ± 2.0**	3.9 ± 2.0
L.smithi	**24.7 ± 6.1**	**19.9 ± 5.2**	**4.8 ± 1.8**	4.5 ± 2.1
L.zelandicum	25.1 ± 3.2	20.2 ± 3.2	4.9 ± 1.5	4.7 ± 2.2
L.fallai	25.4 ± 3.6	19.4 ± 2.9	6.0 ± 1.8	3.6 ± 1.5
L.maccanni	26.2 ± 3.7	22.0 ± 3.0	**4.2 ± 1.5**	**6.3 ± 3.7**
L.telfairi	26.4 ± 3.1	19.7 ± 2.9	6.7 ± 1.7	3.2 ± 1.5
L.n.polychroma	26.5 ± 5.5	21.0 ± 4.4	5.5 ± 1.7	4.1 ± 1.7
L.infrapunctatum	27.3 ± 4.8	19.7 ± 3.9	7.6 ± 1.5	2.6 ± 0.6
L.otagense	27.4 ± 3.3	23.0 ± 3.1	4.4 ± 1.3	5.7 ± 2.0
L.moco	27.7 ± 2.8	22.7 ± 2.5	5.0 ± 1.6	**5.6 ± 4.0**

Table 4.3. Variability (mean ± standard deviation) in the total number of observed nucleotide substitutions and numbers of transitions, and the transition/transversion (Ts/Tv) ratio in relation to the numbers of transversions in the skink data set. There is no significant increase in the number of transitions as the number of transversions increases ($F_{9,368} = 1.81$, $P = 0.065$). The correlation coefficients between numbers of transversions and respectively, the total number of differences and the numbers of transitions are also shown.

No.Tv	No. Obs.	Total	Transitions	Ts/Tv
1	6	18.3 ± 4.9	17.3 ± 5.0	17.3 ± 4.9
2	14	20.7 ± 2.2	18.7 ± 2.2	9.4 ± 1.1
3	40	20.5 ± 5.1	17.5 ± 5.1	5.8 ± 1.7
4	76	22.8 ± 5.7	18.8 ± 5.7	4.7 ± 1.4
5	94	24.2 ± 3.7	19.2 ± 3.7	3.8 ± 0.7
6	70	25.1 ± 2.9	19.1 ± 2.9	3.2 ± 0.5
7	48	26.1 ± 2.7	19.1 ± 2.7	2.7 ± 0.4
8	16	29.4 ± 1.5	21.4 ± 1.5	2.7 ± 0.2
9	8	30.0 ± 2.9	21.0 ± 2.9	2.3 ± 0.3
10	6	30.7 ± 1.0	20.7 ± 1.0	2.1 ± 0.1
All		24.1 ± 4.9	18.9 ± 4.2	4.3 ± 2.5
r^2		**0.26**	**0.03**	

Fig. 4.2. Numbers of transversions in pairwise comparisons of the skink 12S rRNA sequences, plotted against numbers of transitions. Note the variability in numbers of transitions and transversions. The correlation coefficient, r^2, is 0.03.

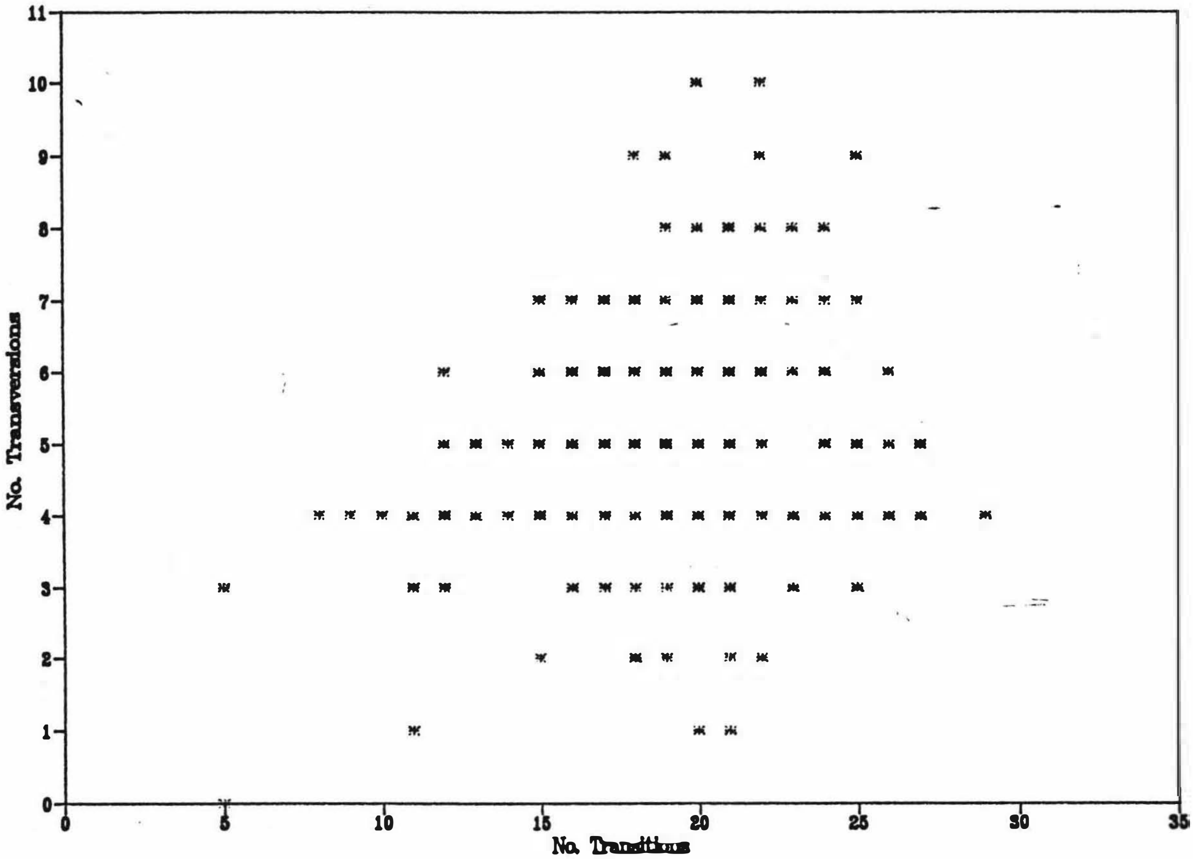


Fig. 4.3. Total number of differences in pairwise comparisons of the skink 12S rRNA sequences plotted against numbers of transversions. There is only a weak correlation between the number of transversions and the total number of observed nucleotide substitutions ($r^2 = 0.26$).

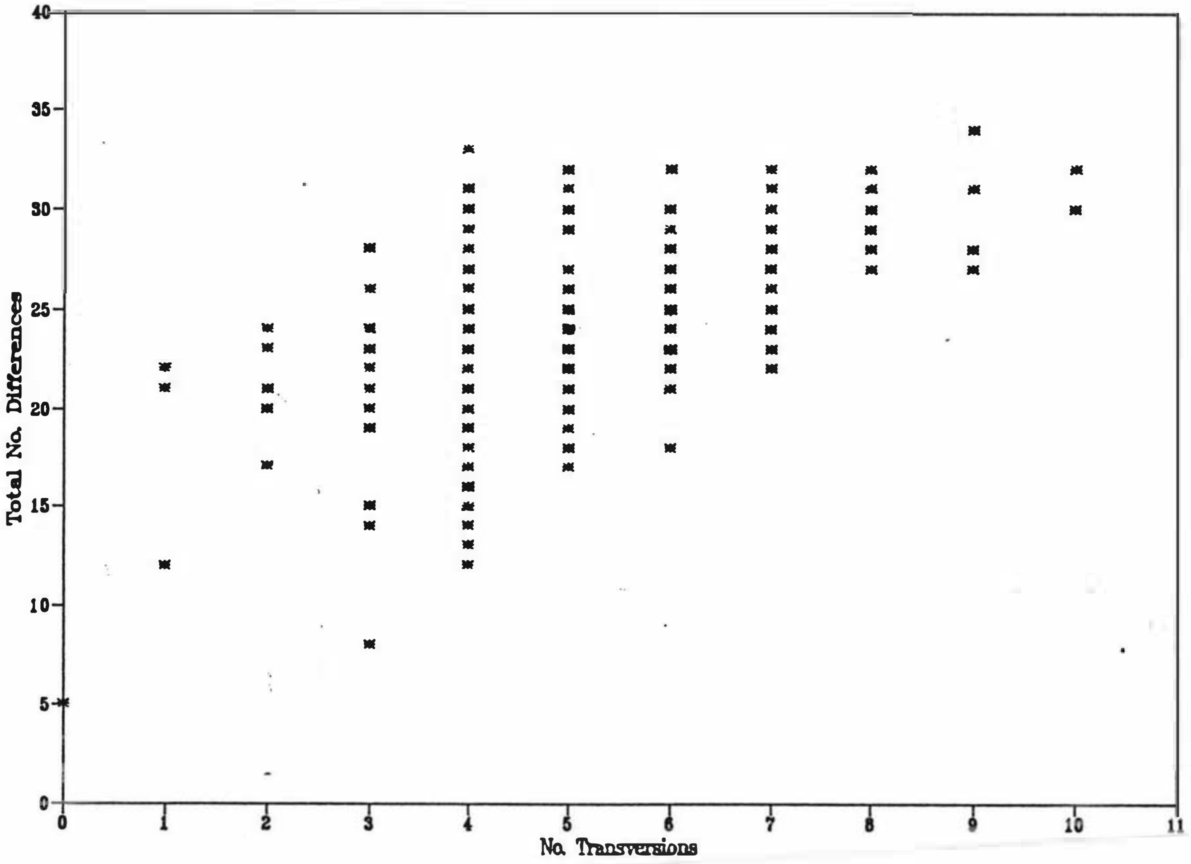


Fig. 4.4. Relationship between numbers of transitions and numbers of transversions for *a.* ratites (12 taxa; $r^2 = 0.40$) and *b.* pecoran bovids (15 taxa; $r^2 = 0.55$). Compare to figure 4.2.

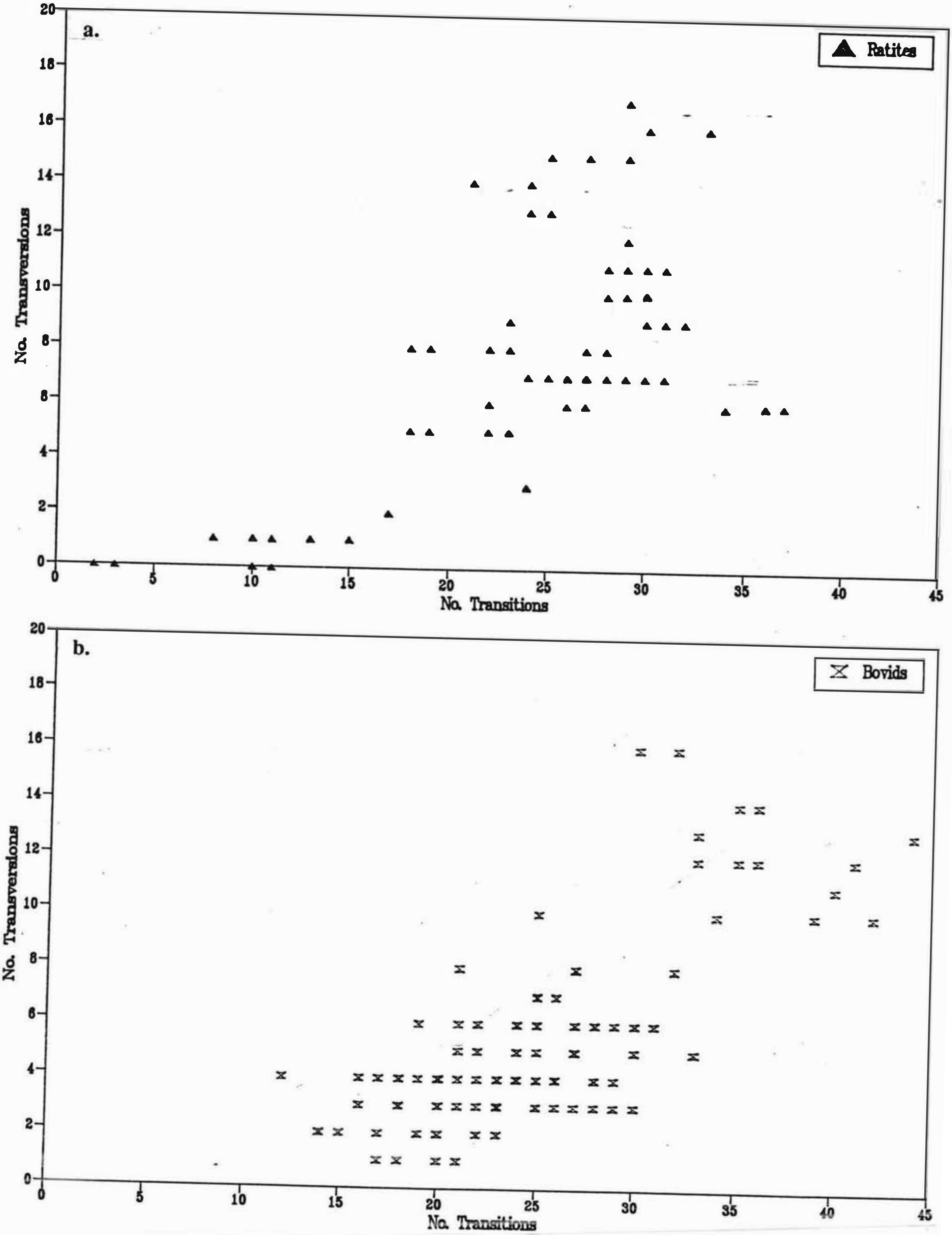
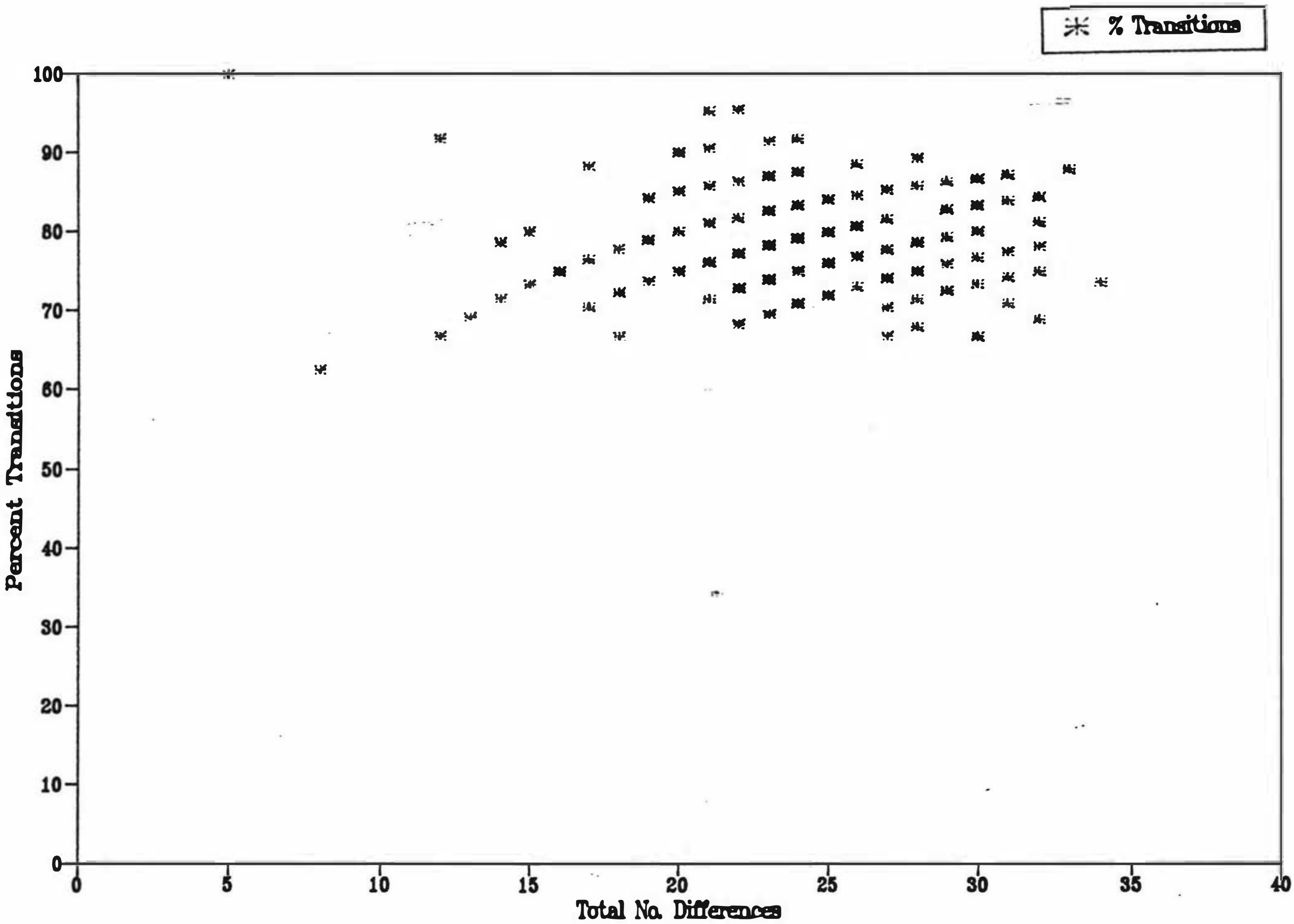


Fig. 4.5. Proportion of transition substitutions in pairwise comparisons of the skink 12S rRNA sequences. Note that total sequence divergence is less than 10%, and that in this data set there is no trend for the proportion of transitions to decrease as the degree of divergence increases ($r^2 = 0.001$).



The expected Ts/Tv ratio if the sequences were saturated can be calculated from equation 11 in Holmquist (1983):

$$\frac{(A_\infty + G_\infty)\,(C_\infty + U_\infty)}{(A_\infty G_\infty)\,(C_\infty U_\infty)}$$

where A_∞, G_∞ etc, are the equilibrium base frequencies. Using the frequencies from table 2.2, the saturated Ts/Tv should be 0.47, well below the smallest observed ratio of 2.6 for *L. infrapunctatum* (Table 4.2).

Although there is no strong trend for the number of transversions to increase as more transitions occur (Fig. 4.2), the Ts/Tv ratio does decline as the number of transversions increases (Fig. 4.6). More importantly, the variation associated with Ts/Tv ratios also declines as the number of transversions increases (Fig. 4.6). This trend is also seen in other data sets (Fig. 4.7) and suggests that this asymptotic decline in Ts/Tv ratio is due to greater variability in the proportion of transition substitutions when only a few transversions occur, rather than being indicative of saturation of substitutions.

Simulations

To examine the extent to which the variability in the skink data set is due to stochastic processes computer simulations were performed to randomly generate sequences. Parameters for the simulations are shown in table 4.4, the key variable being the Ts/Tv ratio. Values for sequence length (384) and number of expected changes (25) were chosen to correspond with the skink data. To generate a similar size transition and transversion matrix as the skinks (Table 4.1b), 200 iterations (runs) of the program were performed (Table 4.4). Longer iterations of 100,000 gave similar results (Table 4.4).

The program creates a random sequence of the specified length. A new sequence is then derived from this with sites of substitutions chosen randomly. All sites in the sequence are free to vary. The probability of each change being a transition is determined by the Ts/Tv ratio read in at the start of the program.

The average number of substitutions between pairs of skink sequences is 25, and the Ts/Tv ratio about four (Table 4.2). In the simulation, when the expected number of changes is set to 25 and the Ts/Tv ratio is 4:1 then both the frequency distributions (Fig. 4.8) and numbers (Fig. 4.9) of transitions and transversions are similar to the skink data (Figs. 4.1 & 4.2). The same pattern of variability in numbers of transitions and transversion is also seen when different numbers of substitutions are allowed (Fig. 4.10), and when the Ts/Tv ratio is altered (Fig. 4.11). The apparent skewness shown in the transition and transversion frequency distribution (Fig. 4.8, see also the skink distributions in Fig. 4.1) is a reflection of sampling error since this disappears when a larger number of simulation runs are performed (Fig. 4.12). The pattern of variability in numbers of transitions and transversions (Figs. 4.9, 4.10) is not due to sampling error however, since increasing the number of runs does not alter the general pattern (Fig. 4.13). This point will be returned to shortly when other data sets are considered.

Fig. 4.6. The relationship between number of transversions and the transition/transversion (Ts/Tv) ratio for the skink data set. Note the decreasing means and variability of the Ts/Tv ratios as the number of transversions increases. (See also Fig. 4.7).

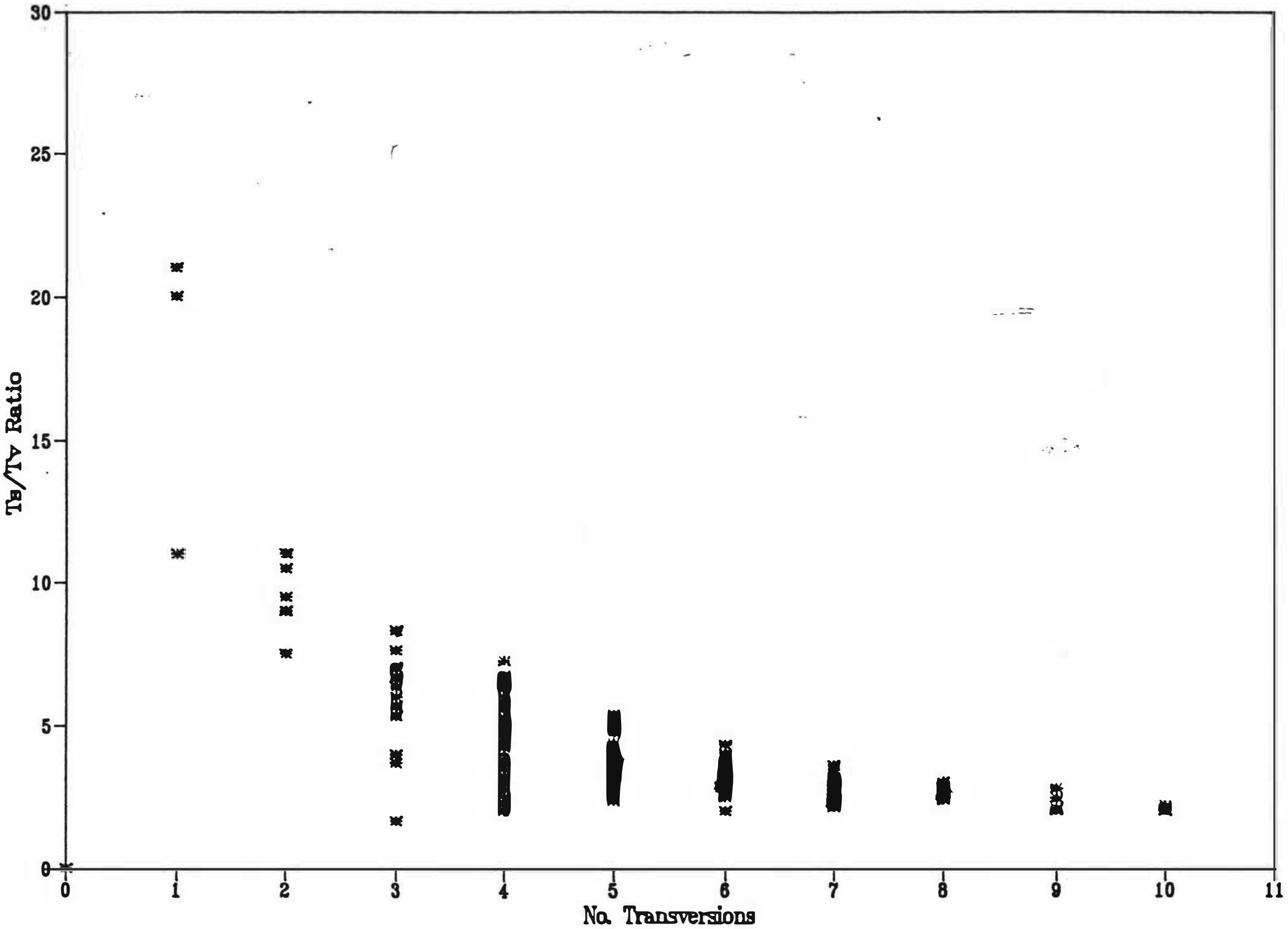


Fig. 4.7. The relationship between number of transversions and the transition/transversion (Ts/Tv) ratio for other data sets. *a.* ratites (12 taxa), *b.* bovids (15 taxa), and *c.* simulated sequences (20 taxa; see Figs. 4.8 & 4.9, and the text). All the data sets show the same pattern of decline in mean and variability in the Ts/Tv ratio as the number of transversions increases.

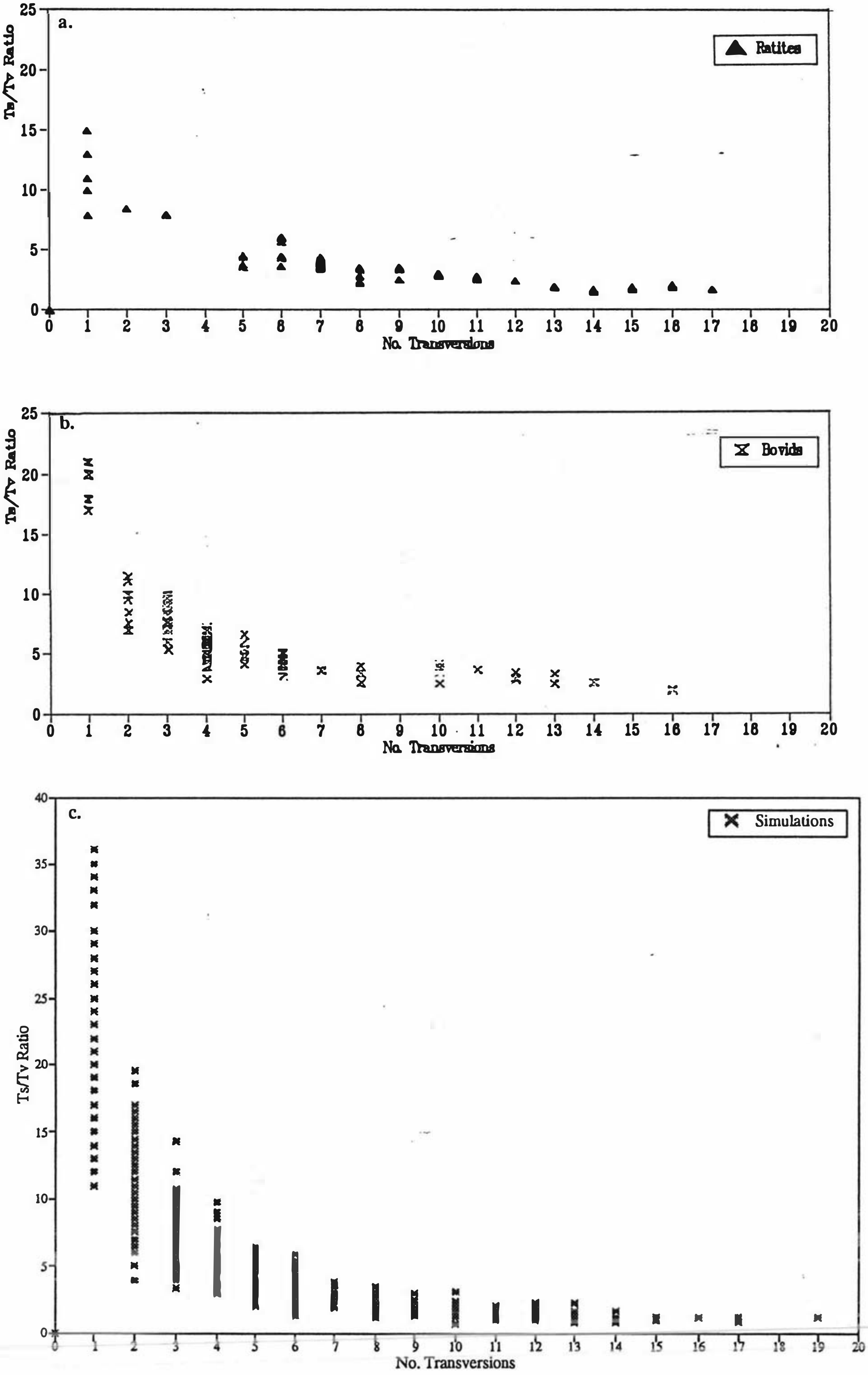


Table 4.4. Parameters used in simulations of transitions and transversions. Values were assigned to be similar to the skink data; sequence length was 384 bases and the number of changes expected to occur was assigned a value of 25. The assigned transition/transversion (Ts/Transversion) value is given on the left and the mean ratio obtained from the simulations is shown in the last column.

Ts/Tv Ratio	No. Runs	Transitions (mean ± Std)	Transversions (mean ± Std)	Mean Ts/Tv
2	200	16.6 ± 3.6	9.0 ± 3.0	1.9
	100,000	16.7 ± 4.0	8.3 ± 2.9	2.0
3	200	19.0 ± 3.8	6.6 ± 2.5	2.9
	100,000	18.7 ± 4.2	6.2 ± 2.5	3.0
4	200	20.1 ± 4.6	5.0 ± 2.1	4.0
	100,000	20.0 ± 4.4	5.0 ± 2.2	4.0
5	200	20.7 ± 4.2	4.3 ± 1.8	4.8
	100,000	20.8 ± 4.4	4.2 ± 2.0	5.0
6	200	21.2 ± 4.5	3.6 ± 2.0	5.9
	100,000	21.4 ± 4.5	3.6 ± 1.9	5.9
7	200	22.2 ± 4.5	3.3 ± 1.8	6.7
	100,000	21.9 ± 4.6	3.1 ± 1.8	7.1
8	200	21.5 ± 4.4	2.8 ± 1.7	7.7
	100,000	22.2 ± 4.6	2.8 ± 1.7	7.9
9	200	22.7 ± 4.8	2.6 ± 1.6	8.7
	100,000	22.5 ± 4.6	2.5 ± 1.6	9.0
10	200	24.0 ± 4.9	2.5 ± 1.4	9.6
	100,000	22.7 ± 4.6	2.3 ± 1.5	9.9

Fig. 4.8. Frequency distributions of transitions (*a*) and transversions (*b*) in simulated sequence data. Sequences are 384 bases in length. Each sequence has on average 25 substitutions relative to other sequences, and a transition/transversion ratio of 4/1. Two hundred simulation runs were performed. Compare these distributions with those of the skink data set in figure 4.1b & c.

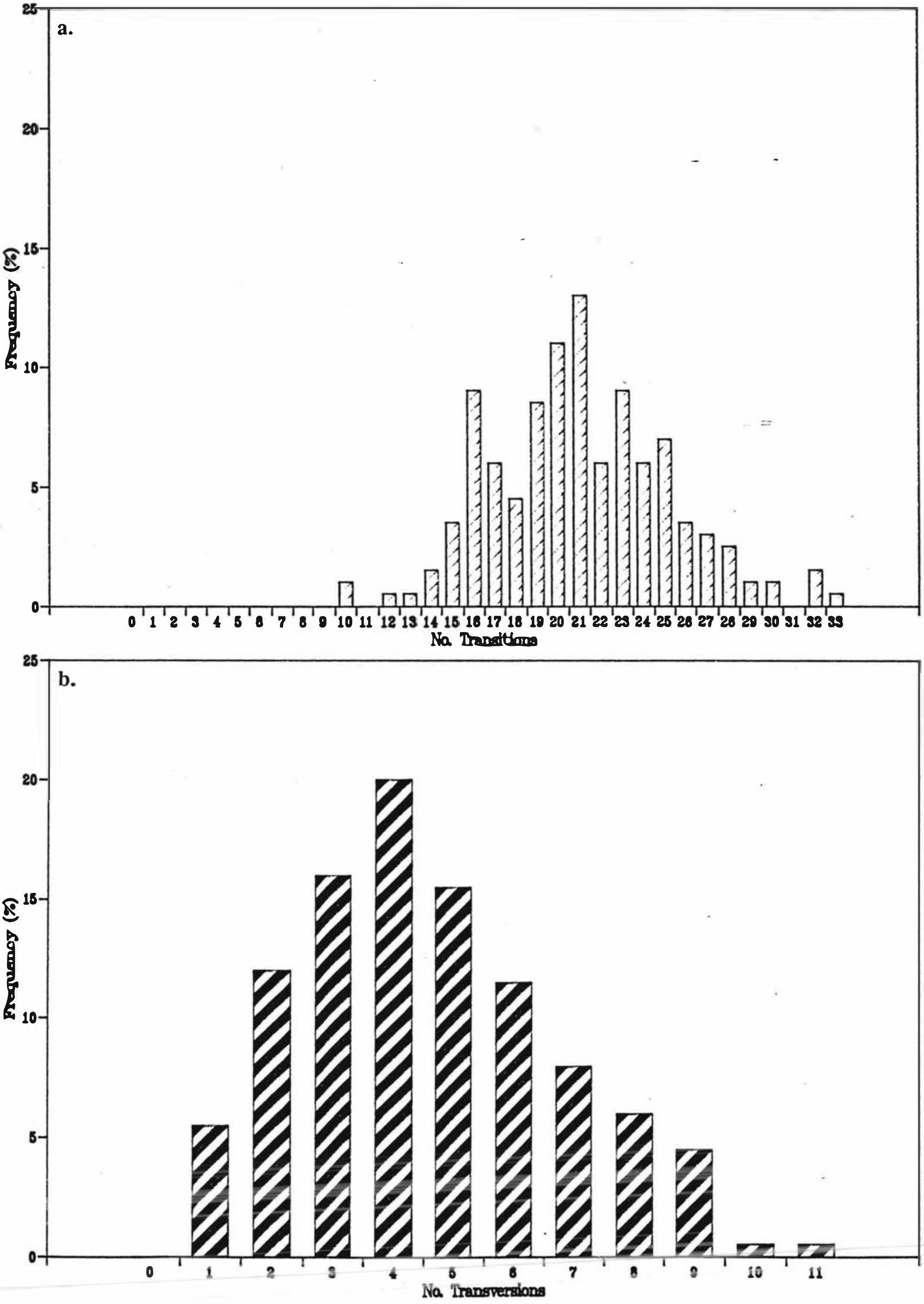


Fig. 4.10. Comparison of the relationship between the numbers of transitions and numbers of transversions for other simulations using, ***a.***40 expected substitutions and, ***b.***55 expected substitutions between each sequence. Sequence lengths were 384 bases, and the transition/transversion ratio was 4/1. Correlation coefficients are, respectively, 0.00 and 0.03. Compare to figure 4.9, noting that the relationship between numbers of transitions and numbers of transversions does not change as more substitutions occur. This differs from the ratite and bovid data sets where there is a trend toward a linear relationship as more substitutions occur (Fig. 4.4).

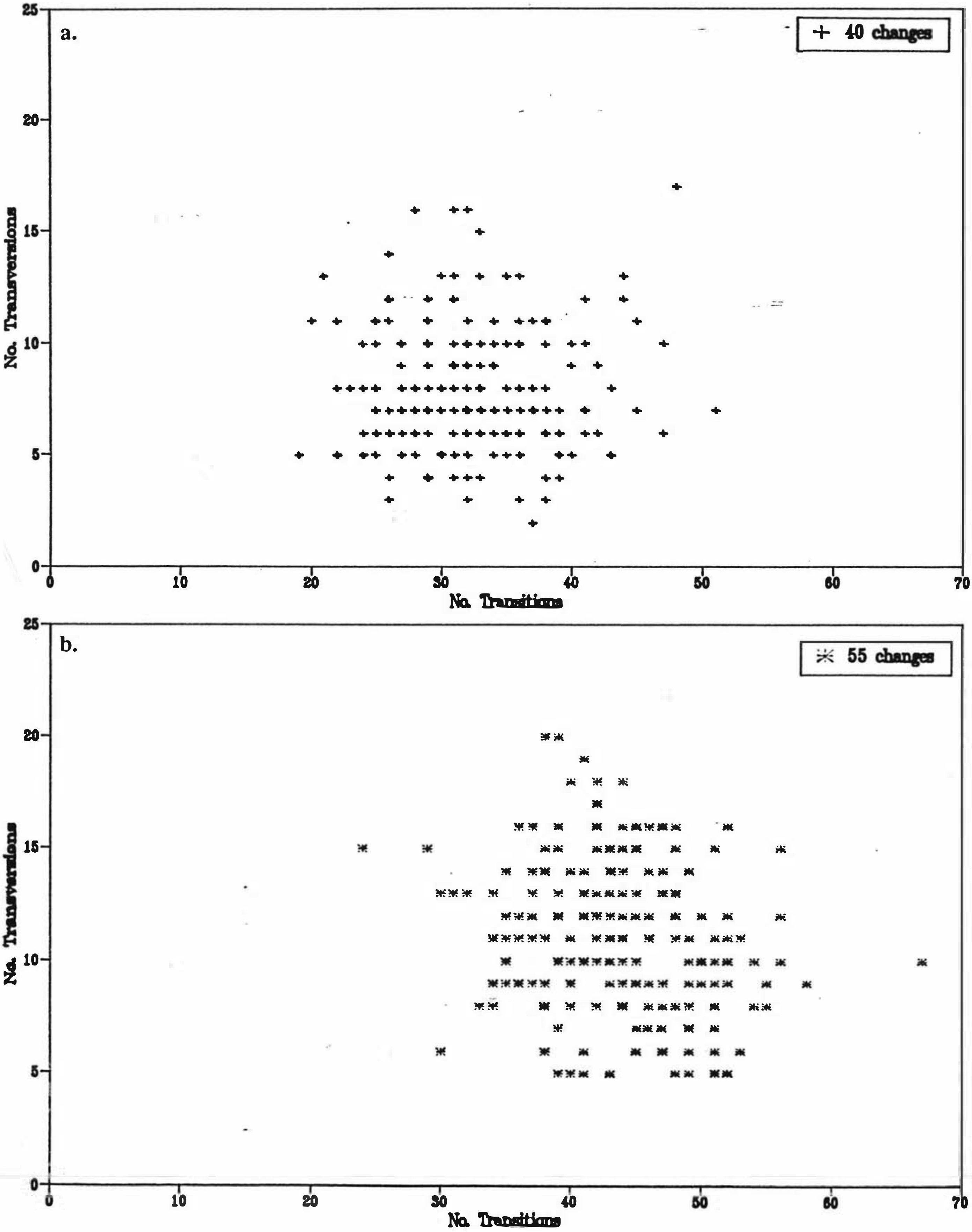


Fig. 4.11. The relationship between the numbers of transitions and numbers of transversions for other simulations using, *a.* a Ts/Tv ratio of 2/1 and, *b.* a Ts/Tv ratio of 8/1. Sequence lengths were 384 bases, and the expected number of substitutions in each sequence was 25. Correlation coefficients are, respectively, 0.024 and 0.002. Compare to figures 4.9 and 4.10, noting the similar patterns of relationships.

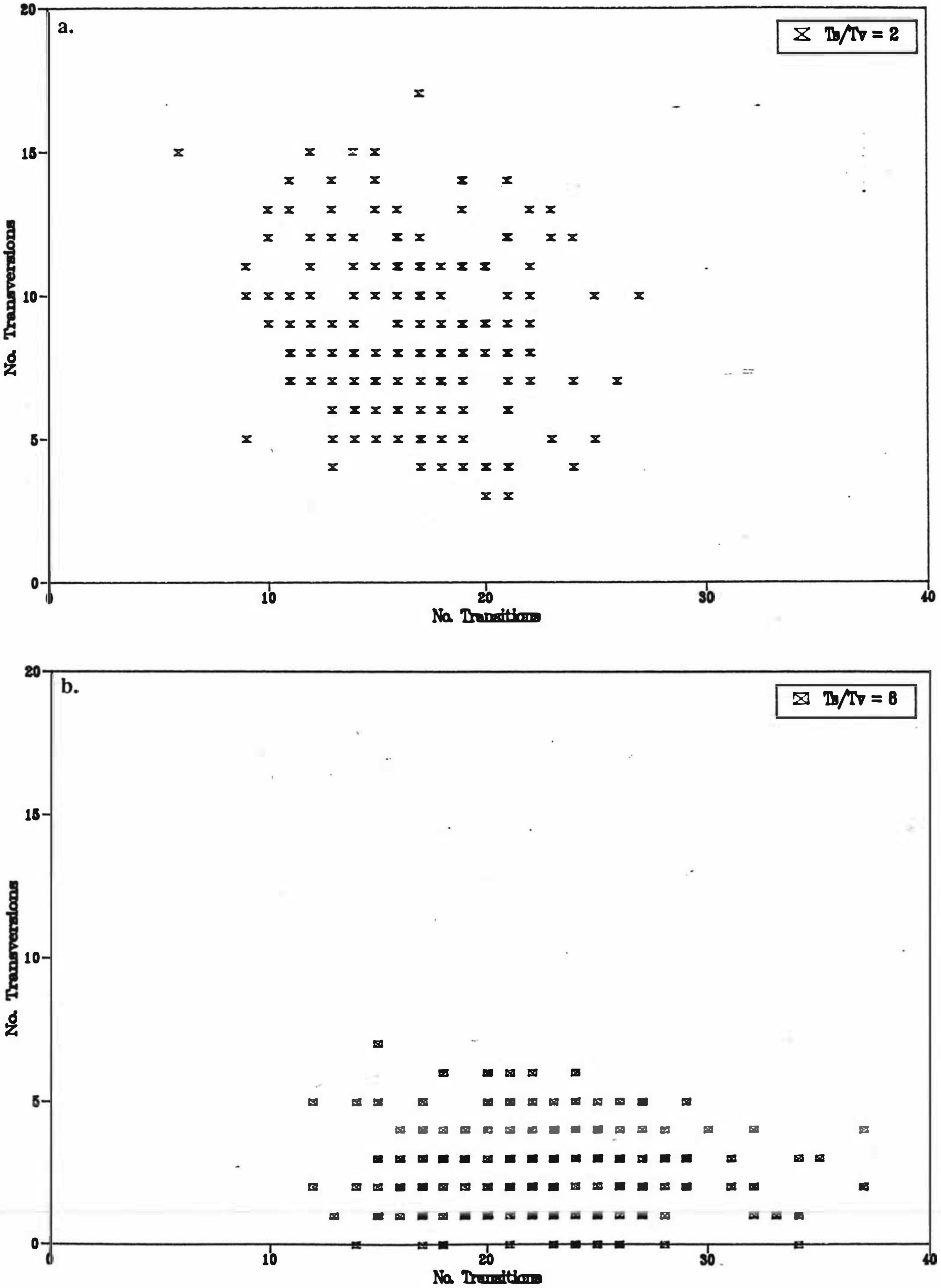


Fig. 4.12. Frequency distributions of transitions and transversions in simulated sequence data for a large number of iterations. In figure 4.8 200 runs were performed, while here the number of runs is 100,000. Increasing the number of simulation runs gives smoother frequency distributions, indicating that sampling error may account for the skewness in figures 4.1 and 4.8.

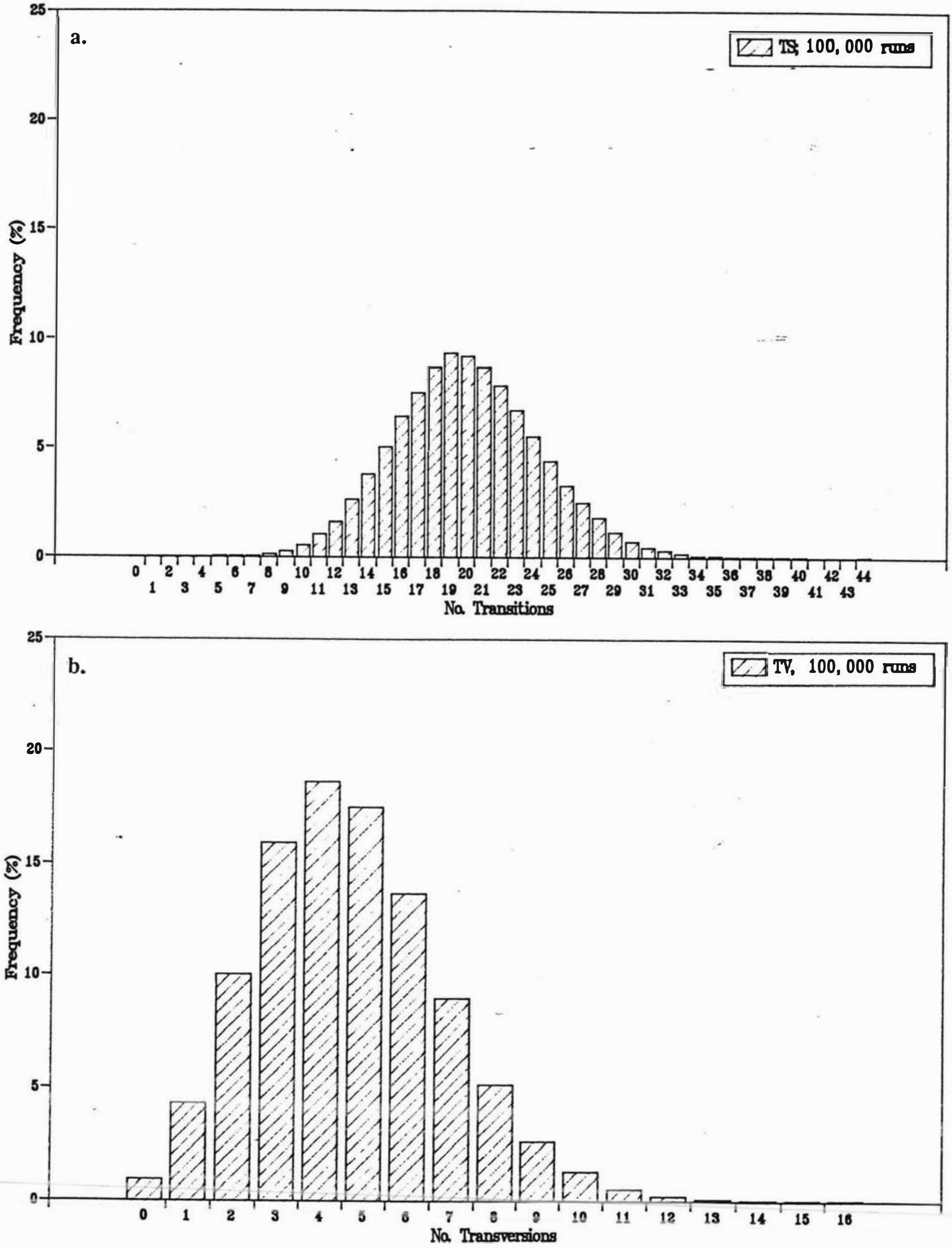
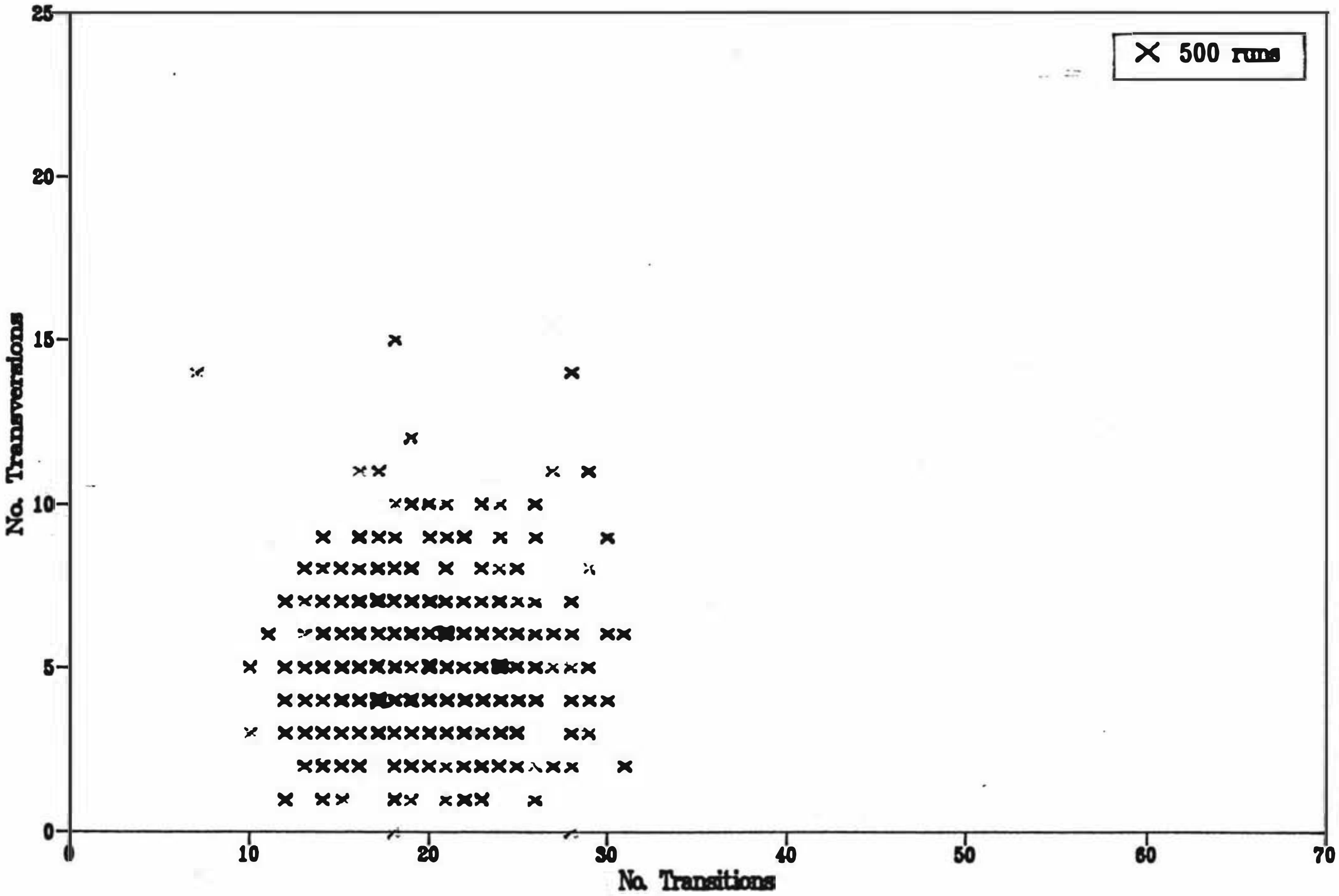


Fig. 4.13. The relationship between numbers of transitions and numbers of transversions in the simulated sequence data set when 500 iterations are performed (larger runs exceeded the capacity of the plotter). Sequences are 384 bases in length, and each sequence has on average 25 substitutions relative to other sequences, and a transition/transversion ratio of 4/1. The correlation coefficient is 0.00, and this pattern of relationships is almost identical to that in figure 4.9 where 200 runs were performed. The lack of a strong correlation between numbers of transversions and numbers of transitions is, consequently, not due to insufficient numbers of comparisons (compare with the ratite and bovid plots in Fig. 4.4).



Estimating Divergence from Transversions

Due to the possibility of saturation of transitions obscuring total sequence divergence, numbers of transversions have been used to estimate the degree of divergence between taxa (Brown *et al.* 1982, Wilson *et al.* 1985, Miyamoto & Boyle 1989, Milinkovitch *et al.* 1993). As noted above however, the numbers of transversions in pair-wise comparisons can be very variable (Figs. 4.2, 4.4, 4.9, 4.10, 4.13). There will, therefore, be a higher variance associated with estimates of the degree of divergence based on transversions than when all substitutions are used. This is illustrated with the simulation data, where the true values for total numbers of differences are known. Multiplying the number of observed transversions by [1 + {Ts/Tv ratio}] gives an estimate for total numbers of differences. For example, if the number of differences is set to be 25 and the Ts/Tv ratio is 4/1, then on average there should be five transversions between each sequence, and 5x[1 + {4}] will give 25 total substitutions. The standard deviation for this estimate of mean numbers of substitutions is however twice as large as that obtained from using the total numbers of observed differences, as shown in figure 4.14. This is true when both the numbers of differences (Fig 4.15) and the Ts/Tv ratio (Fig. 4.16) are varied. Estimates of the mean divergence between taxa are less accurate however when the Ts/Tv ratio is varied (Fig. 4.16)

Comparisons to Other Data Sets

The skink taxa are quite distinct from each other, but does this imply recent and rapid divergence or slower change over a longer period of time? Lack of a fossil record makes it difficult to determine what period of time the observed sequence differences among the skinks reflect. Variation in evolutionary rates between taxonomic groups makes estimates based on a general mtDNA evolutionary rate (Wilson *et al.* 1985, 1987) difficult. This is illustrated in tables 4.5 and 4.6. Ratite and bovid data sets (Table 4.5a & 4.5b, respectively) show generally similar numbers of transitions and transversions for the same region of 12S rRNA, despite having different times of separation (approximately 25 and 80 million years, respectively; Table 4.5).

Xantusiid lizards from the Caribbean and Central America are much more divergent from each other (Table 4.5c), their sequences have many insertion/deletion events (Appendix 2), and substitutions occur in more conserved parts of the molecule (Fig. 3.6b). They therefore either diverged a long time ago or their rate of sequence evolution is high. Based on fossil records, the genera in table 4.5c separated between 35 and 70 million years (the original references for these were not seen, but are discussed Hedges *et al.* 1991). The date of 70 million years is derived from Paleocene fossils of *Paleoxantusia*, which appear to share derived features with *Lepidophyma* and *Xantusia* rather than *Cricosaura* (see Hedges *et al.* 1991). Separation between *Lepidophyma* and *Xantusia* may have occured in the Eocene (35-40 MYA), based on comparisons of *Paleoxantusia* fossils. The phylogenetic relationships of the *Paleoxantusia* are not well known however, and there are few *Xantusia* and *Lepidophyma* fossils (see Hedges *et al.* 1991), so estimates of their divergence times, and hence rates of sequence evolution, are possibly less reliable than those for the birds and mammals.

Fig. 4.14. The means and standard deviations of estimates for the real number of substitutions. One estimate ("Obs.") is based on the total number of observed differences, while the second ("Pred.") uses and the number of observed transversions to determine the total number of substitutions. Simulated sequence data were used, and the mean number of substitutions is expected to be 25. The transition/transversion ratio was set to 4/1, so the expected numbers of substitutions was calculated from transversions by multiplying the number of transversions by 5. The same mean number of differences are obtained when either the total numbers of observed differences or the numbers of transversions are used, but there is a larger standard deviation associated with the estimate derived from transversions. There will, therefore, be greater uncertainty in the accuracy of the estimate of total sequence divergence between taxa when only the numbers of transversions are used (see the text).

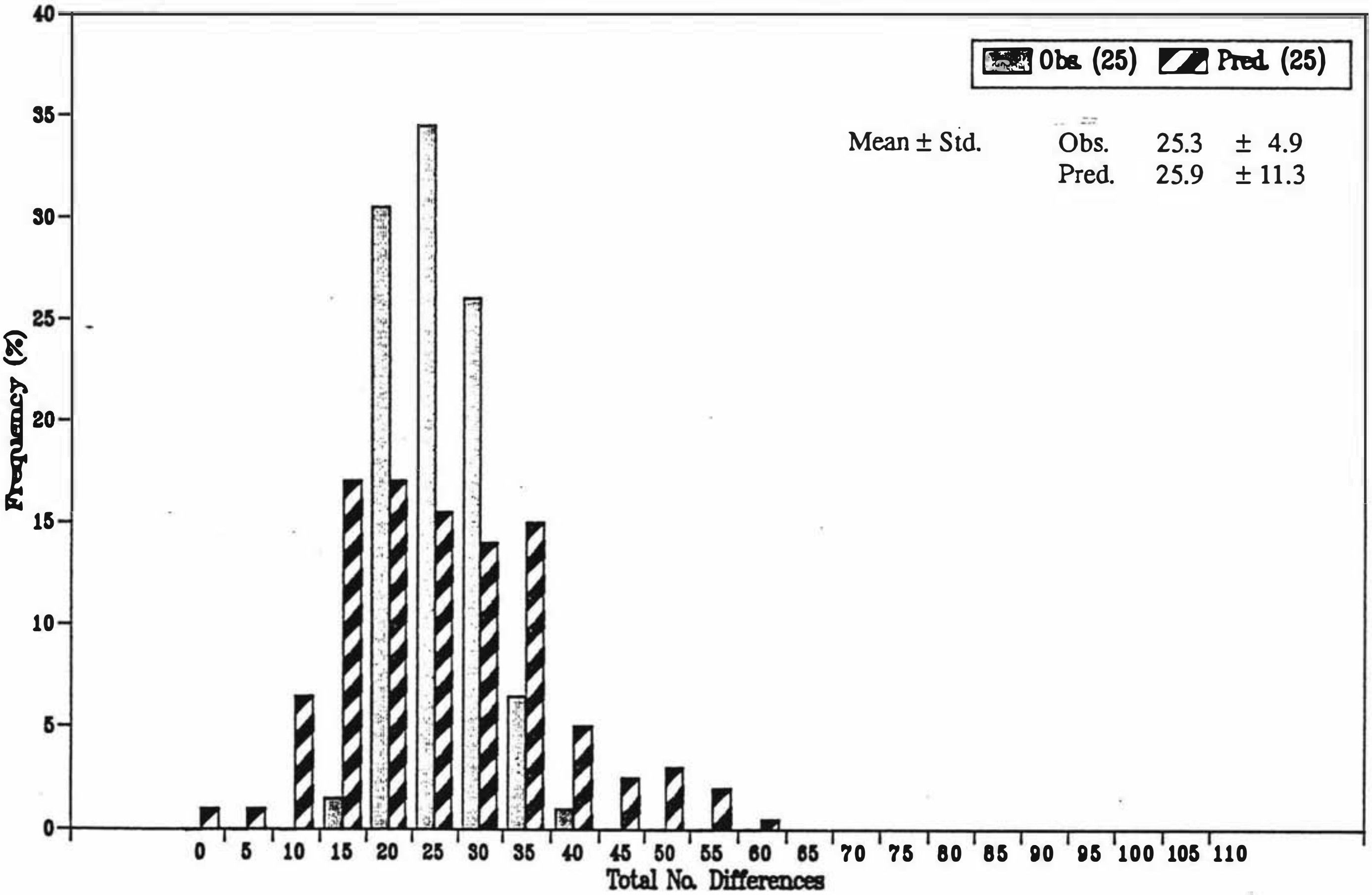


Fig. 4.15. The means and standard deviations for estimates of the real number of substitutions based on the total number of observed differences ("Obs.") and the number of transversions ("Pred."). Simulated sequence data was used, and the transition/transversion ratio was set to 4/1. In *a*, the expected number of substitutions is 40, and in *b* it is 55. Calculations are as described in figure 4.14. The estimate based on the number of transversions has a higher standard deviation than that derived from the observed total numbers of substitutions.

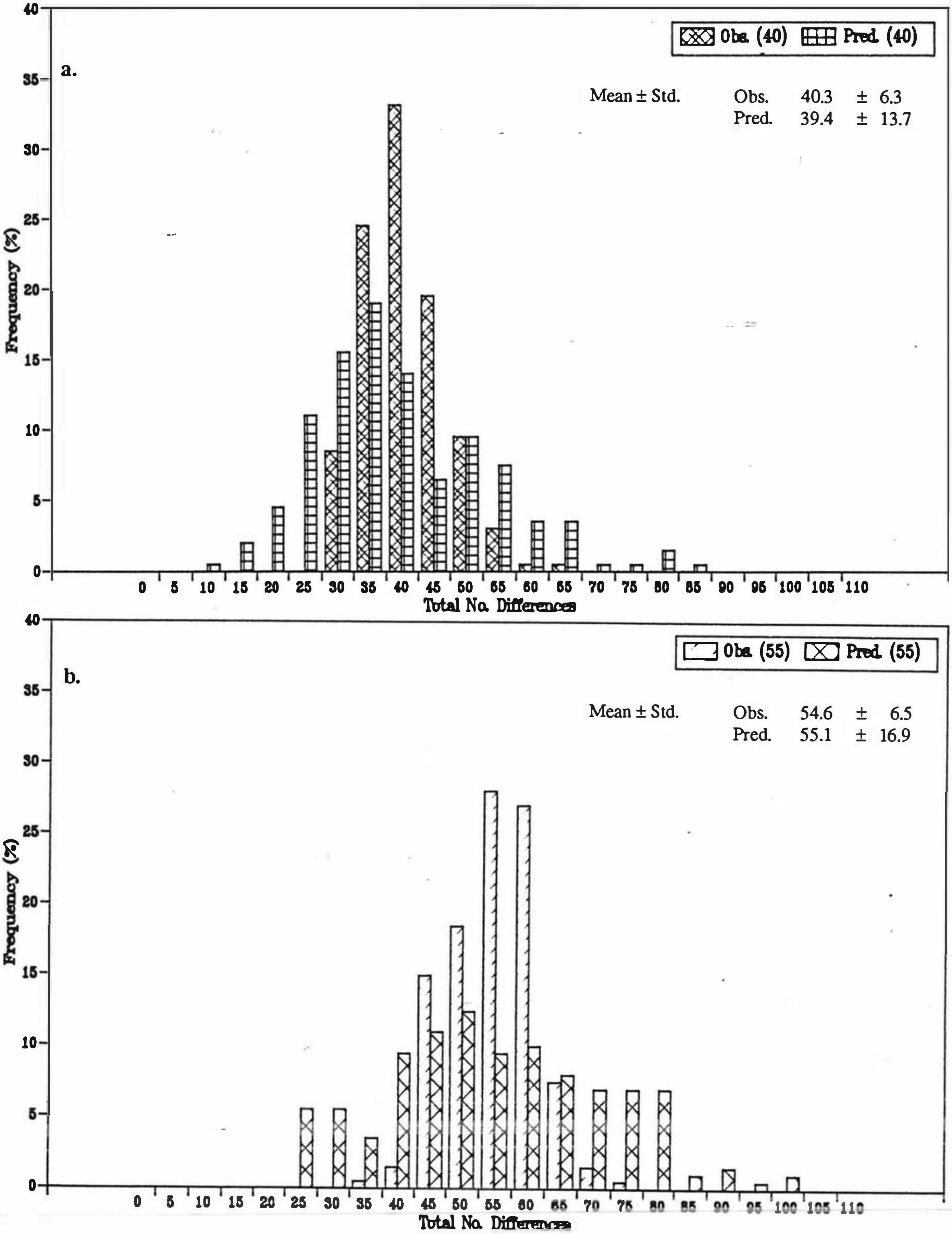


Fig. 4.16. The means and standard deviations for estimates of the real number of substitutions based on the total number of observed differences ("Obs.") and the number of transversions ("Pred."). Simulated sequence data was used, and the expected number of substitutions is 25. In ***a.***, the transition/transversion ratio was set to 2/1, and in ***b.*** it is 8/1. Calculations are as described in figure 4.14, except that transversions are now multiplied by 3 and 9 in ***a*** and ***b***, respectively. Once again (compare with Figs. 4.14 & 4.15) the estimate obtained from numbers of transversions has a higher standard deviation than that derived from observed total numbers of substitutions.

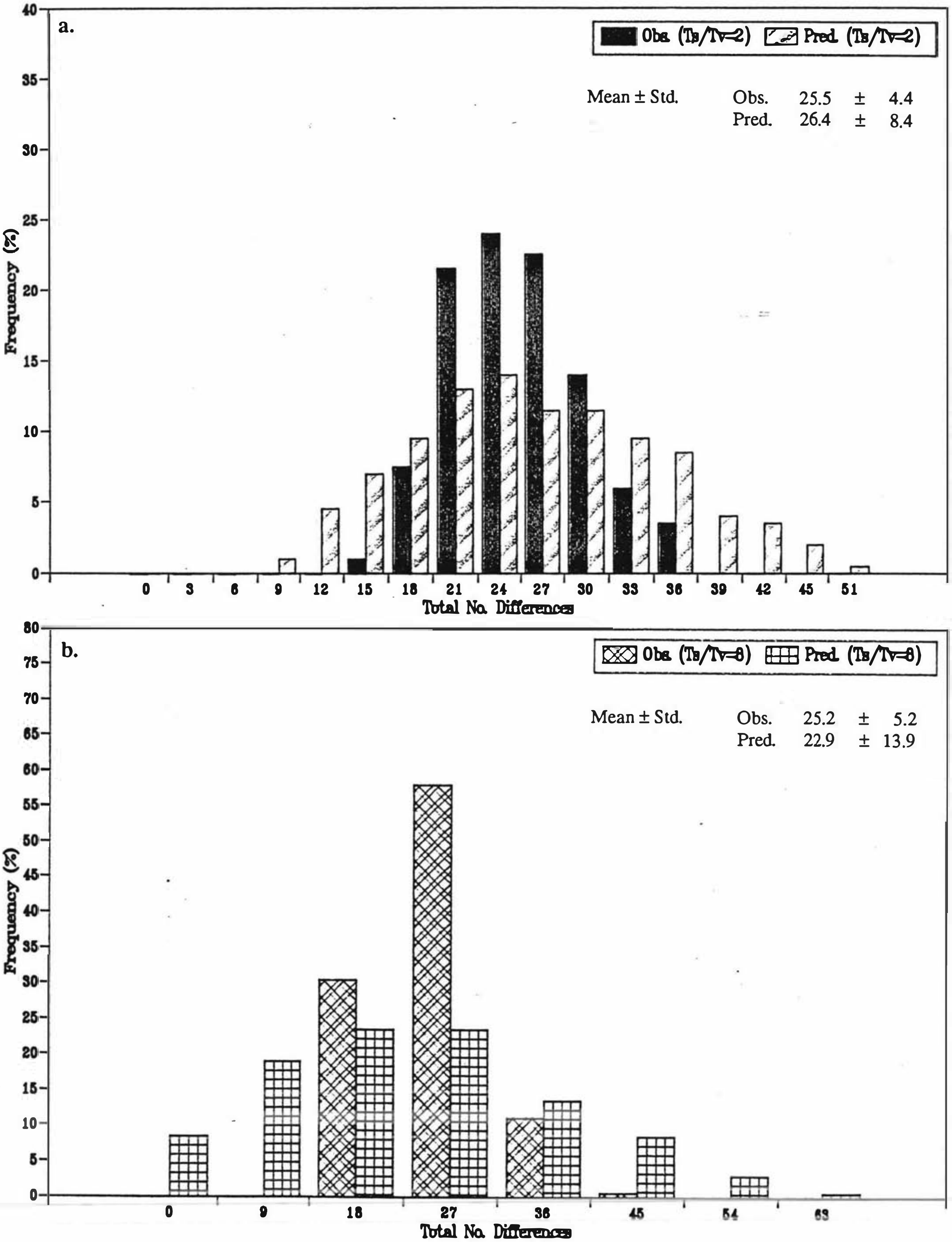


Table 4.5. Distance matrices for 12S rRNA sequences from other vertebrates. The same region of the gene (that bounded by the PCR primers 12SAR and 12SBR) is compared for all groups. Transitions are shown above the diagonal and transversions below. Full species names and GenBank accession numbers are given in Appendix 2.

Table 4.5a. Ratites. Familial divergence times up to 80 million years ago (Sibley & Ahlquist 1981, Cooper *et al.* 1992).

Transversions (below diagonal)\ Transitions (above)

	Ram	**Rda**	**Ost**	**Cas**	**Emu**	**BrK**	**RoK**	**LSK**	**Meg**	**Din**	**Ano**	**Pac**
Rhea amer. (Ram)		10	29	34	36	30	30	31	19	25	27	26
DarwinsRhea (Rda)	0		31	36	37	30	30	31	18	24	26	25
Ostrich (Ost)	7	7		24	24	30	26	27	23	28	23	22
Cassowary (Cas)	6	6	3		17	22	18	19	28	28	26	27
Emu (Emu)	6	6	3	2		27	22	23	27	27	29	28
Brown Kiwi (BrK)	10	10	7	6	6		15	13	29	31	29	28
Roa Kiwi (RoK)	9	9	6	5	5	1		2	29	29	29	28
LSpot Kiwi (LSK)	9	9	6	5	5	1	0		30	30	30	30
Megalapt. (Meg)	8	8	9	8	8	12	11	11		10	11	8
Dinornis (Din)	7	7	8	7	7	11	10	10	1		11	10
Anomalopt. (Ano)	7	7	8	7	7	11	10	10	1	0		3
Pachyornis (Pac)	7	7	8	7	7	11	10	10	1	0	0	

Table 4.5b. Pecoran bovids. Most of these bovids diverged from each other 23-28 million years ago (Kraus & Miyamoto 1991, Allard *et al.* 1992).

Transversions (below diagonal)\ Transitions (above)

	Bta	**Cah**	**Mki**	**Mki**	**Kel**	**Gth**	**Ddo**	**Cem**	**Bot**	**Aem**	**Ovi**	**Mur**	**Hin**	**Aam**	**Tna**
B.tauru		27	23	25	31	31	22	19	29	23	25	25	24	26	33
Ca.hirc	3		14	22	25	19	18	25	20	23	21	24	23	28	39
M.kirki	3	2		20	16	21	17	21	16	14	15	19	18	24	34
K.ellip	3	2	2		22	23	21	23	30	26	25	25	28	30	35
G.thoms	5	4	4	4		26	19	21	23	21	24	27	29	27	40
D.dorca	5	4	4	4	6		21	29	26	27	28	28	30	30	41
Ce.maxw	2	1	1	1	3	3		21	20	22	23	21	22	25	39
Bo.trag	2	3	3	3	5	5	2		23	22	22	21	22	28	37
Ae.mela	4	3	3	3	5	5	2	4		21	29	29	25	25	44
O.virgi	5	4	2	4	4	6	3	5	5		18	13	12	27	33
Mu.reev	5	4	4	4	4	6	3	5	5	2		17	16	30	35
H.inerm	6	5	5	5	5	7	4	6	6	3	3		17	27	33
C.unico	5	4	4	4	4	6	3	5	5	2	2	3		27	35
A.ameri	7	6	6	6	8	8	5	5	7	8	6	9	8		32
T.napu	13	10	10	12	14	10	11	13	11	12	14	15	14	16	

Table 4.5c. Xantusiid lizards. Divergence of *Cricosaura* and Xantusiid lineages approximately 70 million years ago, *Lepidophyma* and Xantusiids 40-35 million years ago (see Hedges *et al.* 1991).

Transversions (below diagonal)\ Transitions (above)

	Cty	**Les**	**Xbo**	**Xhe**	**Xvi**	**Xri**
C.typica		48	44	42	40	40
Le.smithii	55		48	48	48	45
X.bolsonae	57	38		30	29	28
X.henshawi	57	42	18		22	23
X.vigilis	55	39	11	11		11
X.rivers.	51	37	9	11	4	

Table 4.5d. Great Apes (Hixson & Brown 1986). Orangutan-Gorilla divergence estimated at 13-15 million years (Miyamoto *et al.* 1988), and Gorilla-Chimpanzee-Human at 4-7 million years (Miyamoto & Goodman 1990).

Tv (below diagonal)\ Ts (above)

	Ora	**Gor**	**PCh**	**CCh**	**Hum**
Orangutan		19	24	25	21
Gorilla	1		11	10	10
PygmyChimp	0	1		3	9
CommonChimp	0	1	0		11
Human	0	1	0	0	

Table 4.6. Comparison of estimated times of divergence among the skinks based on rate estimates for the same 12S rRNA fragment from other vertebrate taxa (see Table 4.5). Numbers of differences between selected taxa from tables 4.5a-4.5d are shown and the approximate nucleotide changes per miliion years are calculated from suggested divergence times. These rates are then applied to numbers of differences observed in the skink data set. The large differences in rates between the different data sets illustrates that rates can vary between taxonomic groups. Estimates of divergence times for the skinks based on any of these comparisons are therefore likely to be unreliable (see text).

Taxa[1]	**No.**[2] **Diff.**	**Est.**[3] **Time**	**Changes per**[4] **Million Years**	**Estimated time**[5] **if Differences of** 25	30	35
Ost-Ram[a]	36	80	0.5	50	60	70
Cri-Xri[b]	91	70	1.3	19	23	27
Les-Xri[c]	82	35	2.3	11	13	15
Bta-Aam[d]	31	25	1.2	21	25	28
Ora-Hum[e]	21	14	1.5	17	20	23
CCh-Hum[f]	10	5	2.0	12.5	15	17.5

[1] Comparisons between:

[a] Ostrich & *Rhea americana* (Table 4.5a)

[b] *C. typica & X. riversiana* (Table 4.5c)

[c] *L. smithii & X. riversiana* (Table 4.5c)

[d] *Bos taurus & A. americana* (Table 4.5b)

[e] Orang-utan & Human (Table 4.5d)

[f] Common chimpanzee & Human (Table 4.5d)

[2] Number of observed differences bewteen taxa.

[3] Estimated times of divergence in millions of years (see Table 4.5).

[4] Number of substitutions/million years calculated from Number of Differences and Estimated Divergence Times.

[5] Times of divergence (in million years) based on rates in previous column and applied to a range of observed numbers of nucleotide differences in skink pair-wise comparisons (see Table 4.2).

Approximate rates of evolution for this region of 12S rRNA are presented in Table 4.6, using sequence differences and times of separation from tables 4.5a-d. These values are then used to calculate times of separation for skink taxa when between 25 and 35 nucleotide substitutions occur (Table 4.6). The rates of change for the 12S rRNA sequence vary between the different groups, so it is difficult to determine times of separations for skinks based soley on these comparisons, though they could have diverged 70 million years or more ago (Table 4.6).

While the xantusiid lizards are also small lizards, using their rates of 12S rRNA evolution (Table 4.6) as estimates for a skink rate is liable to be inaccurate. As noted above, times of separation of the xantusiids may be imprecise. In addition, the xantusiids have many differences between them (Table 4.5c), whereas the skinks have comparatively few (Table 4.1b), so the error associated with extrapolating rates of sequence evolution from xantusiids to skinks is likely to be large. Other ways of estimating times of separation of the skinks will be discussed in Chapter Five.

Changes in the Context of Secondary Structure

One quarter of the sites in the skink sequence are variable (Fig. 4.17). For the sites which do change it is important to establish if there are patterns in the rate at which they accumulate mutations. Rapidly changing sites or regions are expected to be most informative for relatively closely related taxa, while greater resolution for more divergent taxa can be obtained from consideration of more conservative sites (Simon 1991, Dixon & Hillis 1992).

In the skinks most of the transversions, and the most variable positions (in terms of number of taxa which differ), occur in the unpaired portions of the most variable regions (S26, S34, L38′ & S46; Fig. 4.17). Four transversions occur in helices, but none of them have a corresponding compensatory change in the other member of the base pair (Fig. 4.17). Eight sites had both transitions and transversions, and more than half the taxa are variable at four of these sites (positions 101, 203, 204 & 206; Table 3.1, see also Fig. 4.17), suggesting that these locations may be sites of multiple changes. The effect that these highly variable sites have on phylogenetic analyses is investigated in Chapter Five.

Five pairs of taxa have fewer than 15 differences between them (*L. microlepis*+*L. smithi*, *L. grande*+St. Is. Green, *L. inconspicuum*+*L. notosaurus*, *L. n. polychroma*+*L. n. nigriplantare*, and *L. infrapunctatum*+*La. guichenoti*; Table 4.1a). There is a trend for these pairs to differ at the most variable regions (S26, L38′ and S46; Fig. 4.18). However, *L. n. polychroma* and *L. n. nigriplantare* differ by 13 substitutions but some of these occur in the more conserved regions of the molecule (S2′ for example; Fig. 4.18). In addition, some of the most variable sites, position 101 for example, accumulate differences only between more distantly related taxa (Fig. 4.19). Other regions also tend to accumulate changes as the number of differences between taxa increases (e.g., L30′, S34, SL40, and L45′; Fig. 4.19).

Similar analyses of closely related ratites suggest that regions S26, L34′ and SL40 are the first to change in these birds (Fig. 4.20a). These regions are also the most variable in comparisons between more distantly

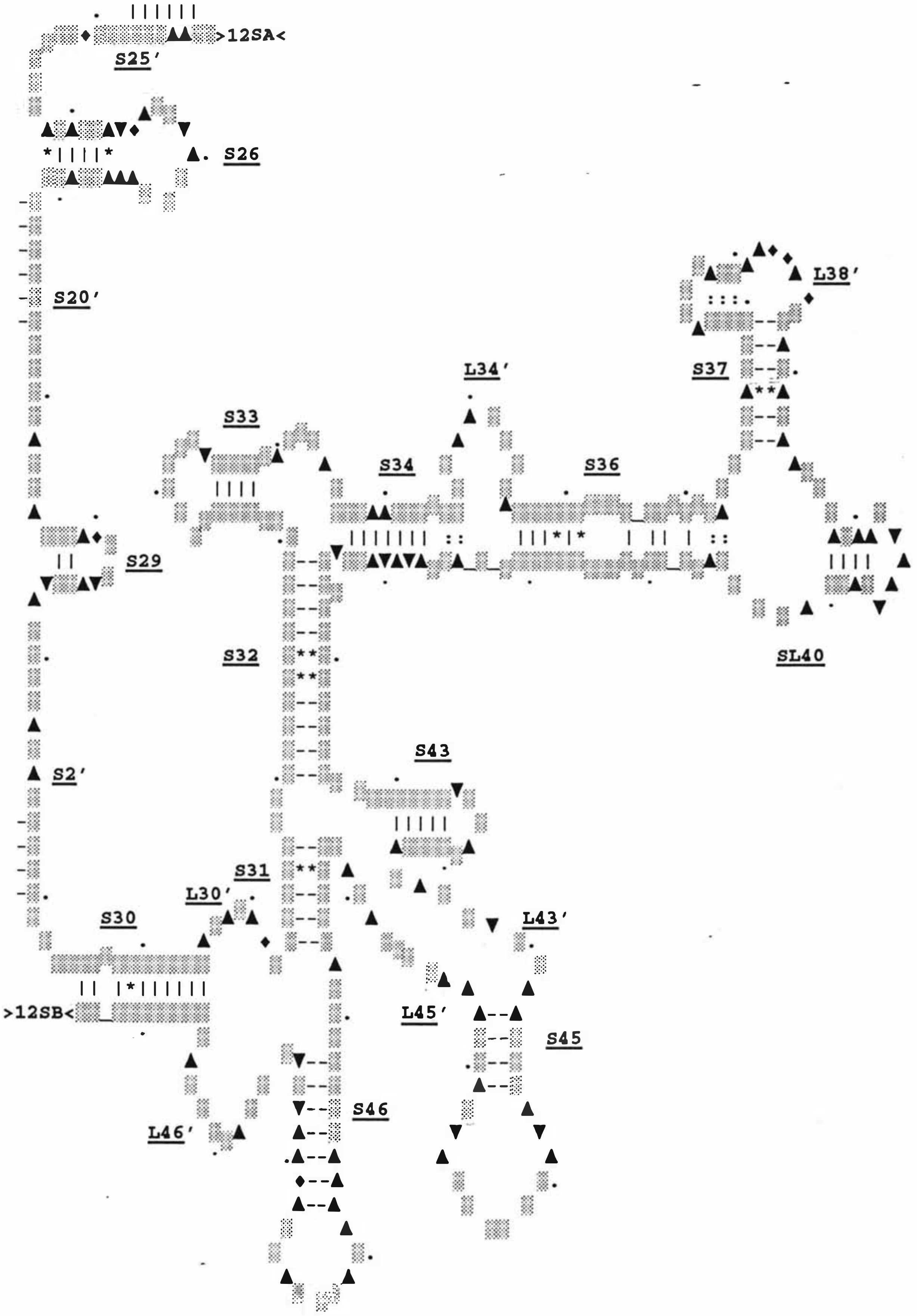


Fig. 4.17. Variable sites in the skink 12S rRNA domain III. Constant sites are shown as ▒, transitions as ▲, and transversions by ▼. Sites at which both transitions and transversions occur are indicated by ◆. Base pairings are shown by - and | (and * for G*U pairs), while a colon (":") denotes less certain bonds. Every tenth base is marked by ".". Regions of the molecule are identified as either paired (S) or unpaired (L) as described in Chapter Three.

Fig. 4.18. Location of nucleotide substitutions in 12S rRNA sequences for pairs of taxa which have only a small number of differences between them. The sequence was divided into regions as described in Chapter Three. Most of the substitutions for these pairs of taxa occur in S26, L38′, and around helices S43-S46. The *L. n. polychroma+L. n. nigriplantare* pair are unusual in that differences between them can occur in some of the more conserved regions of the molecule (see Figs. 3.5 & 4.17). The pairs of taxa and numbers of observed substitutions between them are:

Lmi vs Lsm = *L. microlepis* & *L. smithi* (5 substitutions).
Lgr vs SIG = *L. grande* & Stewart Island Green (8).
Lin vs Lno = *L. inconspicuum* & *L. notosaurus* (12).
Lfr vs Lag = *L. infrapunctatum* & *Lampropholis guichenoti* (12).
Lnp vs Lnn = *L. n. polychroma* & *L. n. nigriplantare* (13).

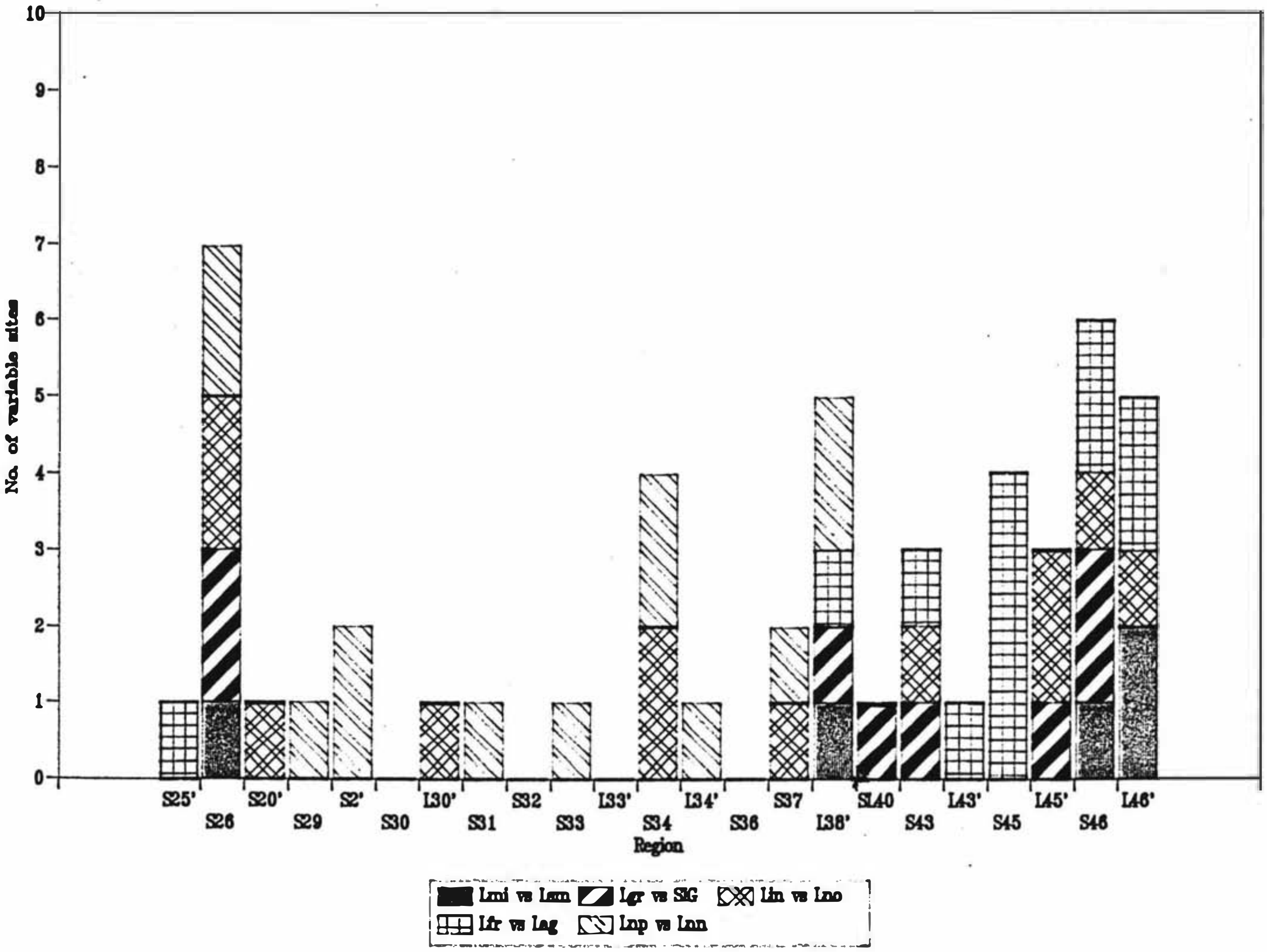


Fig. 4.19. Comparison of regions of variability in the 12S rRNA sequences for pairs of taxa which have few ("close") or many ("far") substitutions between them. The data for taxa with few differences between them is the same as presented in figure 4.18, though the y axis now represents the proportion of sites in the molecule which are variable. Comparisons between pairs of taxa which have larger numbers of substitutions between them ("far") were done in the following way. From the "close" data set the locations of nucleotide substitutions between *L. n. polychroma* and four of the other taxa (*L. microlepis* [22 substitutions], *L. grande* [24], *L. inconspicuum* [29], and *L. infrapunctatum* [34]) were noted. The total numbers of differences in each region for these comparisons were then used to calculate the proportion of variable sites. Note that region S26 has a similar proportion of variable sites when both "close" and "far" comparisons are made. L38′ however, is relatively more variable among taxa which have few numbers of differences between them, while S34 and L46′ have higher proportions of substitutions when more distantly related taxa are compared. Different regions of the skink 12S rRNA molecule therefore accumulate substitutions at different rates.

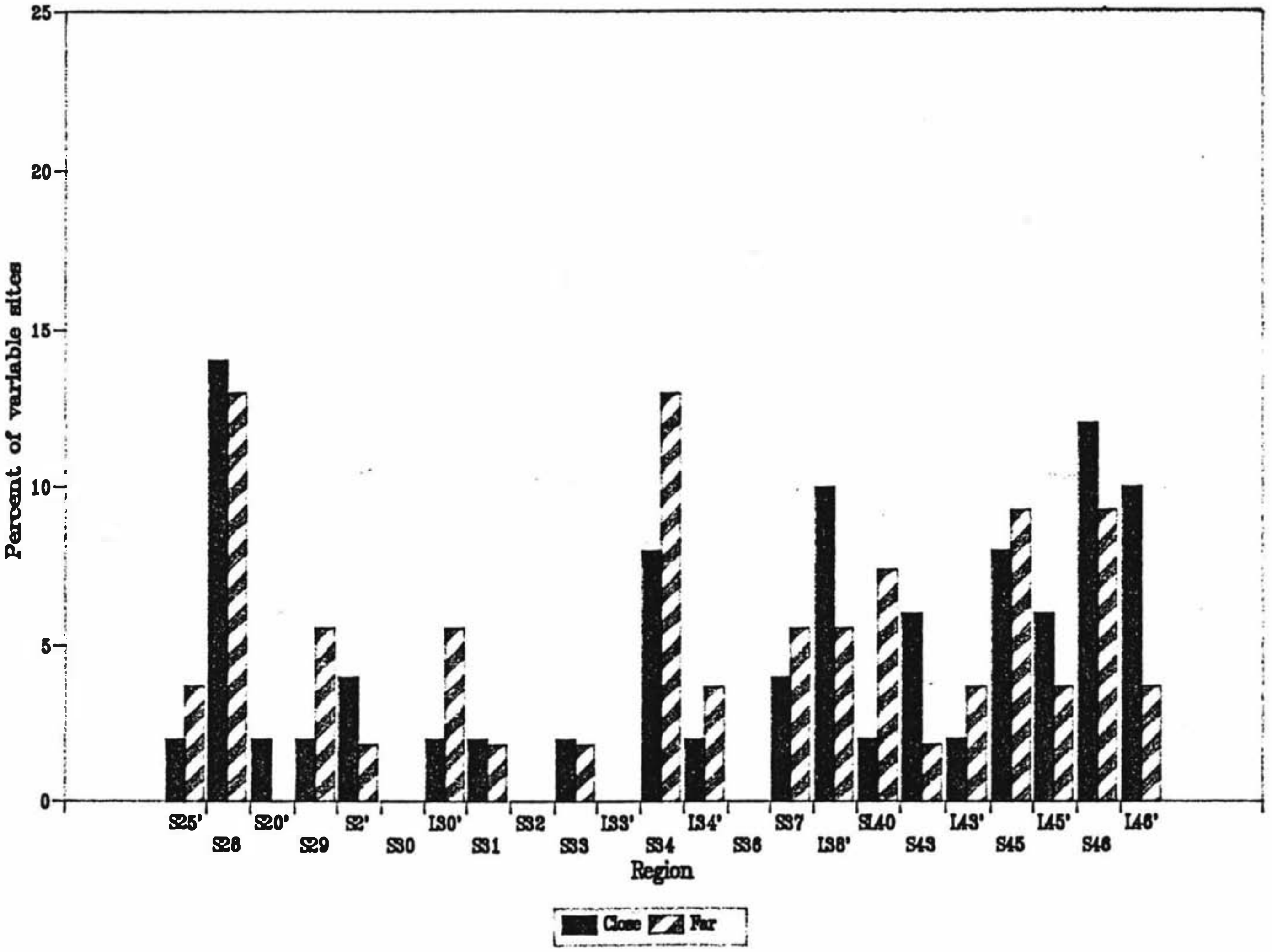


Fig. 4.20a. Comparisons of regions of variability in the 12S rRNA sequence for closely and more distantly related ratites. Calculations are described in figure 4.19. For closely related ratites the numbers of differences between the following pairs of taxa were counted;

Rhea americana & *Pterocnemia pennata* (Darwin's rhea) [10 subsititutions]
Apteryx haastii (Roa kiwi) & *A. owenii* (Little spotted kiwi) [2 substitutions]
Megalapteryx didinus & *Dinornis novaezealandiae* [11 substitutions]
Dinornis novaezealandiae & *Anomalopteryx didiformis* [11 substitutions]
Anomalopteryx didiformis & *Pachyornis elephantopus* [3 substitutions]

For comparisons between more distantly related ratites the *A. haastii* sequence was compared to each of the four moa sequences (see Table 4.5a).

As in the skink sequences (Fig. 4.19), S26 is one of the most variable regions in both closely and more distantly related ratite taxa. SL40 however is more variable between more closely related ratites than it is in skinks. Note that the region L38′ has very few substitutions in the ratites, in contrast with its variability in the skinks (Fig. 4.19).

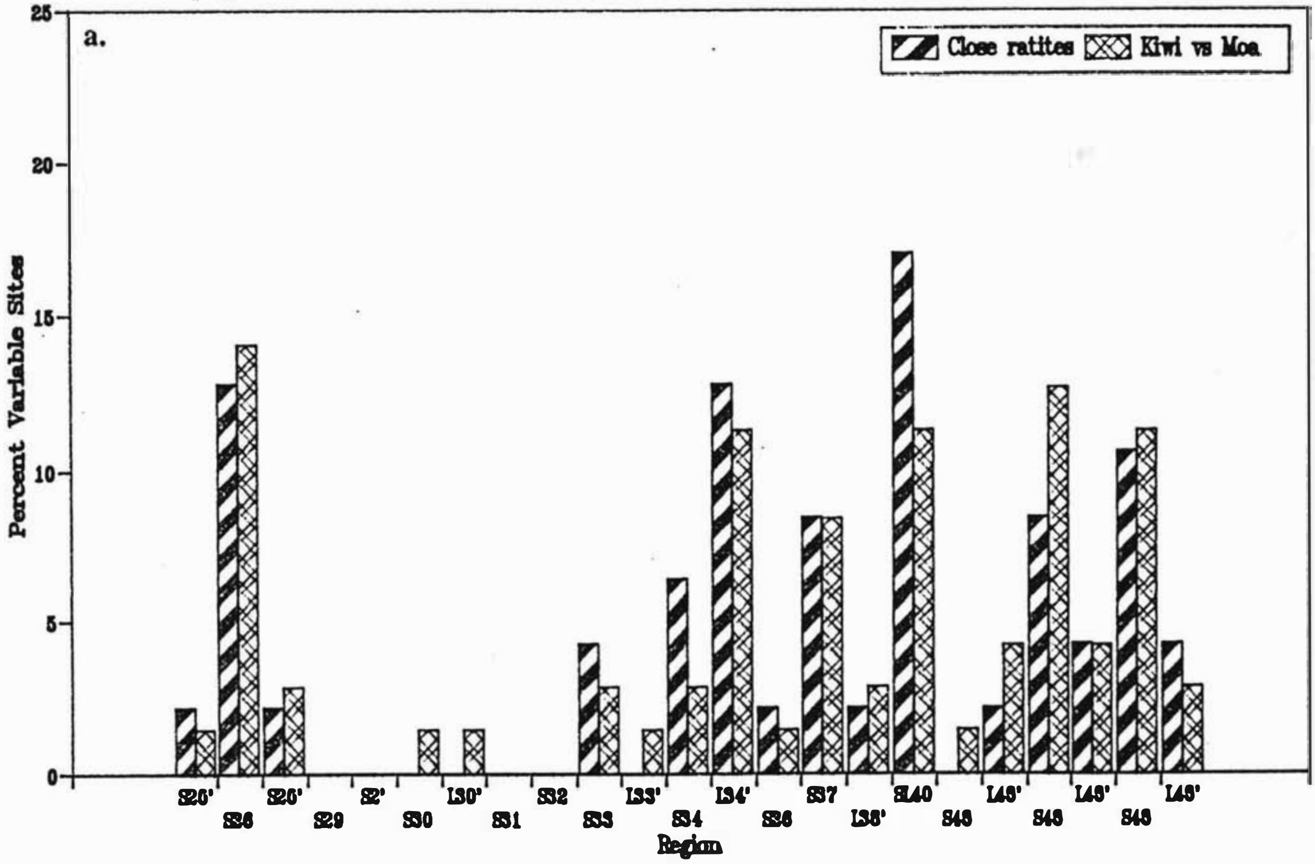
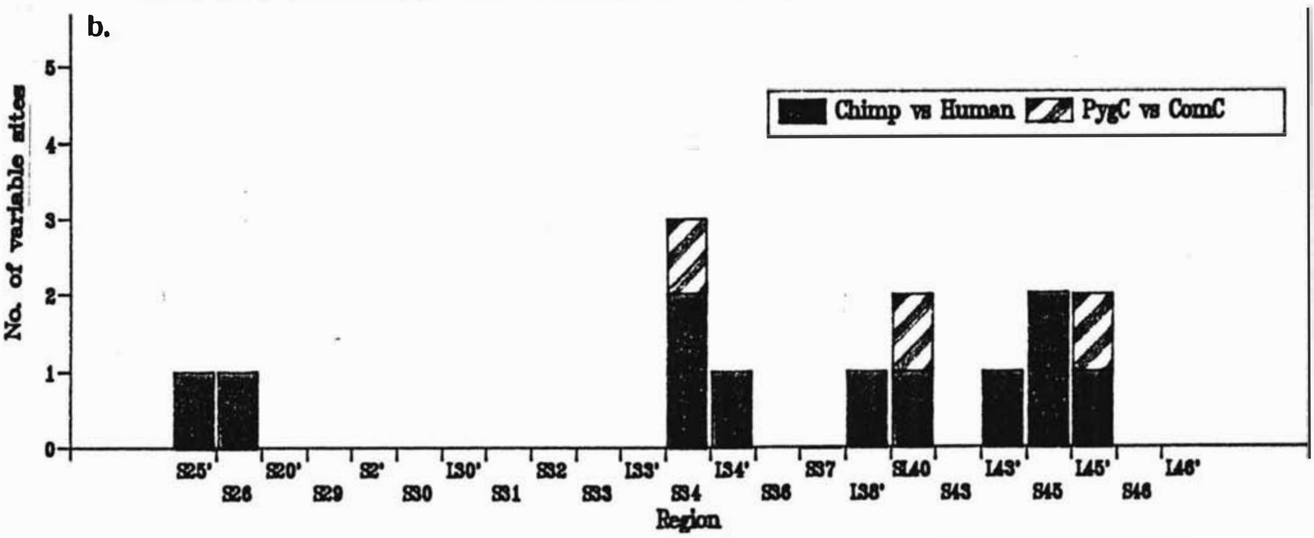


Fig. 4.20b. Comparisons of regions of variability in the 12S rRNA sequence for the most closely related great apes; the two chimpanzee species (pygmy chimpanzee and common chimpanzee; 3 sequence differences) and the common chimpanzee and human (11 substitutions; see Table 4.5d). There appears to be a tendency for substitutions to occur in regions S34, SL40, S45 and L45′ first.



related ratites as well (Fig. 4.20a), so it may be difficult to differentially weight regions for taxonomic comparisons. Among the great apes, regions S26, S34, SL40, S45 and L45' vary (Fig. 4.20b). Similar comparisons were not made in the bovid data set since taxa differ from each other by more than 15 substitutions (Table 4.5c).

DISCUSSION

The 12S rRNA sequences from the skinks appear unusual because of the weak correlation between numbers of transitions and numbers of transversions (Fig. 4.2). A bias in favour of transitions is observed however, as has been observed for other groups and sequences (Fitch 1967, Vogel 1972, Wilson *et al.* 1985, Jukes 1987, Moritz *et al.* 1987). The cause of this bias is not clear though. Differences in the extent of the transition bias occur in mtDNA (see for example Vawter & Brown 1986, Thomas & Beckenbach 1989, DeSalle *et al.* 1987), and transition biases are found in nuclear sequences as well (e.g., Fitch 1967, Jukes 1987), so the absence of a mis-match repair system in mitochondria (Wilson *et al.* 1985, Schaaper & Dunn 1987) may in itself be insufficient to account for a biased rate of fixation of transitions.

The variability in the proportions of transitions and transversions does not seem to be due to saturation of transition mutations. A common feature of mtDNA sequence evolution is the decline in the proportion of transitions as time since separation increases (Wilson *et al.* 1985, Moritz *et al.* 1987, Miyamoto & Boyle 1989). The skink taxa have relatively few differences between them (less than 10%), and forty two percent of the substitutions in the skink data set are singletons. The proportions of transitions also do not show a tendency to decline as more differences accumulate (Fig. 4.5), which suggests that the degree of divergence between the skink taxa has not been obscured by many unobserved changes.

The relative decline in numbers of transitions in skinks is apparent however when the Ts/Tv ratio is plotted in relation to the number of transversions (Fig. 4.6). This is a common trend though (Fig. 4.7), and the fact that the skink Ts/Tv ratios (Table 4.2) were much larger than the asymptotic value of 0.47 indicates that saturation of fixed mutations has not occurred.

The variation in the numbers of transitions and transversions among the skinks (Fig. 4.2) may be attributed in part to the stochastic nature of the substitution process. There is variability in both the ratites and bovids when fewer than 30 transitions occur (Fig. 4.4). As more transversions accumulate variability decreases (Table 4.3, Figs. 4.4, 4.6, 4.7), which also suggests that the variability is related to having a relatively small number of changes. The observation that there may be a relatively high number of transversions in comparisons between taxa with few differences, but not when more divergent taxa are compared (see for instance Stewart Island Green; Table 4.1b) could be related to sampling error.

The simulation studies also indicated that numbers of transversions can vary in relation to the number of transitions (Fig. 4.9). However, the observation that this variability did not decrease as more substitutions occurred (Fig. 4.10), or as the Ts/Tv ratio was varied (Fig. 4.11), suggests that there is a difference between the simulation process and that which generated, for instance, the ratite pattern of differences. The sequences produced for the simulations were generated by a "star tree", or "tumbleweed", model:

where all sequences are derived from the same point source (the initial starting sequence). In contrast, phylogenies such as the ratites are usually formed by a more consecutive branching order as shown here:

The patterns of substitution in the skinks seem to fit the star tree model quite well. Many of the skink taxa have similar numbers of nucleotide substitutions between them, and the pattern of variation in numbers of transitions and transversions (Fig. 4.2) is also similar to the simulation results (Fig. 4.9). These observations suggest that the skinks (including the Australian *La. guichenoti* and the Mauritian *L. telfairi*) diverged from each other too rapidly for many phylogenetically informative changes to occur in this portion of the 12S rRNA. The Pecoran bovids diverged relatively rapidly from each other, over a period of five million years (Kraus & Miyamoto 1991, Allard *et al.* 1992). The plot of transitions and transversions for the skinks is more similar to the simulated data than to the bovid pattern (Fig. 4.4b) however, indicating that the skinks may have diverged relatively more rapidly than the bovids.

Rates of mtDNA Evolution

Reliable estimates of times of divergence of the skinks cannot be obtained by comparison to other data sets (Table 4.6). Restriction enzyme digestion studies of mtDNA suggested a general rate of evolution of between 1-2% sequence difference per million years for primates and several other mammalian groups (Wilson *et al.* 1985, 1987), but the applicability of this rate to both specific genes, such as the slowly evolving 12S rRNA, and to other groups has not been validated (Vawter & Brown 1986, Moritz *et al.* 1987, Thomas & Beckenbach 1989, Goddard *et al.* 1990). Rates of change for specific sequences are also lineage dependent (Britten 1986, Gillespie 1986, Goddard *et al.* 1990, Palmer 1990, Satta & Takahata 1990). The slow rate of change in the ratite sequences, in comparison with the bovids (Table 4.6), supports the view that rates of bird sequence evolution may be slower than those of mammals (Kessler & Avise 1985), though Shields & Wilson (1987) suggested that geese have a mtDNA evolutionary rate similar to mammals.

Rates of evolution could be affected by efficiency of DNA repair (Britten 1986), cell division rates (Wu & Li 1985), generation time (Wu & Li 1985, Li *et al.* 1987), and metabolic rate (Martin *et al.* 1992, Palumbi & Martin submitted). Recent studies have suggested that poikilothermic vertebrates may have slower rates of mtDNA evolution than birds or mammals (Thomas & Beckenbach 1989, Avise *et al.* 1992, Martin *et al.* 1992, Martin & Palumbi submitted). These studies examined aquatic groups only (salmon, turtles and sharks), and the applicability to other groups such as terrestrial amphibians and reptiles is less certain. Some newts and salamanders however appear to have slow rates of sequence evolution (Larson & Wilson 1984, Wallis & Arntzen 1989), but the sequence data from xantusiid lizards (Table 4.6) could indicate that poikilotherms can have rates of sequence evolution as high as some homeotherms.

Slower rates of evolution in poikilotherms may be related to their lower basal metabolic rate. A high metabolic rate (as in many birds and mammals) may result in a faster turnover of mtDNA, and consequently an increased chance of DNA replication errors (Martin & Palumbi submitted). High body temperatures may also lead to greater levels of oxidative damage in DNA (Shigenaga *et al.* 1989). Some lizards, and other reptiles, however may not have a single basal metabolic rate, (e.g., Werner & Whitaker 1978; see also Schmidt-Nielsen 1986). If the skinks do have a slower rate of mtDNA evolution than mammals then this implies that many of the skink taxa diverged from each other much earlier than the separation of the orangutan from the other great apes, more than 15 million years ago (see Tables 4.5d, 4.6). This period of time contrasts sharply with the suggestion, based on morphological comparisons, of 5 million years separation between New Zealand and Australian *Leiolopisma* (Hardy 1977).

Allozyme data (Daugherty *et al.* 1990b, C.H. Daugherty & G.B. Patterson pers. comm.) and immunological studies (Baverstock & Donnellan 1990, Hutchinson *et al.* 1990) also indicate much greater times of separation of skinks than morphological features imply. Skinks have no fossil record in New Zealand (Bull & Whitaker 1975) and are poorly represented in fossil faunas elsewhere as well (Carrol 1969, Molnar 1991). Rates of skink molecular evolution cannot therefore be directly calibrated with geological time. The dynamics of reptilian molecular evolution have not been investigated so it may be inappropriate to extrapolate rates from other groups, such as birds or mammals. Reptilian groups with more substantial fossil records, such as crocodilians and iguanids (Carroll 1969, Estes & Price 1973, Pregill 1989), require DNA sequencing studies so that rates of molecular evolution in reptiles can be investigated in more detail. The use of other information to infer times of divergence for the skinks will be considered in the next chapter after phylogenetic analyses of the sequences.

Estimating Degree of Divergence with Transversions

The variability in the number of transversions implies that estimates of total sequence divergence based on numbers of transversions (Brown *et al.* 1982, Wilson *et al.* 1985, Miyamoto & Boyle 1989, Milinkovitch *et al.* 1993) may have a high level of uncertainty (Figs. 4.14, 4.15, & 4.16). More comprehensive simulation experiments and analyses than those presented here however are required to examine this problem in detail.

Regions of Variability

Two points emerge from comparison of changes between closely related taxa (or those with the fewest number of differences). The first is that certain regions do have a tendency to vary first (Figs. 4.19, 4.20). Helix 26, part of domain II, is very variable within and between vertebrate groups (Table 3.3), but still retains its helical structure. Helix 34 in skinks is also variable, more so than some of the unpaired regions (Fig. 4.17). These two helices are not primary points of contact for ribosomal proteins, nor do they appear to be sites of tRNA binding (Gutell *et al.* 1985, Noller *et al.* 1990). Helix 33 in contrast is well conserved (Fig. 4.17, and see also Table 3.3), a fact also noted by Simon (1991) for cicadas. This helix appears to be a point of contact for ribosomal protein S19 (see Stern *et al.* 1989, Ehresmann *et al.* 1990, Noller *et al.* 1990) and so the conserved nucleotides may be associated with this.

The second point is that different groups of vertebrates may have different centres of variability. L38′ is very variable in skinks, xantusiids and bovids but not in ratites or the great apes (Table 3.3, Fig. 3.6). These latter two groups show more variability in SL40 and S45, respectively (Figs. 3.6 & 4.20). The fact that different groups tend to vary in different regions suggests either that there may be little functional significance to these patterns of variability, or that functional constraints differ between groups. Differences in sites and frequencies of variability between groups will be affected by how many taxa are examined for each group, and also by how closely related they are, so comparisons of more species and groups are required to determine if there are taxonomic patterns of regional variability.

Skink taxa with larger numbers of differences between them accumulate substitutions in regions S29, L30′, S34 and SL40, as well as S26 (Fig. 4.19). A similar pattern occurs among the ratites (Fig. 4.20). Half of the eight most variable sites in skinks occur in S26 and L38′ (Fig. 4.17), but the variable site (position 101) in L30′ tends to differ only between more distantly related taxa (for example between *L. n. polychroma* and *L. infrapunctatum*; Table 3.1).

L. n. polychroma and *L. n. nigriplantare* differ from each other in some of the more conserved regions of the molecule (Fig. 4.18), despite the fact that they have relatively few differences between them (Table 4.1a). While changes in more conserved regions may imply a more distant relationship between these two taxa, this is not supported by morphological or genetic information (Daugherty *et al.* 1990b), nor by analyses of the sequence data (see Chapter Five). Errors in PCR or sequencing could explain why these two taxa differ in more conserved regions, but this seems unlikely since at least two individuals were sequenced for both *L. n. polychroma* and *L. n. nigriplantare* (Table 2.1) and several amplification and sequencing reactions were performed for each sequence. The locations of the differences between them may just reflect the fact that not all sites in "more conserved" regions are equally conserved, just as some sites in "more variable" regions (e.g., position 207 in L38′, Fig. 4.17) can be more conserved than their neighbours.

The effects on phylogenetic analyses of both a transition substitution bias and different levels of variation bewteen sites in the sequence are investigated in the next chapter.

Chapter Five: Signals in the Data and Phylogenetic Analyses

Aligned macromolecular sequences contain sites of variability, the patterns of which (the "signals") can be evaluated by clustering or phylogenetic algorithms. Different phylogenetic analysis algorithms use differing amounts of this information and have different optimality criteria for evaluating it and constructing evolutionary trees (see Felsenstein 1988, Swofford & Olsen 1990, Nei 1991, and Penny *et al.* 1992 for reviews). With biological data conflicting signals, which may arise from, for instance, convergence, can prevent taxa being placed into mutually exclusive groups. Such signals can complicate analyses, but they may be reconciled. As an example, a part of the skink data set yields the following signals (some of which are underlined):

L. notosaurus	$\mathrm{aa\underline{au}a\underline{a}uguaugua\underline{c}}$
L. lin/chl.	$\mathrm{aagca\underline{a}uauauguau}$
L. suteri	$\mathrm{aag\underline{u}auuauauguau}$
L. n. nigriplantare	$\mathrm{aa\underline{a}cauuguaugua\underline{c}}$

The signals in this region provide support for *L. notosaurus* grouping with all of the three other taxa. *L. notosaurus* has the most signals in common with *L. n. nigriplantare* however, and in this example these two would have the strongest relationship. Quantifying the signals supporting different sets of relationships in this way indicates the support, and hence reliability, for phylogenetic relationships.

The signals present in the skink 12S rRNA sequence are examined in this chapter using the Hadamard conjugation, a new method for spectral analysis (Hendy & Penny 1993). This method shows that with New Zealand skinks the data set contains many conflicting signals and a completely resolved phylogeny cannot be obtained with the available information. Furthermore, differential weighting of both transversions and more variable sites in the molecule has little effect in clarifying the skink relationships. Rapid diversification of the skinks appears to be the cause of many of the conflicts in the data. Three hypotheses to account for the origins of New Zealand skinks are proposed.

Spectral Analysis of DNA Sequences

The Hadamard conjugation uses a discrete Fourier transform to adjust for unobserved nucleotide changes in DNA sequences (Hendy & Penny 1993). Application of this method gives a "**spectrum**" which provides a quantitative measure of conflicting signals in the data which can support different phylogenetic hypotheses. A unique property of the Hadamard conjugation is it's "invertibility" which allows investigation of the properties in the data with respect to their fit to explicit assumptions of the model. The model has three essential elements (Penny *et al.* 1992, 1993), namely

a tree,
a mechanism for sequence change, and
probabilities of changes (lengths) along branches of the tree.

Given the model it is possible with the Hadamard conjugation to predict the data that would perfectly fit the tree. The number of patterns in the observed data is compared to the number of patterns in the predicted data and statistically evaluated. A statistically poor fit between the observed and predicted data can lead to an incompletely resolved set of relationships. That is, the model is a poor explanation of the observed data. Details of the Hadamard conjugation are shown in Box 5.1.

There are two principal programs for the Hadamard conjugation:

PREPARE - which reads sequences in a variety of formats (interleaved, block, Nexus), and can calculate base composition, and frequencies of patterns in the sequences. Output files can be prepared suitable for analysis by Hadtree, PAUP, and Phylip.

HADTREE - reads the partition frequency data from Prepare, corrects for multiple changes (using Cavender's 1978 model), and searches for an optimal tree using the closest tree criterion (Box 5.1; Hendy & Penny 1989). It also has the ability to use other optimality criteria, such as parsimony.

Both four- and two-character state analyses can be done using Hadtree. The four-character state model uses Kimura's (1981) three parameter model of evolution (one class of transition mutations, and two classes of transversions) to analyse signals on the basis of all four types of nucleotides. **Quadripartitions** of the data are created in this way, grouping taxa which share patterns of *A*, *C*, *G*, or *U*, respectively at a site. Storage limitations currently restrict four-character state analysis to 10 or fewer taxa. Two parameter models divide the signals into **bipartitions**, segregating for example taxa having a purine (*A* or *G*) from those having pyrimidines (*C* or *T*) at a specific site, as shown in Box 5.2. Currently up to 20 taxa can currently be analyzed using the two-character state procedure. The order of taxa in the data file does not influence the results.

In this chapter the skink data set of 20 taxa (Table 3.1) is analyzed using the two character state condition. The "sum of seven" option (Box 5.2) was selected to find all possible signals. Use of the "AT/GC" and "AC/GT" options give similar spectra and results. Too few transversions occur to use the "R/Y" option.

What is a Bipartition and How is it Represented?

Taxa can be grouped into bipartitions with 0, 1, 2, ..., n taxa. The number of occurrences for each bipartition are summed for the sequence and the frequency of the bipartition is calculated by dividing by the total sum of all bipartitions in the data set (including the bipartition which contains all the taxa; i.e. constant sites). The frequencies of bipartitions are called **signals** and the collection of signals for a sequence is referred to as a **spectrum** (Hendy & Penny 1993). Individual signals in the spectrum may be referred to as a **spectral signal** in this thesis. Bipartitions with only one taxon in them correspond to pendant branches on a tree - the branch leading only to that taxon. The other bipartitions (excluding the one with all taxa in it) are potential internal branches (or edges) in the phylogenetic trees.

Box 5.1. Interpretation of components of the Hadamard conjugation. The Hadamard conjugation operates on the signals in observed data and a model (tree + mechanism + probabilities of change). It's invertible properties allow the prediction of patterns in data that would perfectly fit the evolutionary model. It also permits correction for unobserved changes in sequence data before such data is either viewed directly (as the γ spectrum) or best-fitted to a tree, so corrections are independent of the tree. Probabilities of observing changes along edges of a tree (***p***) are specified by the model. Corrections to ***p*** for unobserved changes at sites are then made to generate inferred partition values, γ, or expected edge lengths in the optimal tree. The Hadamard transform, ***[H]***, converts these values to ρ, a vector which represents corrected path lengths (distances between taxa in the tree) for subsets of taxa in the tree. The correction for unobserved changes is then reversed to generate observed distances, ***r***. The Hadamard transform is then reversed, $[H]^{-1}$, to produce ***s***, the vector containing expected frequencies of observed patterns in all possible trees. With real data the process is reversed by going from s to γ. The closest tree optimality criterion uses the γ values to select the best fit between the real data to a tree that has edge lengths ***p***.

Model
↕
p (probabilities of change on edges)
↕ (corrections for unobserved changes)
γ (corrected estimate of edge lengths)
↕ ***[H]***
ρ (corrected path lengths between all taxa)
↕ (uncorrect for unobserved changes)
r (uncorrected distances between all taxa)
↕ $[H]^{-1}$
s (uncorrected estimate of edge
↑ lengths in all possible trees).
↑
Sequence Data

Box 5.2. Options for two-character state analysis of sequence data using the Hadamard conjugation. Comparisons between taxa are performed for each site in the sequence. The tilde (~) corresponds to the mathematical expression "not".

1. *A/~ A*	Group taxa with adenine at a site and group those which have a different nucleotide.
2. *G/~ G*	Group taxa with guanine at a site.
3. *C/~ C*	Group taxa with cytosine at a site.
4. *T/~ T*	Group taxa with thymine (or uracil).
5. *AC/GT*	Group taxa with adenine or cytosine at a site and group taxa with guanine and thymine (or uracil).
6. *AT/GC*	Group taxa with adenine or thymine.
7. *R/Y*	Compare on the basis of transversions only (AG/CT).
8. *All 7*	Performs each of the above analyses individually.
9. *Sum of 7*	Performs the first 7 comparisons and sums the frequencies of the signals.

These internal edge bipartition signals are ranked in order of descending frequency. The "strongest signal" has the highest frequency, and means that a relatively large number of sites in the sequence support the grouping of the taxa in this bipartition. If this signal is in the tree, its branch would have the largest internal length. Conflicting signals however may mean that the strongest signal is not included in a resolved tree. This is illustrated later in the Chapter.

Each bipartition can be uniquely identified by giving each taxon a binary number and assigning the bipartition the sum of the taxa it contains. For example, with five taxa the binary numbers 1, 2, 4, 8, and 15 describe each of the five taxa respectively (the last taxon's number is one less than its real binary number so that all bipartition numbers are unique). The bipartition corresponding to a grouping of the first two taxa can be described as either (1+2) = 3, or as (4+8+15) = 27. By convention the smaller of the two numbers is used to label the bipartition (Penny *et al.* 1993). A second example illustrates why the last taxon is identified by a number one less than its true binary number. The bipartition of the first four taxa corresponds to (1+2+4+8) = 15, which is the same as saying that the fifth taxon is in a bipartition of its own.

Lack of Resolution in the Skink 12S rRNA Data Set

Analysis of the 20 skink taxa does not yield a completely resolved tree. A graphical representation of the spectrum (Fig. 5.1) shows that there is much conflict in the data, most of the bipartition frequencies are the same. There are only four relatively distinct and strong signals (Fig. 5.1). However, for 20 taxa there are $\{2^{19} - 21\}$ or 524,267 possible bipartitions, but only 66 occur among the skinks. For a functional gene not all bipartitions will be present because of functional constraints, but such a small proportion of bipartitions in the skink data set suggests that the sequences do contain phylogenetic history. A data set with no conflicting signals would, for 20 taxa, produce 17 signals, each representing an internal edge of the phylogenetic tree (in addition there will also be 20 other signals corresponding to each pendant branch).

Spectral Analysis of other Vertebrate 12S rRNA Data Sets

To investigate whether the skink 12S rRNA molecule was unsuitable for the study, spectra were determined for two other vertebrate 12S rRNA data sets. The ratite data set (12 taxa) gives a resolved phylogeny using other methods (Cooper *et al.* 1992), while the Pecoran bovid data set produces a less resolved tree (Kraus & Miyamoto 1991, Allard *et al.* 1992). The ratite 12S rRNA sequences have a greater number of strong spectral signals and a resolved phylogeny is produced (Fig. 5.2a). The bovid data set however produces a spectrum similar to the skinks' and relationships cannot be completely resolved (Fig. 5.2b).

Parsimony and Neighbor-Joining Analyses of the Skink Data Set

Spectral analysis of the ratite and bovid sequences confirmed the results from previous phylogenetic analyses (see Cooper *et al.* 1992, Allard *et al.* 1992). However, to confirm that aspects of the Hadamard conjugation were not a cause of lack of resolution among the skink sequences, maximum parsimony

Fig. 5.1. Spectral analysis of 382 bases of 12S rRNA from 20 *Leiolopisma* and related taxa. In this example the bipartitions are ranked, rather than each bipartition being identified by it's unique number. The taxa in the four most well supported bipartitions are identified;

Lmi = *L. microlepis*
Lfr = *L. infrapunctatum*
Cae = *C. aenea*
Lze = *L. zelandicum*
Lsm = *L. smithi*
Lag = *La. guichenoti*
Lfa = *L. fallai*
Lmo = *L. moco*

Note that most of the other bipartitions have similar amounts of support in this data set. No fully resolved tree is produced.

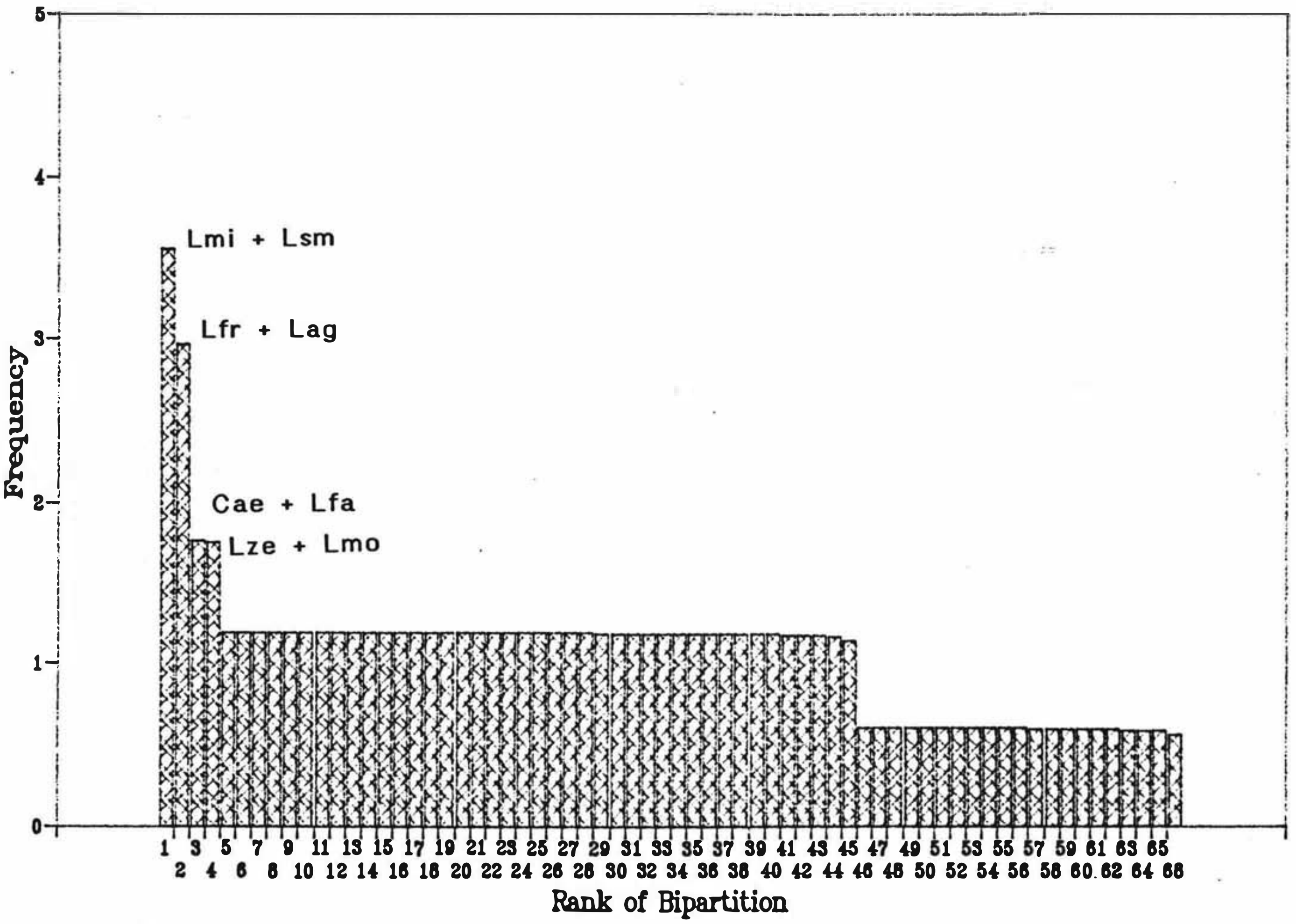


Fig. 5.2a. Spectral analysis of 380 bases of 12S rRNA from 12 ratite birds (data from Cooper *et al.* 1992). A resolved tree is produced using HADTREE and is shown in the upper right corner of the figure. Compare this spectrum with that of the skinks in figure 5.1. In particular, note the different relative frequencies of bipartitions in the ratite data set. Full species names and sequences are given in Appendix 2.

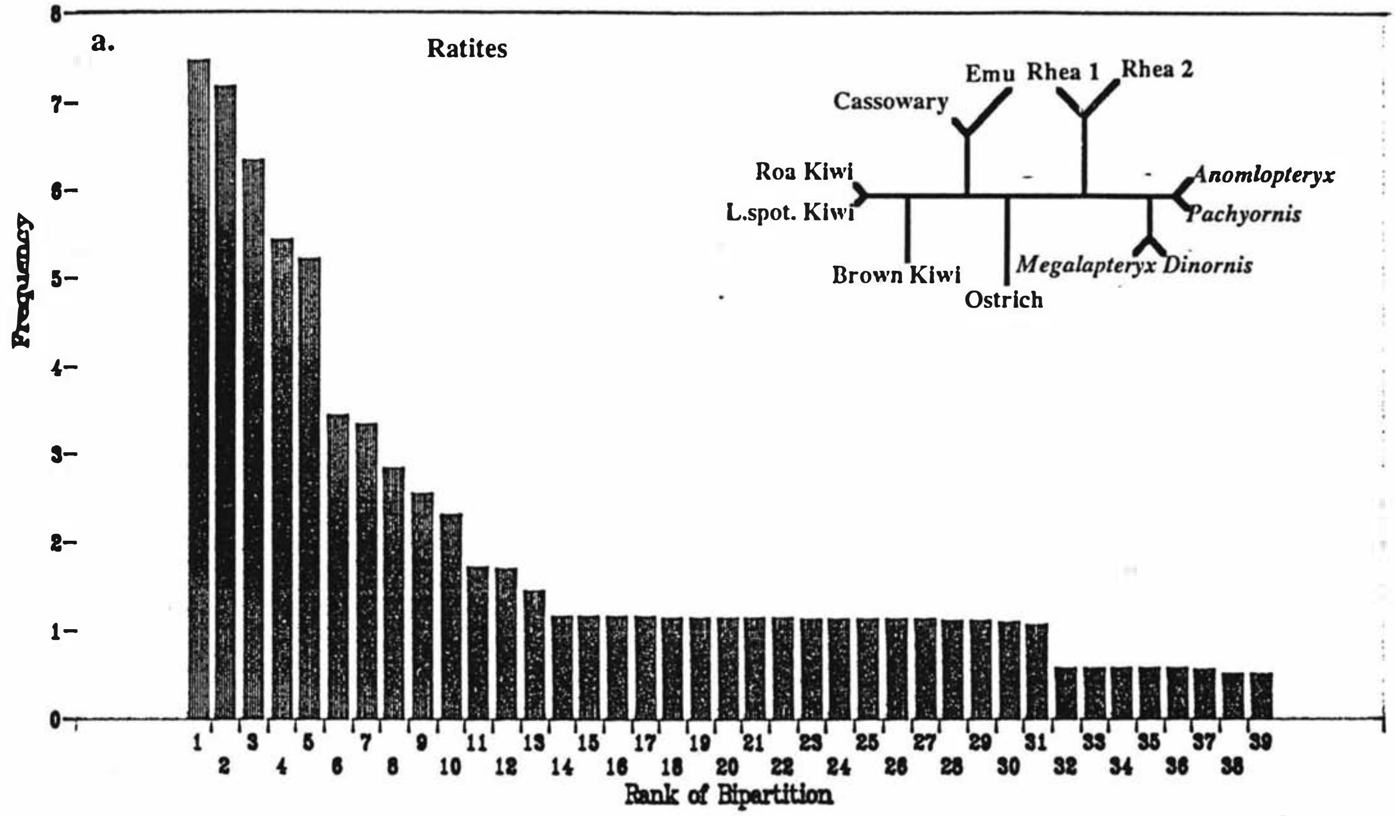
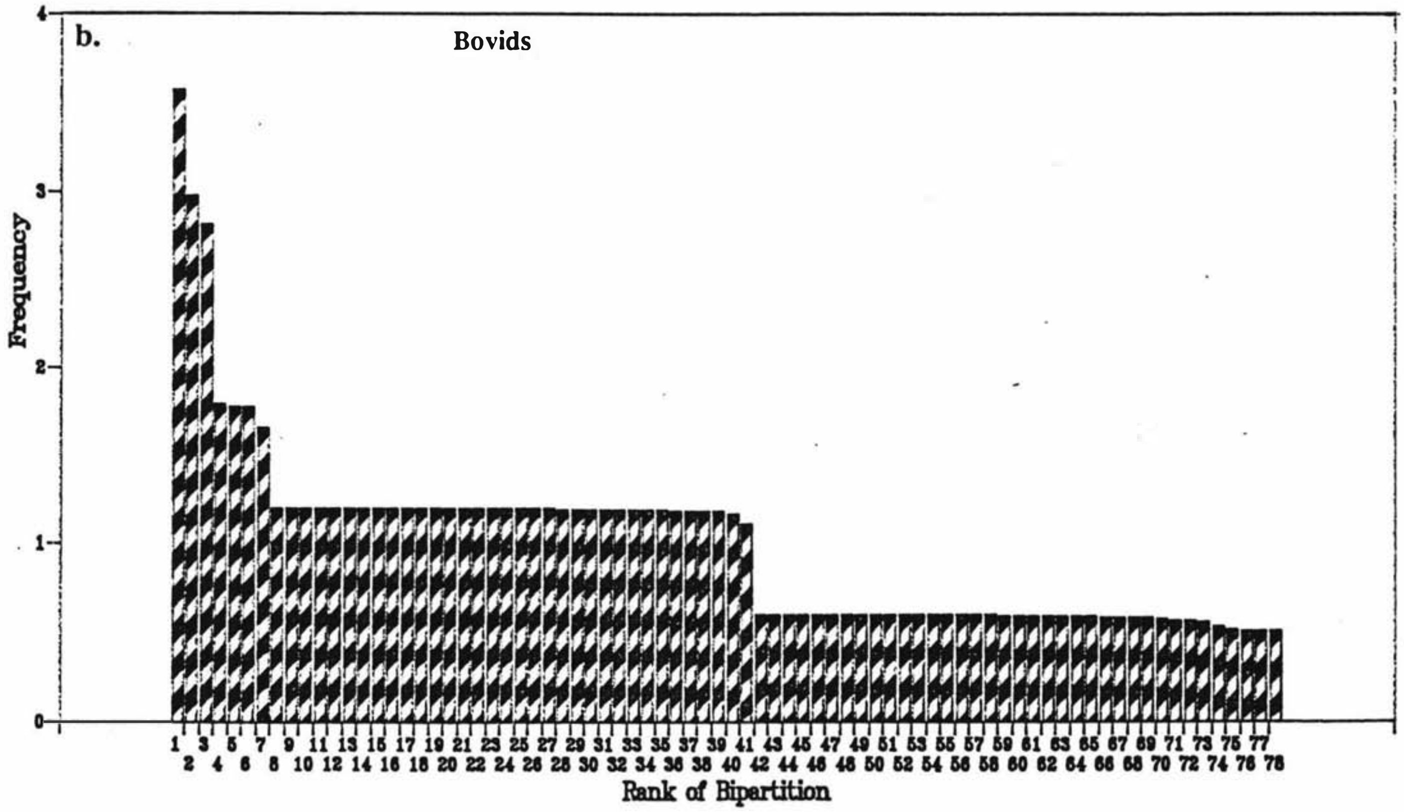


Fig. 5.2b. Spectral analysis of 387 bases of 12S rRNA from 15 bovids (data from Kraus & Miyamoto 1991 and Allard *et al.* 1992). A fully resolved tree is not produced by HADTREE. As with the skinks, many of the bipartitions have similar frequencies. The bovid data set is listed in Appendix 2.



(Swofford 1991) and neighbor-joining (Saitou & Nei 1987) analyses were also performed. Both of these latter two methods give similar results (Figs. 5.3 & 5.4). Internal branches are short and most of these are collapsed when bootstrapping is performed. The unbootstrapped trees are presented in figures 5.3 and 5.4 however since they at least provide clues for possible relationships among the taxa. Both parsimony and neighbor-joining produce similar estimates of relationships of the skinks (Figs. 5.3 & 5.4).

Partially Resolved Relationships Using the Hadamard Conjugation

Although a completely resolved set of relationships for the *Leiolopisma* is not obtained, the spectrum does indicate partially resolved relationships (Fig. 5.5). In this case every taxon is linked to at least one other but an unambiguous branching order for all taxa cannot be determined. The four strongest signals in the spectrum (Fig. 5.1) correspond to the pairs *L. microlepis+L. smithi, L. infrapunctatum+La. guichenoti, L. fallai+C. aenea* and *L. zelandicum+L. moco* respectively, though support for the latter two is comparatively weak. The same clustering pattern of the first three of these pairs is also evident in the parsimony (Fig. 5.3) and neighbor-joining trees (Fig. 5.4).

It is not the purpose of this thesis to compare in detail the Hadamard conjugation with other tree reconstruction algorithms, and more detailed comparisons between the trees are not considered here. Discussion of the Hadamard conjugation in relation to other methods can be found in Penny *et al.* (1991, 1992).

Weighting of Characters

The preceeding analyses treated all sites and types of changes in the sequences equally. Differentially weighting characters or regions of the sequence can be used to take account of different rates of change or violations in assumptions used in the model. However deciding what to weight and appropriate weighting values is often subjective and problematical (Swofford & Olsen 1990, Cracraft & Helm-Bychowski 1991, Mindell 1991). Weighting of sites on the basis of type or frequency of change have been suggested.

Transversions often accumulate less rapidly than transitions in DNA sequences (see Jukes 1980, Wilson *et al.* 1985, Avise *et al.* 1987, Moritz *et al.* 1987). This slower rate may mean that they are less likely to be obscured by multiple substitutions than transitions, and so may be more phylogenetically informative when comparing distantly related taxa. Giving transversions more phylogenetic importance by weighting them is therefore sometimes used (see Mindell 1991). A second weighting scheme takes account of the fact that, for rRNA sequences, fixation of mutations in base-paired regions may not be independent. If one member of a base pair changes, selection for the maintenance of the pair bond could increase the chance of fixation of a complemenatry change in the other member of the bond. Paired regions may therefore be weighted differently from unpaired regions in phylogenetic analyses (Mindell & Honeycutt 1990). A third method, as discussed by Simon (1991), is that regions of different levels of sequence variability in a molecule may be more informative for different depths of phylogenetic

Fig. 5.3. Consensus of nine equally parsimonious (shortest) trees for the 382 bases of 12S rRNA sequence from 20 *Leiolopisma* and related taxa. The tree shown is a 50% majority rule tree, that is, only associations of taxa which occur in at least half the trees are indicated. Lengths of edges are proportional to the number of changes along each edge. Note that many of the internal edges are short relative to pendant branches. Thick lines in the tree denote those branches which do not collapse after 10 bootstrap replications (more could not be performed because of the long computational time required). PAUP version 3.0s was used for the analyses.

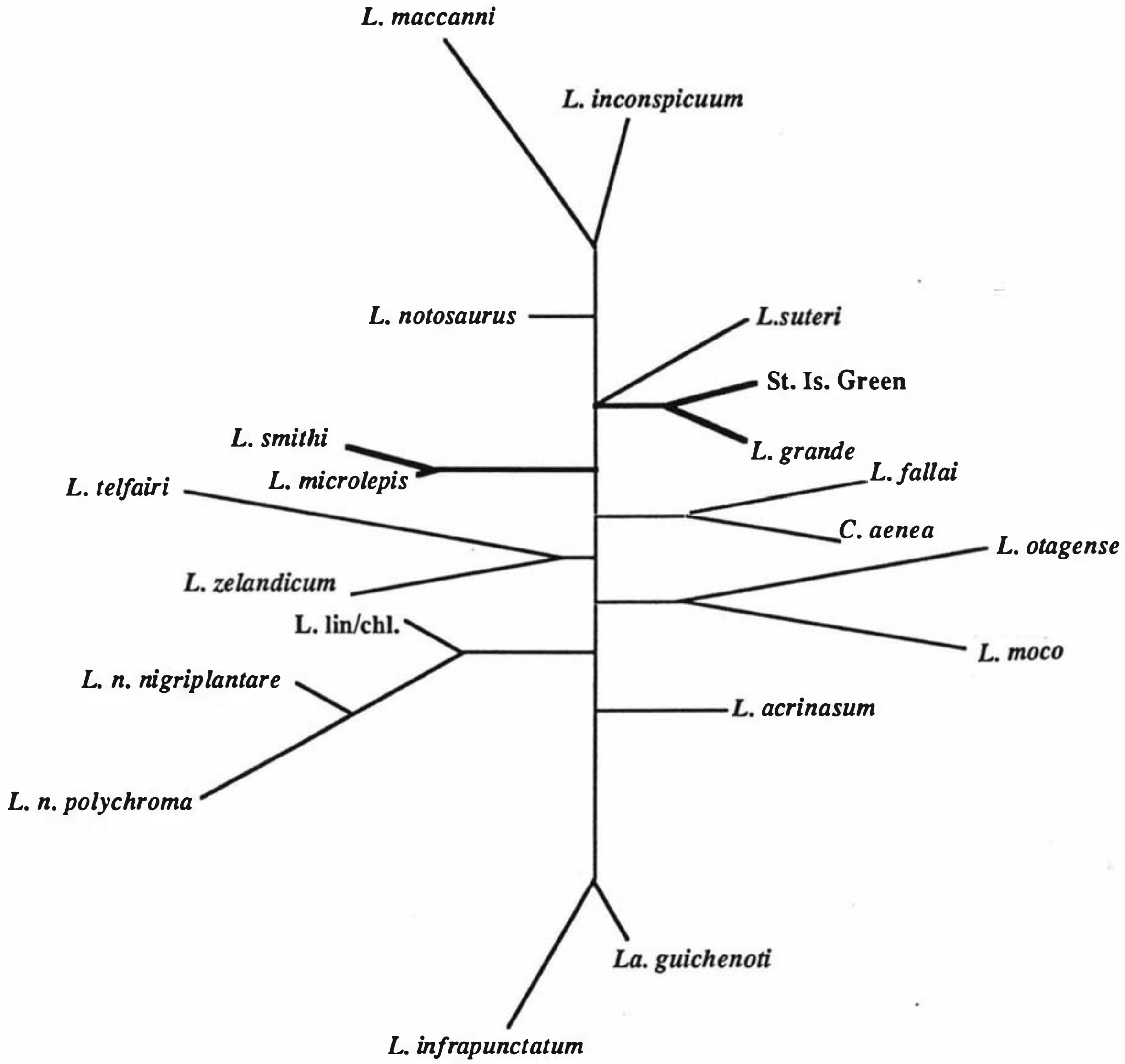


Fig. 5.4. Neighbor-joining tree for the 382 bases of 12S rRNA sequence from 20 *Leiolopisma* and related taxa. (Phylip version 3.4 was used). Percentage values on internal edges correspond to the number of times that edge occurred in 100 bootstrap replications. Only four of the internal edges occur in more than 80% of the bootstrapped trees. Lengths of branches are proportional to the probability of change along that branch. Note that internal edges are relatively short in comparison to the pendant branches. The tree is unrooted. Compare this tree with the parsimony tree in figure 5.3 and the Hadamard tree in figure 5.5.

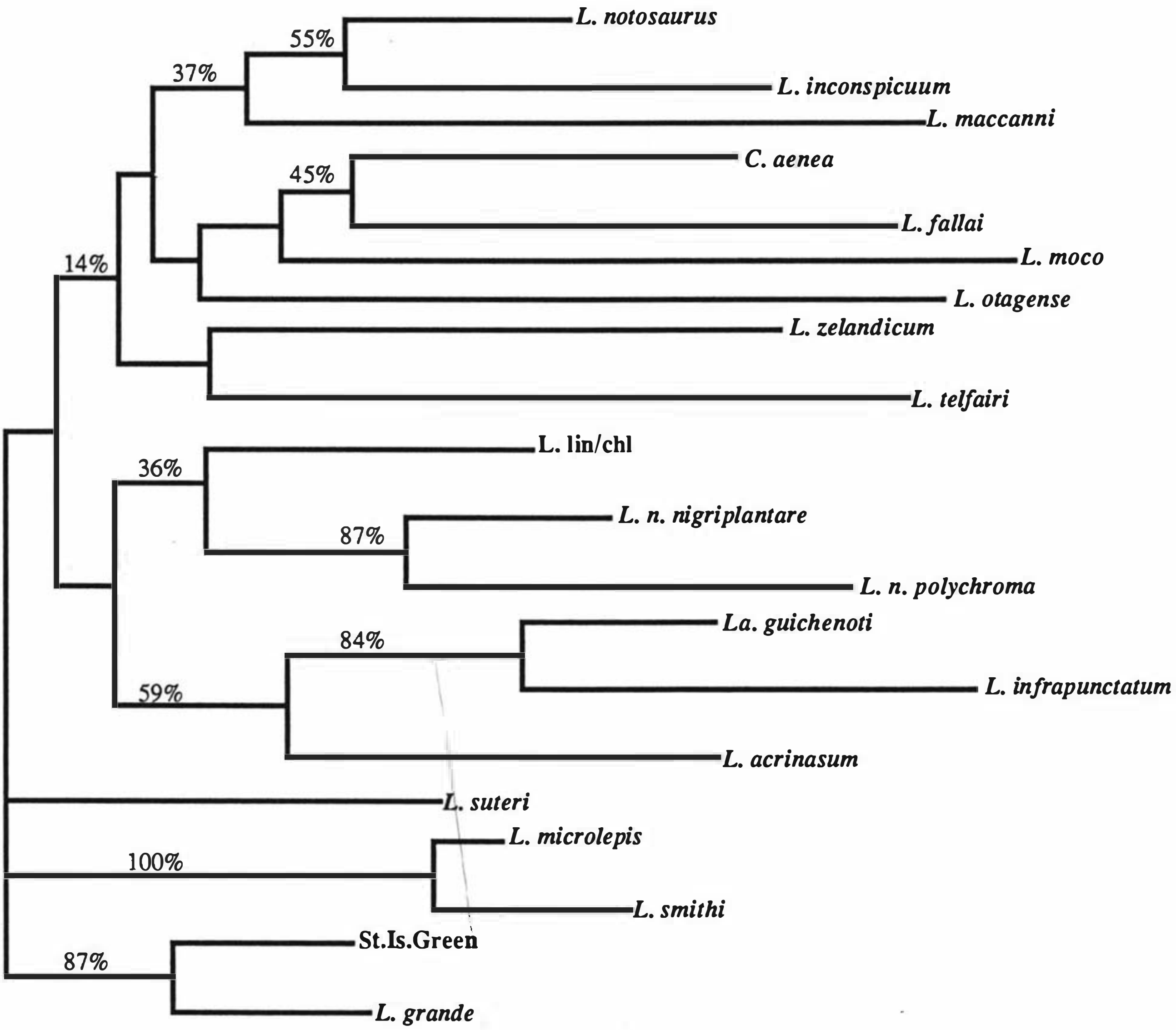
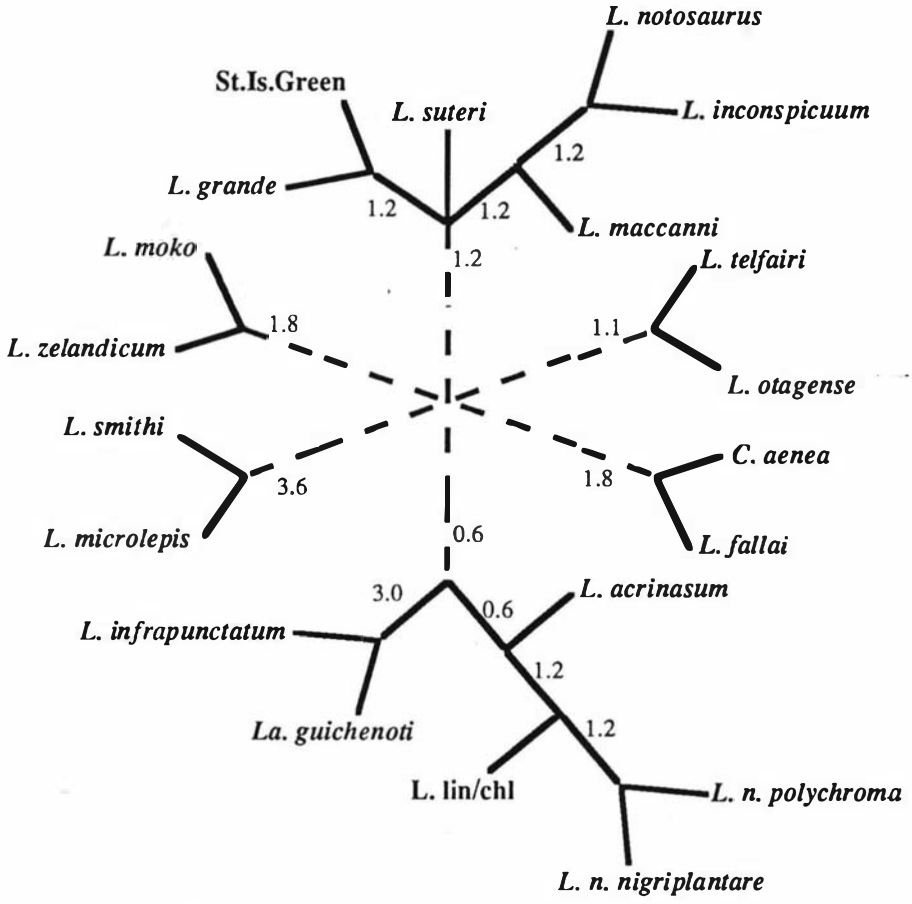


Fig. 5.5. Inferred phylogenetic relationships for the 20 skink taxa based on spectral analysis of 382 nucleotides from the 12S rRNA gene. The frequency of each grouping of taxa (bipartition) is shown next to the branch leading to that group. The clusters cannot be reconciled into a single phylogenetic tree and this is indicated by the broken lines. Low frequency values indicate that few shared nucleotide substitutions occur to support that bipartition and the true phylogenetic relationships for groupings of such taxa are therefore uncertain. Transitions and transversions are given equal weighting. Branch lengths are not proportional to the degree of divergence between taxa.



"The Gods of the earth and sea
Sought thro' nature to find the Tree;
But their search was all in vain:
There grows one in the Human Brain."
(Blake)

branching and could be weighted differently to reflect this. These three weighting options were investigated for the skink sequences to determine if they enhanced or reduced signals and lead to better phylogenetic resolution.

Weighting of Transversions

Transitions outnumber transversions in the skink data set by, on average, four to one. A four-fold weighting factor applied to transversions did not greatly alter the strongest signals in the spectrum but it did change some of the other signals (Fig. 5.6). The relationships between *L. grande* and St.Is. Green, and between *L. n. polychroma* and *L. n. nigriplantare* are lost for example, because many of the differences between these pairs of taxa are transversions (Table 3.1). When the subtrees for this differential weighting are drawn it can be seen that *L. grande*, *L. acrinasum* and *L. otagense* cannot be linked to any other taxa (Fig. 5.7), in contrast to the more resolved relationships in the equally weighted analysis (Fig. 5.5). Given this result, and the fact that high numbers of transversions do not signify more distant relationships (Table 4.3, Fig. 4.2), transitions and transversions are given equal weighting in all further comparisons.

Weighting of Paired Regions

Wheeler & Honeycutt (1988) reported that for both animal and plant groups, analysis of paired regions in 5S rRNA were phylogenetically misleading whereas phylogenies derived from unpaired regions were in more agreement with relationships inferred from morphological data. This they attributed to selection maintaining base pairings in rRNA. They suggested that a change in one member of a base pair increases the chance of fixation of a complementary change in the other base pair, and Wheeler & Honeycutt consequently recommended assigning weights of 0.5 to nucleotides involved in pairings; that is, a change at an unpaired site can be considered twice as informative as a change in a nucleotide which is involved in a base pair. This level of weighting is only appropriate however if complete compensation occurs in paired regions, but this is not the case for skinks (Table 3.6) or many other groups (Smith 1989, Hedges *et al.* 1990, Mindell & Honeycutt 1990, Hillis & Dixon 1991, Simon 1991, Dixon & Hillis 1992). Nevertheless, paired regions can be phylogenetically informative. Dixon & Hillis (1992) examined relative weighting of paired regions for 28S rRNA and found that a weighting factor closer to 1.0 than 0.5 was appropriate for their study since more changes in paired regions were uncompensated than compensated.

At least 54% of the nucleotides in the skink sequences are involved in base pairing (Fig. 3.3), and 39% of the variable sites occur in paired regions (Table 4.17). A high proportion of changes have both pairing partners changed and pairing is maintained (Table 3.7).

Dixon and Hillis (1992) adopted a linear scaling approach to weighting paired regions. With this method the weighting given to paired nucleotides is calculated by comparison to the number of changes expected by chance and with no compensation. Following their scheme, a relative weighting of 0.71 is obtained for the paired regions in the skink molecule (Table 5.1). This equates to a weighting of

Fig. 5.6. The effect of weighting transversion substitutions four-fold higher than transitions in the skink data set. The major difference from the equally weighted spectrum (Fig. 5.1) is that with differential weighting there is less support for the *C. aenea+L. fallai* and the *L. zelandicum+L. moco* bipartitions. Minor differences occur in the strength of support for other bipartitions, but as in figure 5.1 most of the bipartitions have similar levels of support. Resolution of the skink relationships is not improved by giving transversions greater phylogenetic importance than transitions (see also Fig. 5.7).

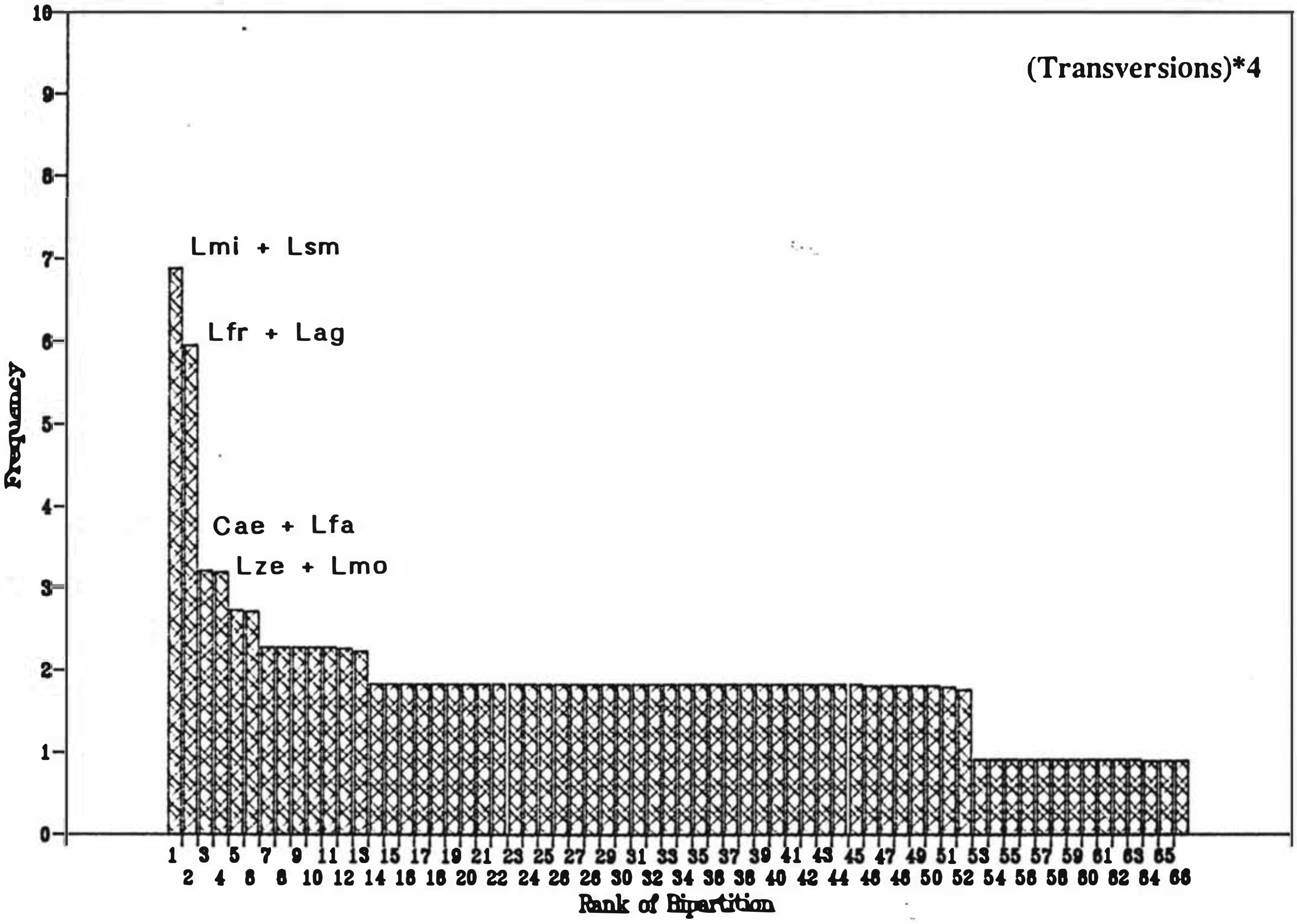


Fig. 5.7. Inferred phylogenetic relationships for 20 skink taxa based on spectral analysis of 382 nucleotides from the 12S rRNA gene. The frequency for each bipartition is shown next to the branch leading to it. Transversions are weighted four times higher than transitions. Compare these relationships and signal strengths to Fig. 5.5. Note that using this weighting scheme, *L.grande*, *L. acrinasum* and *L. otagense* cannot be placed with other taxa , and that *L. n. nigriplantare* now groups with *L. telfairi* rather than with *L. n. polychroma.*

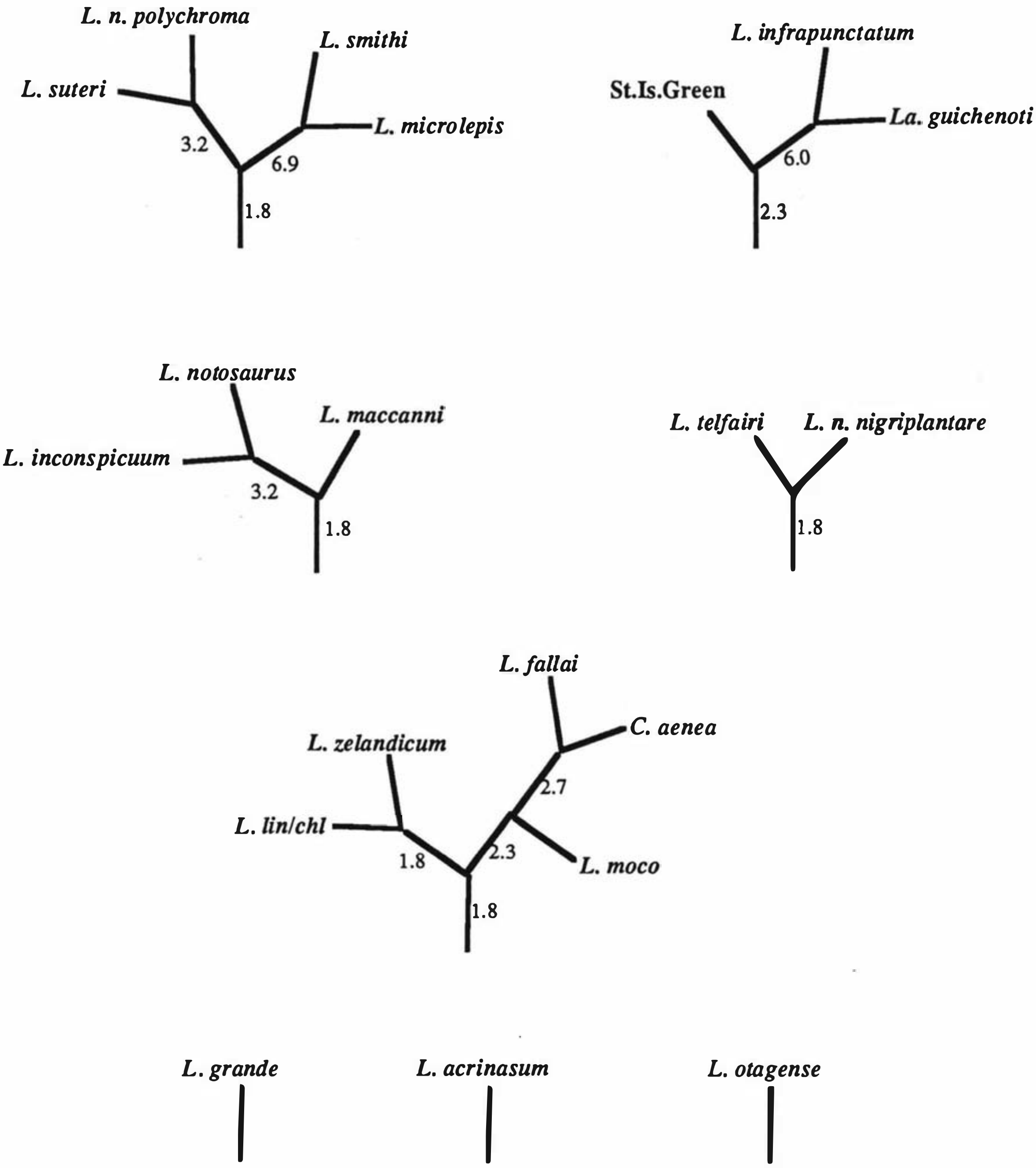
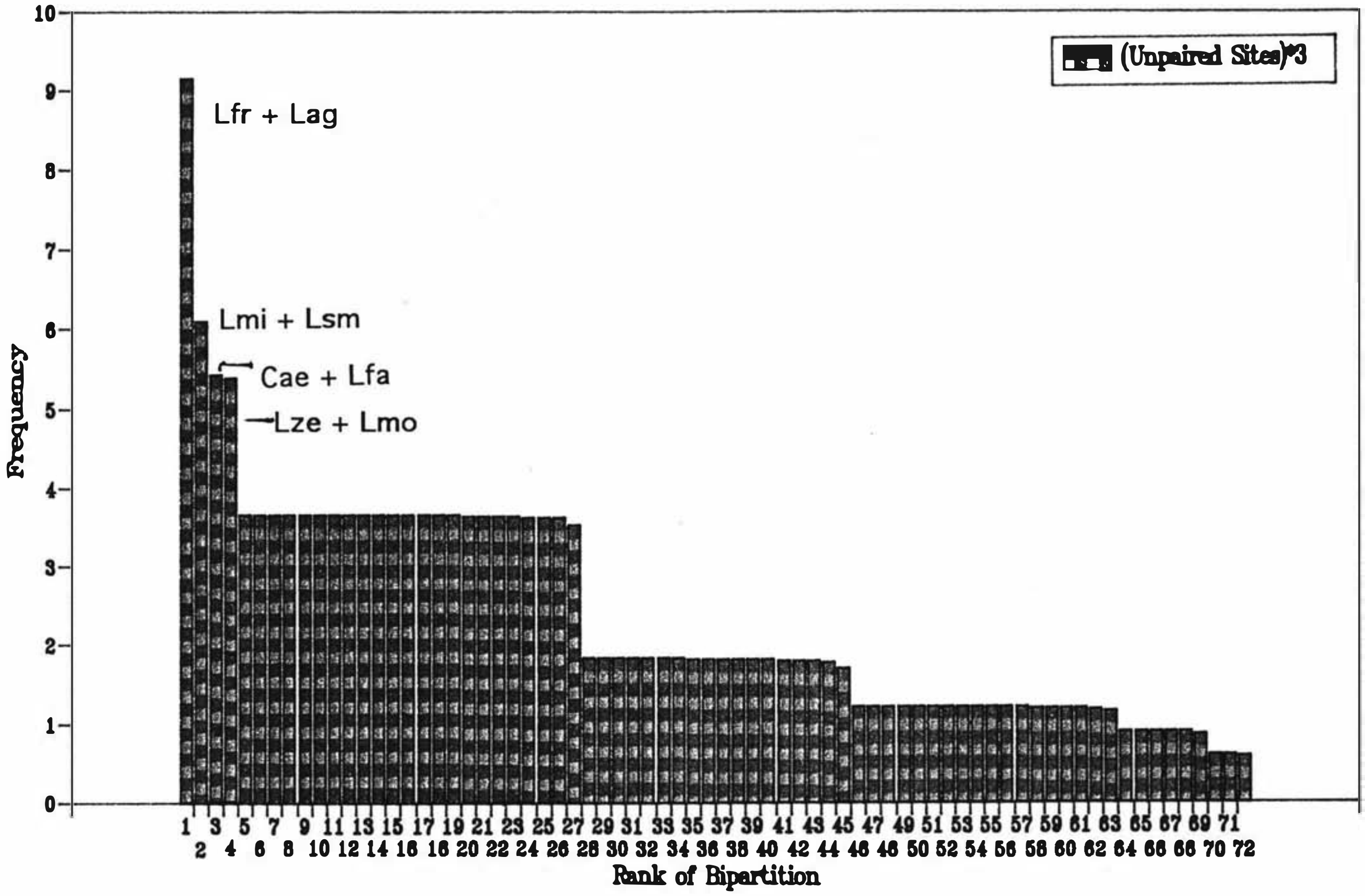


Table 5.1. Determining a relative weighting factor for phylogenetic analysis of paired regions in the skink 12S rRNA fragment. ~~Expected and observed numbers of changes in paired regions are from Table 4.6~~. If changes are random all base substitutions will be independent and therefore no differential weighting should be applied. Alternatively, if all changes in paired regions were compensatory, then each substitution should be given a weighting of 0.5. Changes in the skink helices fall nearly halfway between the two extremes.

	Random Expectations	**Observed**	**Complete Compensation**
No. Changes	15.3	56	85
	↓	↓	↓
% Dependence	0 %	58.4%	100 %
	↓	↓	↓
Relative Weighting	1.0	**0.71**	0.5

Fig. 5.8. The effect of a three-fold higher weighting factor for substitutions in unpaired regions of the skink 12S rRNA sequence three-fold higher than changes in paired regions (see text and Table 5.1). The bipartition of *L. infrapunctatum+La. guichenoti* is now the most strongly supported, indicating that substitutions supporting this bipartition occur mostly in unpaired regions. This is confirmed by examining the sequence alignment (Table 3.1). In contrast, substitutions supporting the *L. microlepis+L. smithi* bipartition are more common in paired regions (Table 3.1), and the spectral signal for this bipartition has decreased (compare with figure 5.1). No fully resolved tree is produced with this weighting scheme.



approximately 3:1 for unpaired versus paired regions. The spectrum obtained using this value differs little in general from that obtained by equal weighting - no fully resolved tree is produced (Fig. 5.8). The four major signals remain, though the signal for the bipartition of *L. infrapunctatum+La. guichenoti* is now the strongest, while the signal for *L. microlepis+L. smithi* is reduced. The three sites supporting the *L. infrapunctatum+La. guichenoti* bipartition are all in unpaired regions, while two of the three positions which support the *L. microlepis+L. smithi* bipartition are in helix 34 (see Table 3.1). The signals ranked from 5 to 27 in figure 5.7 are proportionally higher than in the unweighted spectrum, implying that they are derived from unpaired sites (compare Fig. 5.8 to Fig. 5.1). The Hadamard spectrum and a relative weighting scheme are therefore an easy way to see the extent to which paired regions contribute to bipartition signals.

Weighting of More Variable Sites

Eight positions in the skink data set have both transitions and transversions (Fig. 4.17). These sites are potentially rapidly changing positions which could be useful for resolving relationships amongst closely related taxa but could mislead more distant phylogenetic relationships because of unobserved substitutions (Mindell & Honeycutt 1990, Simon 1991). This does not however appear to be the case for the skinks. The taxa which have the fewest numbers of differences between them also tend to group together in figure 5.5, so small numbers of nucleotide substitutions between taxa seems to be a good indicator of closeness of relationships (see also the neighbor-joining tree, Fig. 5.4). Taxa classified on this basis do not always differ at the most variable sites. *L. microlepis* and *L. smithi* for instance, differ from each other at five positions, but only one of these (position 204) is classed as a highly variable site (Table 3.1).

Down-weighting the eight most variable sites by a factor of two or four has little effect on the spectrum (Fig. 5.9, compare to Fig. 5.1). However, excluding these eight sites entirely from the analysis results in the loss of 14 (21%) bipartitions (Fig. 5.10). These lost bipartitions represent groups of seven, eight or more taxa, rather than pairs of taxa, indicating that removal of these sites has only a minor effect on resolution of pairs of taxa. The reduction in the number of bipartitions when the most variable sites are removed does suggest though that these sites are a major source of conflicting signals. A resolved tree is still not produced however.

The Effect of Constant Columns in Sequence Analysis

The correction for unobserved changes in the Hadamard conjugation assumes that all sites are free to vary. This is not the case for functional genes (Fitch & Margoliash 1967, Shoemaker & Fitch 1989). As shown in Chapter Three, one third of the region of the 12S rRNA molecule under scrutiny here is conserved across the vertebrates. Assuming that all sites are free to vary will mean that corrections for unobserved changes will underestimate the true numbers of substitutions. This is because the frequency of change **per site** is lower if averaged across the whole sequence. If only a proportion of the sites can change then the actual frequency of substitutions at variable sites is higher, and so the probability of multiple changes should be higher. Lengths of some potential internal edges in the tree will then be longer and may result in greater resolution.

Fig. 5.9. The effect of giving a lower weighting to the eight most variable sites in the skink 12S rRNA sequence. (Both transition and transversion substitutions occur at these sites). Weighting these positions either two-fold (*5.9a*) or four-fold (*5.9b*) lower than the other sites in the sequence has little effect on the relative differences between bipartitions. The weighting of sites was done by simply duplicating, prior to analysis, the 374 less variable sites once (5.9a) or thrice (5.9b) with respect to the other 8 sites. Consequently, the frequencies of the bipartitions are proportionally higher than in the original spectrum (Fig. 5.1).

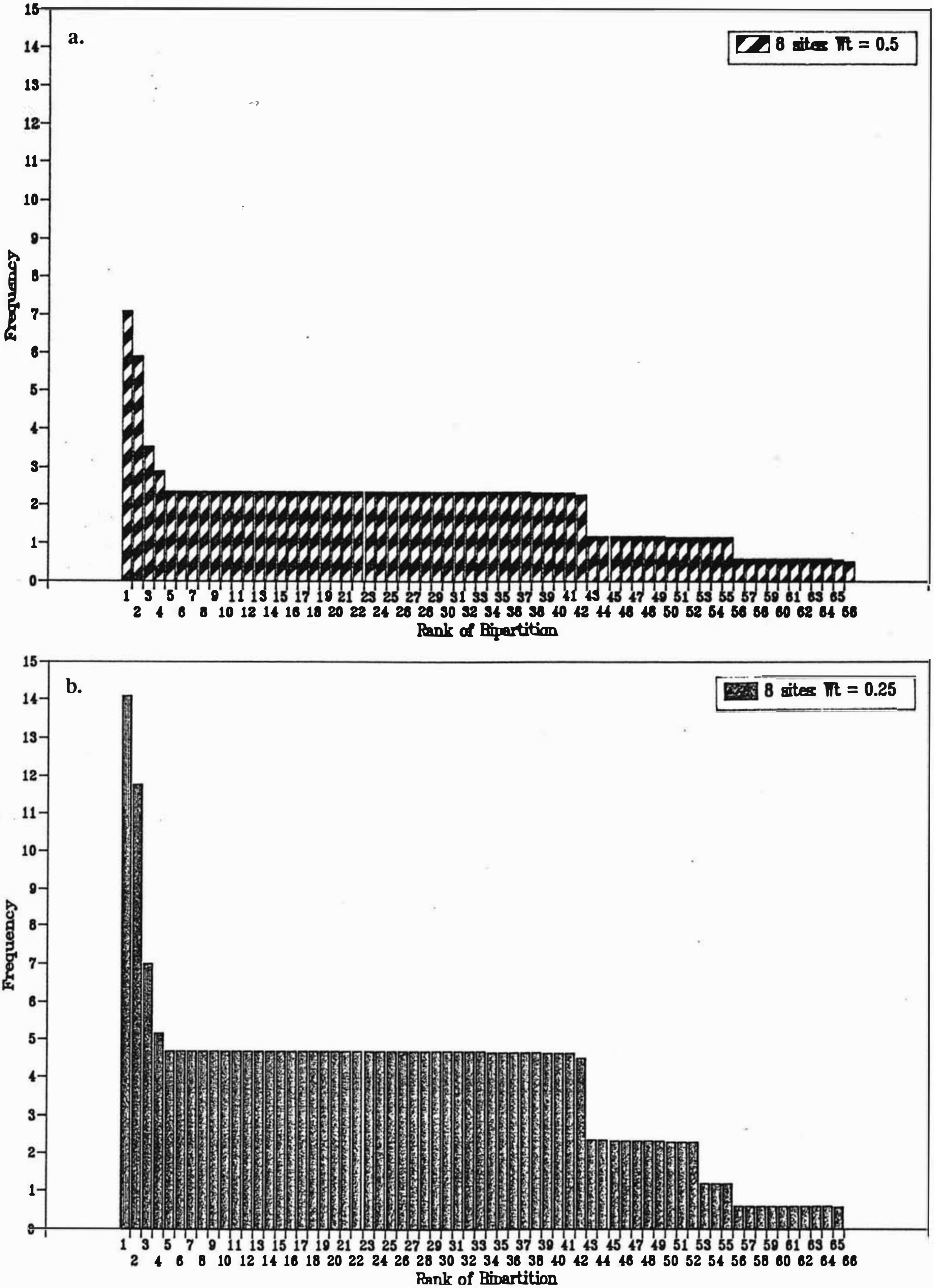
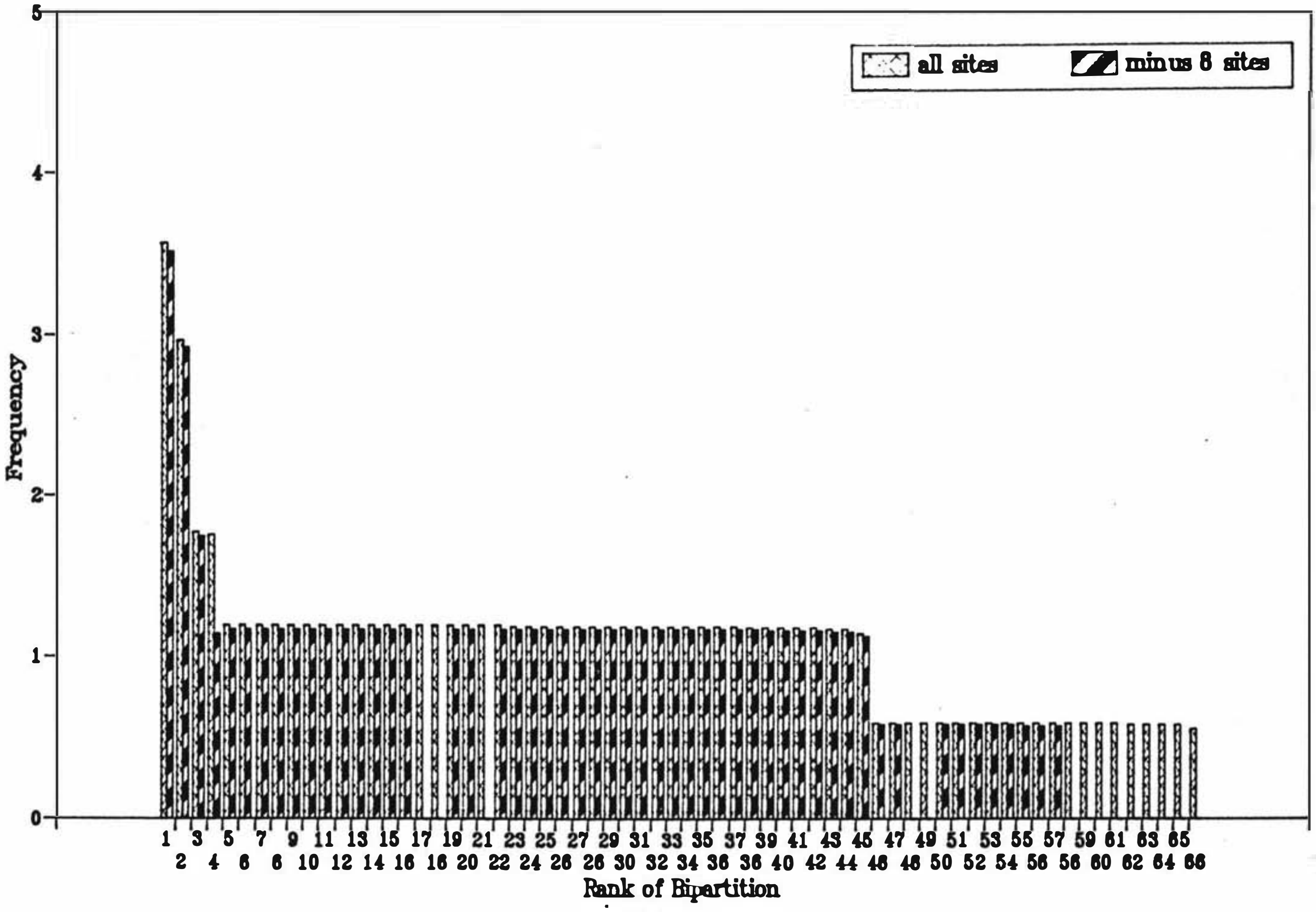


Fig. 5.10. The effect of removing the eight sites in the skink 12S rRNA data set which have both transition and transversion substitutions. Both the original spectrum (from Fig. 5.1) and the edited sequence spectrum are shown. Removal of the 8 sites has a greater effect than just giving them a lower weight in analyses (Fig. 5.9). Support for 14 bipartitions is lost when the eight most variable sites are removed, but the frequencies of other bipartitions are largely unchanged. The lost bipartitions generally contain eight or more taxa and so resolution of the relationships of more closely related taxa (those with few nucleotide differences between them) does not appear to be affected. Removal of the most variable sites, and consequent reduction in the number of bipartitions, does however indicate that such sites may be contributing noise to the analyses of more distantly related taxa.



The presence of an invariant core in the molecule and its affect on the Hadamard transform can be approximated by reducing the number of constant sites in the data. As the number of columns is reduced an expectation is that the strength of the signals should increase since there will be a greater correction for unobserved changes. Signals whose strength is independent of the number of constant columns are likely to be spurious and have little phylogenetic information.

The Effect on Skink Phylogeny of Weighting of Constant Columns

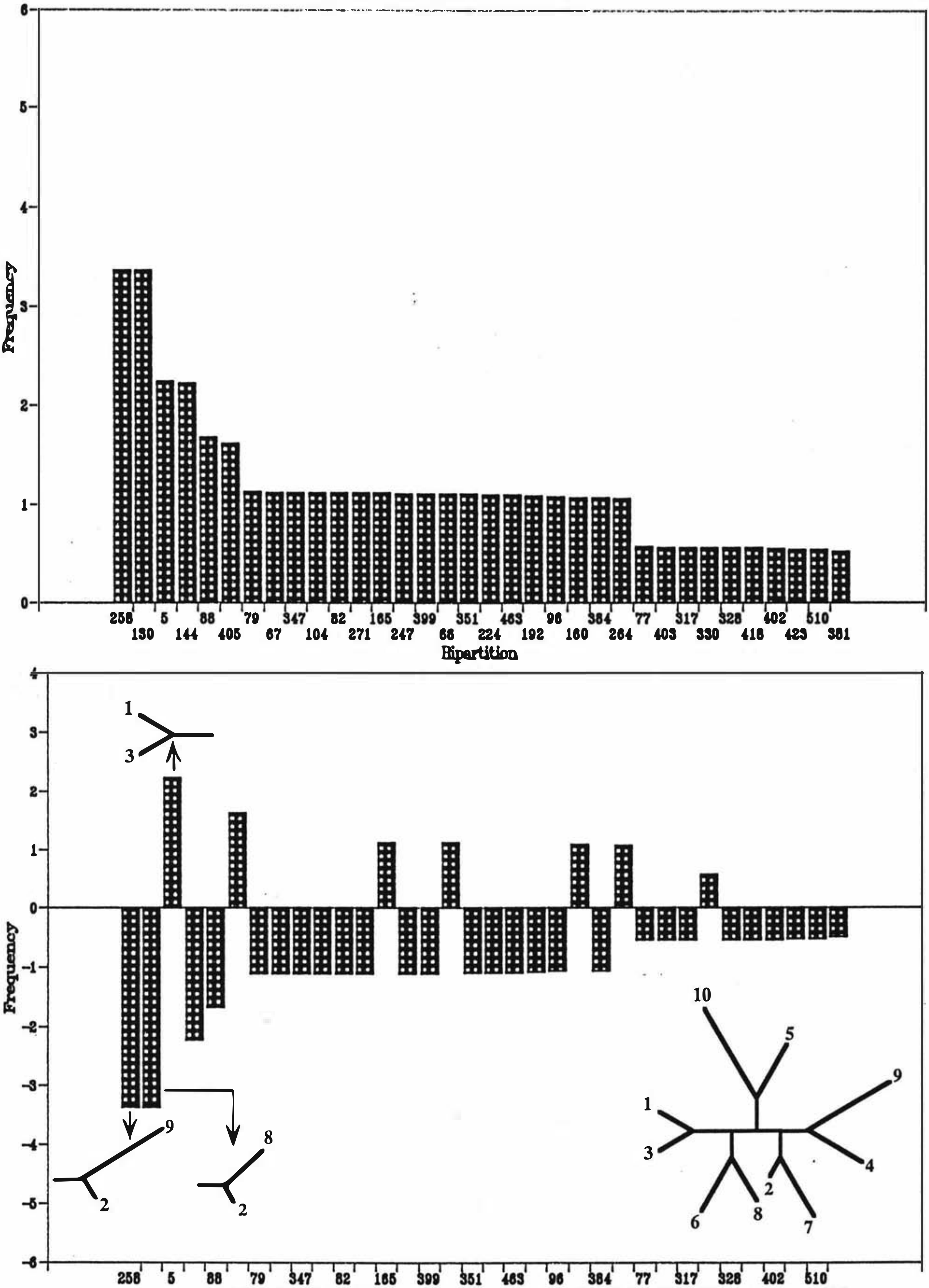
To examine this problem a subset of 10 skink taxa were chosen so that the effects are easier to detect. The taxa were chosen because they illustrate an extreme case where the two largest spectral signals are not included in the optimal tree (Fig. 5.11; see also Penny *et al.* 1993). Are some of the conflicting signals in this data set a result of inaccurate corrections for unobserved changes?

The frequencies of each bipartition in a data set are calculated by the PREPARE program. This information is written into a separate file which is then used by HADTREE to generate the spectrum. It was simpler to adjust for constant columns by changing parameters in this frequency file than to remove constant columns from the original data set. Only two numbers need to be changed to achieve this; one represents the total number of columns of data, and the other the number of these columns which represent constant sites in the data. The effect of progressively removing constant columns (1/3, 1/2, and 2/3) prior to the Hadamard conjugation was investigated for the 10 taxa data set. Figure 5.12a shows that as more constant sites are removed most of the bipartition frequencies increase slightly, though some do not. The increase in size of the bipartition frequencies is relatively small (Fig. 5.12a), and all of the seven original internal edges in the optimal tree are retained even when two-thirds of the constant sites have been removed (Fig. 5.12b). This indicates that the majority of the inferred phylogenetic relationships are robust and not due to invalid an assumption in the model (namely, that all sites are free to vary).

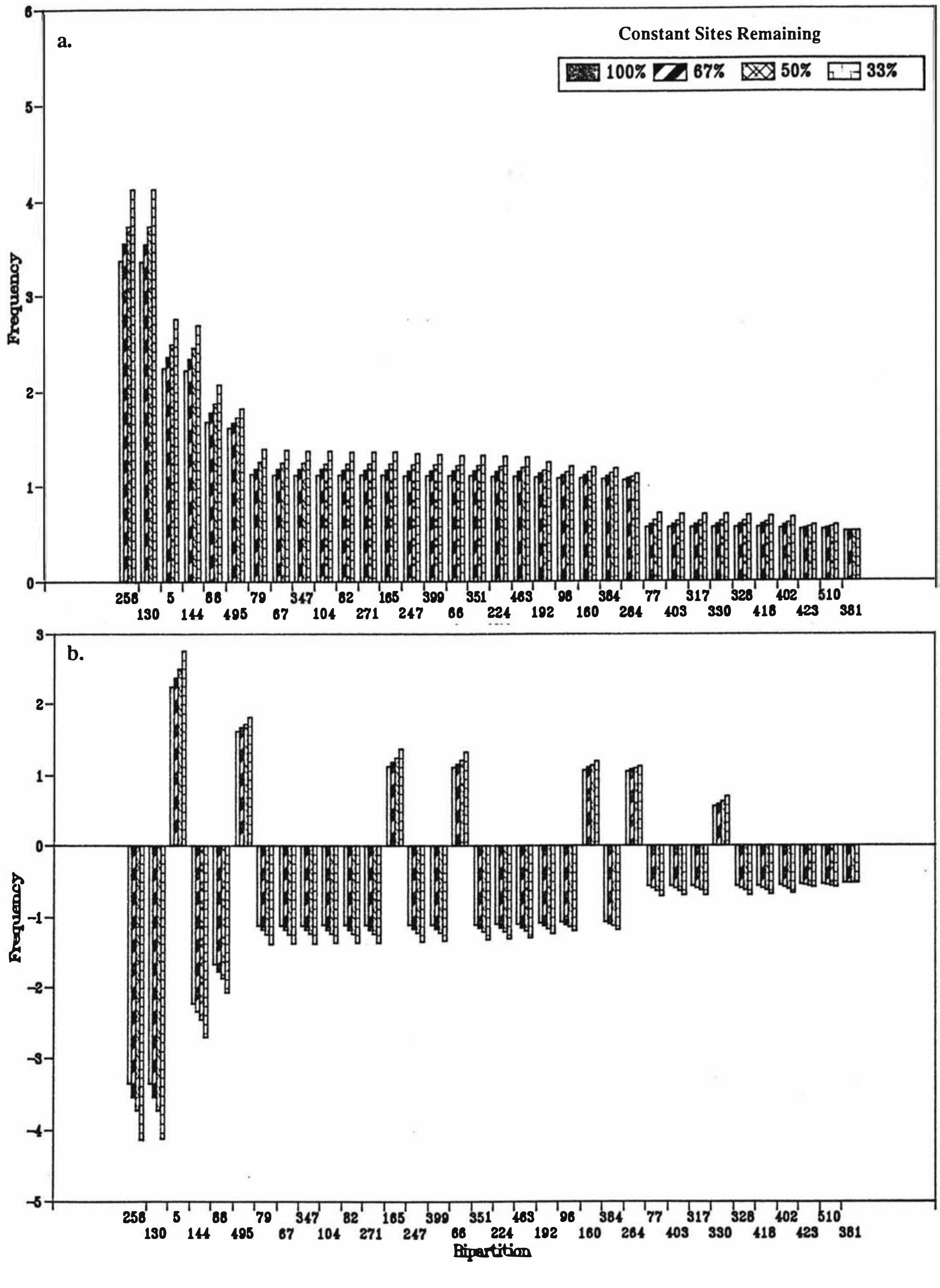
The last few bipartitions in the spectrum in figure 5.12 have little change in their frequencies as more constant sites are removed, suggesting that they are probably spurious signals caused by the correction for unobserved changes. None of these signals in fact exist as observed changes, they are all produced by the correction for unobserved changes.

Summary of the Effect of the Weighting Schemes on the Skink Phylogeny

Adjusting the analyses to take account of a transition bias, differences in frequency of changes between paired and unpaired regions, and different frequencies of nucleotide substitution do not enhance resolution of the skink phylogenetic relationships. There are no large changes in the spectral signals for individual bipartitions which suggests that the bipartitions with the most support do represent relatively close phylogenetic relationships rather than being artifacts due to noise or limitations of analyses.



1. *St. Is. Green (1)*
2. *L. lin/chl (2)*
3. *L. grande (4)*
4. *L. microlepis (8)*
5. *C. aenea (16)*
6. *L. inconspicuum (32)*
7. *L. zelandicum (64)*
8. *L. maccanni (128)*
9. *L. n. polychroma (256)*
10. *L. otagense (512)*

a.
Constant Sites Remaining
100%
67%
50%
33%
Frequency
0
1
2
3
4
5
6
256
130
5
144
88
495
79
67
347
104
82
271
165
247
399
66
351
224
463
192
96
160
384
264
77
403
317
330
328
418
402
423
510
381
b.
Frequency
3
2
1
0
-1
-2
-3
-4
-5
256
130
5
144
88
495
79
67
347
104
82
271
165
247
399
66
351
224
463
192
96
160
384
264
77
403
317
330
328
418
402
423
510
381
Bipartition

Spectral Analysis of Simulated Sequences

The preceeding analyses have demonstrated that lack of phylogenetic resolution among the skink taxa is not due to the HADTREE program, nor to insufficient variable sites, nor biases in sites or types of substitutions. In Chapter Four it was shown that randomly generated sequences gave a similar pattern of transitions and transversions as the skink data (Figs. 4.2 & 4.9). In this section spectral analyses of randomly generated sequences are performed and compared to the skink spectrum (Fig. 5.1).

Using the program described in Chapter Four, sets of twenty random sequences were made. These sequences were 384 nucleotides long, and had an average of 25 total changes and five transversions between each sequence, corresponding to the mean differences between the skink taxa (Table 4.2). Spectral analysis of these sequences gave spectra like the one shown in figure 5.13a. This is similar to the skink spectrum (Fig. 5.1) in that there is no step-wise decrease in signal frequency (as in Fig. 5.2), and there are large numbers of signals with the same frequencies. There are differences however between the skink and simulated sequence spectra. The skink spectrum had only 66 signals but the simulated sequence spectrum (Fig 5.13a) has 157. There are also more variable sites (75% as compared with 26% in the skinks) and more singleton changes (62% versus 42%) in the simulated sequences than in the skink sequences.

The simulated data have an important difference from the skink data however - every site is free to change. In the vertebrate sequence only two-thirds of the molecule appears to be free to vary (Fig. 3.5). To more closely emulate the skink data set the simulation was modified so that the 25 changes occurred in 264 bases (two-thirds the length of the original sequence). The new spectrum (Fig. 5.13b) is generally the same as that when all 384 sites are free to vary (Fig. 5.13a), but the proportion of singleton changes (46%) is now more similar to the frequency of singletons in skink data set. When invariant sites are taken account of therefore, the simulated data describe the pattern of variation in the skink data quite well. The implications of this are discussed later in this chapter.

Relationships of Northern and Southern New Zealand *Leiolopisma*

The relationships for all of the taxa are poorly resolved (Fig. 5.5), but can more information be obtained from subsets of the taxa? The New Zealand species can be sub-divided into two groups, based on their occurrence north or south of the Nelson-Marlborough region (Table 5.2; see Pickard & Towns 1988). Biogeographic connections exist between the southern North Island and Nelson-Marlborough, reflecting their connection during the Pleistocene (Fleming 1980, McGlone 1985). Clines in allozyme frequency for skinks also occur across Cook Strait supporting this past continuity (C.H. Daugherty pers. comm.). *L. n. polychroma* is widely distributed and so is included in both groups. *L. n. nigriplantare* from the Chatham Islands shares close genetic similarity to *L. n. polychroma* (Table 4.1, Fig. 5.1, Daugherty *et al*. 1990b), and so is also included in both northern and southern groupings. Members of the *L. lineoocellatum/chloronotum* complex occur in both the north and the south, and along with *L. telfairi* and *La. guichenoti* are included in both subsets as well.

Fig. 5.13. Spectral analysis of 20 randomly generated sequences (see Chapter Three for details of the simulation program). ***a.*** Shows a sample spectrum obtained when sequences are 384 bases long, the mean transition/transversion (Ts/Tv) ratio is 4/1, and an average of 25 substitutions occur (as in the skink data set). As with the skink spectrum (Fig. 5.1), there are many bipartitions of similar frequency, but the simulated sequence spectrum has more bipartitions. The star-tree model which generated the simulated sequence data is shown in the top right corner of the figure. In ***b.*** the spectrum of another simulated spectrum is shown. In this example the effect of having sites not free to vary is approximated by reducing the sequence length by one-third, to 264 bases, while still having on average 25 substitutions and a Ts/Tv ratio of 4/1. The overall spectrum is similar to that in 5.13a, though more bipartitions now occur.

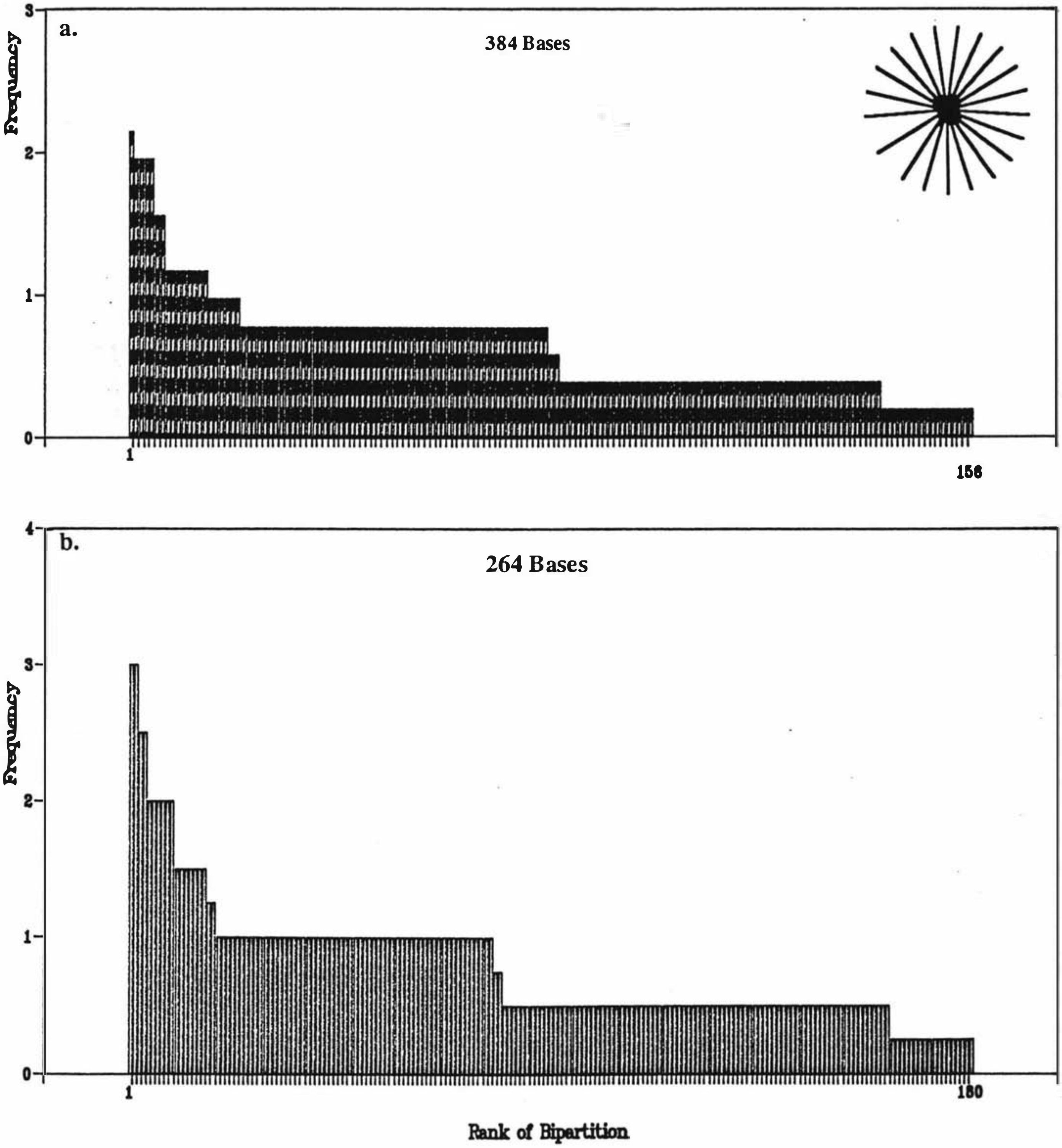


Fig. 5.14. Spectral analyses of 12S rRNA sequences from ***a.*** 11 skink taxa which occur in northern New Zealand, and ***b.*** 10 skink taxa which occur in southern New Zealand. The Australian *Lampropholis guichenoti* and the Mauritian *Leiolopisma telfairi* are also included in both data sets. All the spectral signals are shown in these examples, but the only labelled bipartitions are those corresponding to the branches leading to individual taxa (these signals are independent of the tree). The other bipartition, representing potential internal edges in trees, are identified in figures 5.15 and 5.17. There are two major points to note in figure 5.14. Firstly, the number of changes on branches leading to individual taxa varies greatly. Secondly, the differences between internal edge bipartitions are smaller in the spectrum for the southern taxa than in the northern taxa, indicating poorer resolution of the relationships of the southern taxa (see also Figs. 5.16 & 5.18).

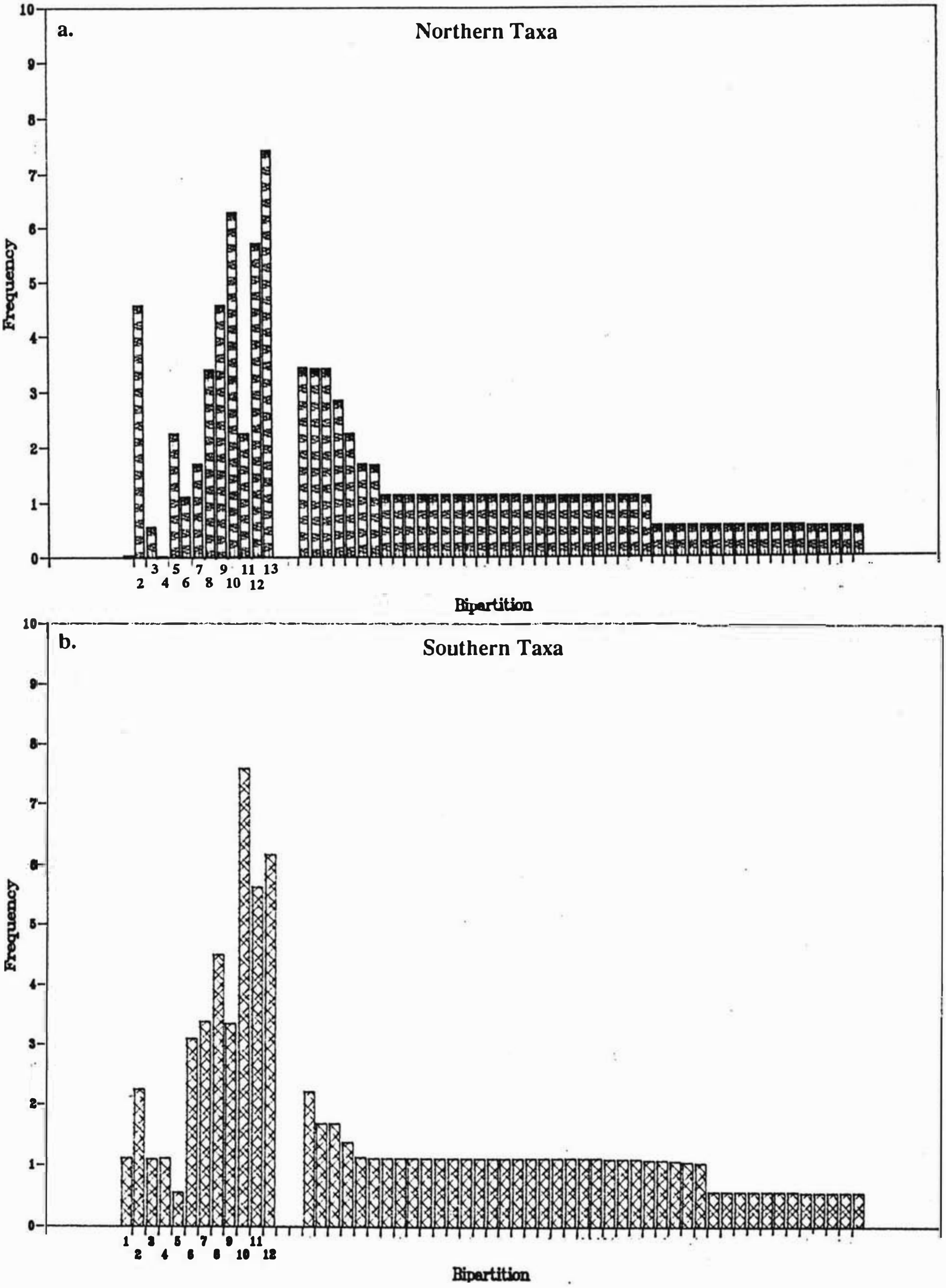


Table 5.2. The New Zealand *Leiolopisma* taxa for which 12S rRNA sequence data is available subdivided on the basis of present geographic distribution (see Pickard & Towns 1988). Stewart Island taxa (*L. notosaurus* and St.Is. Green) are included with the South Island species. Taxa occuring north of Nelson are listed as being in the North Island. *L. n. polychroma*, *L. n. nigriplantare*, and *L. lineoocellatum/chloronotum* are included in both islands (see text).

South Island	North Island
L.n. polychroma	*L.n.polychroma*
L. n. nigriplantare	*L. n. nigriplantare*
L. lin/chl.	*L. lin/chl.*
L. grande	*L. zelandicum*
St.Is. Green	*L. infrapunctatum*
L. maccanni	*L. microlepis*
L. inconspicuum	*L. smithi*
L. notosaurus	*L. suteri*
L. otagense	*L. fallai*
L. acrinasum	*L. moco*
	C. aenea

Spectral Analysis of Skinks from Northern New Zealand

The northern New Zealand skinks have stronger and more structured spectral signals than the southern group (compare Figs. 5.14a & 5.14b), indicating that there is less ambiguity in the phylogenetic relationships of northern taxa. This is also shown by the relatively longer internal branch lengths in the optimal tree for the northern taxa (Fig. 5.15).

The Complex Relationships of *C. aenea*

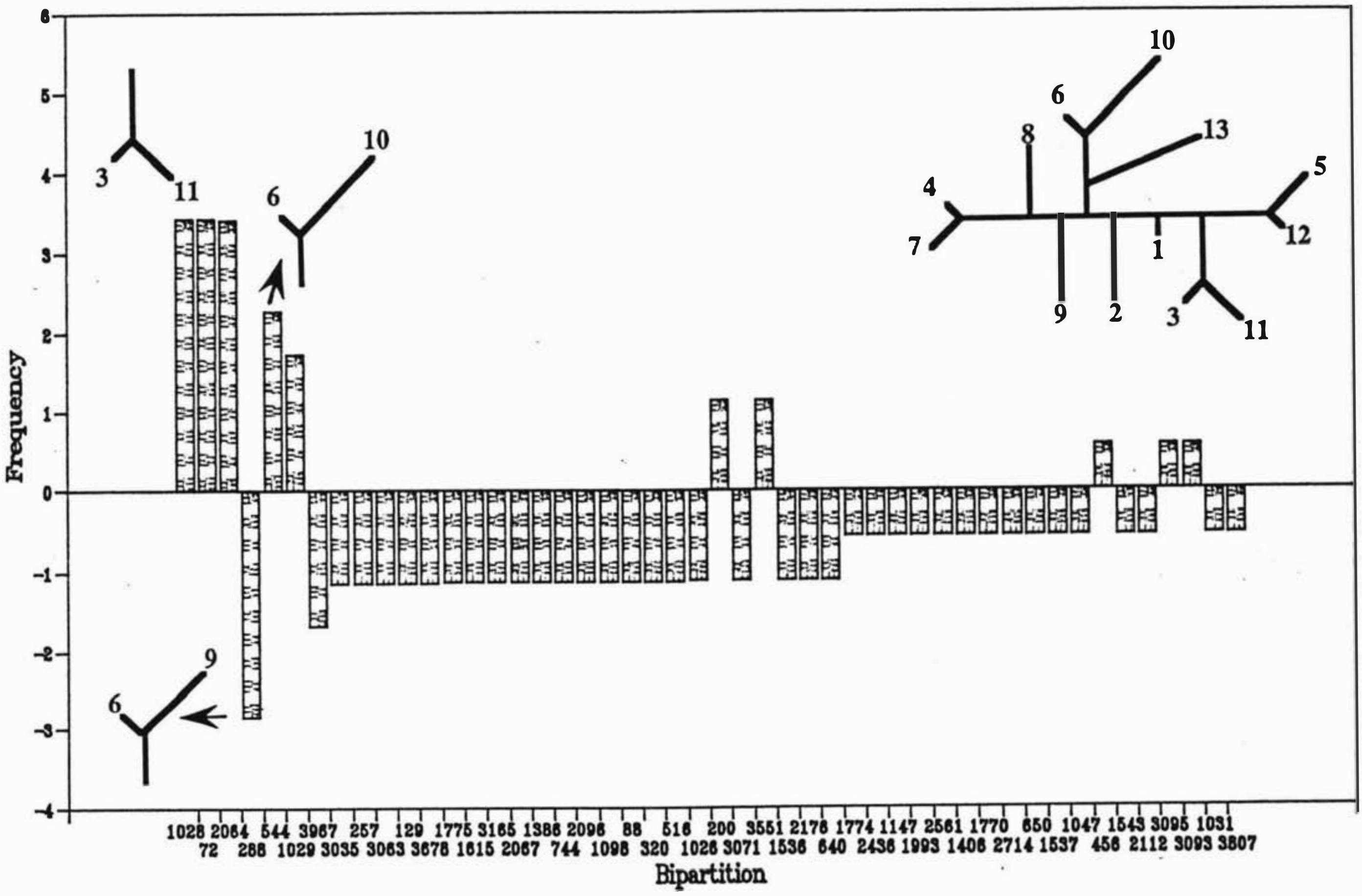
C.aenea clusters with *L. telfairi* in this example (Fig. 5.15), though in the spectra for all 20 taxa (Fig. 5.1), *C.aenea* groups with *L. fallai*. *C.aenea* and *L. fallai* differ by only 17 differences, and the bipartition of these two taxa is supported by three shared substitutions (at positions 67, 73 & 140; Table 3.1). These substitutions are in the relatively conserved S2′ and L33′ regions (Figs. 3.3 & 4.17), which could imply *C.aenea* and *L. fallai* share a common ancestor. The bipartition of *C. aenea* and *L. telfairi* is also supported by three substitutions (positions 194, 263, & 331; Table 3.1), one of which is in the tetra-loop of stem 38. In addition to these bipartitions, *C. aenea*, *L. fallai*, and *L. telfairi* share other substitutions among themselves and with other taxa. One substitution (at position 291) is shared by *C. aenea*, *L. fallai*, and *L. telfairi* (along with *L. suteri* and *L. moco*; Table 3.1). Three observed substitutions group *C. aenea* non-exclusively with *L. fallai* (positions 30, 35, & 64), while *C. aenea* and *L. telfairi* have a substitution in common with three other taxa at position 309 (Table 3.1). This illustrates the complexity of the relationships among some of the skinks.

The case of *C. aenea* serves to demonstrate another feature of HADTREE. The bipartition favouring *C. aenea+L. fallai* (#288) has about the same strength of support as that favouring *C. aenea+L. telfairi* (#544; Fig. 5.15). An option in HADTREE allows specific bipartitions to be forced into the optimal tree. When the *C. aenea+L. fallai* bipartition is forced however, no resolved tree is produced. This indicates that the pattern of sequence relationships in this data set are better explained by *Cyclodina* having a more distant relationship to the New Zealand *Leiolopisma*.

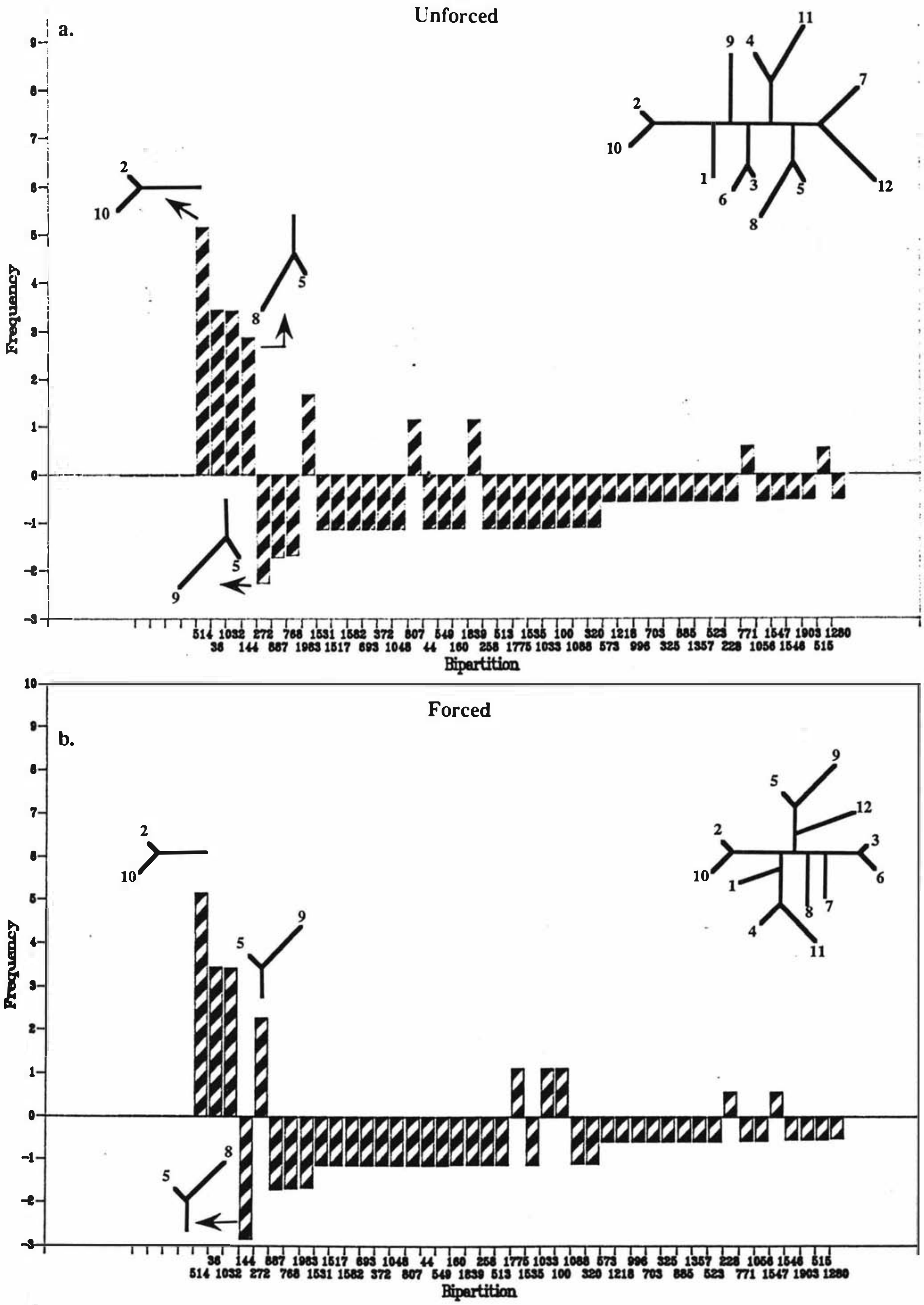
C. aenea can however be made to group with *L. fallai* if *L. lin/chl* is omitted (Fig. 5.16a). The *L. lin/chl* individual used in this study was collected from Otago, the main range of *L. chloronotum* (see Pickard & Towns 1988), so there is a rationale for excluding it for this example. Forcing the *C. aenea+L. telfairi* bipartition (#272) into the tree changes six of the nine internal edges, and some of the internal edges in the tree shorten (Fig. 5.16b). Given that some of the shared nucleotide substitutions grouping *C. aenea* both with *L. fallai* and with *L. telfairi* are at relatively well conserved sites, the phylogenetic relationships of *C. aenea* are uncertain.

Why *L. lin/chl* affects the grouping of *C. aenea* is not clear. Exclusion of *L. lin/chl* also increases the strength of the *L. n. nigriplantare+L. n. polychroma* bipartition (compare figures 5.15 & 5.16). The conflicting signals associated with inclusion of *L. lin/chl* were also noted in the analyses shown in figure 5.11

Fig. 5.15. Bipartitions included in the optimal tree for 11 northern New Zealand and two overseas skink taxa. (Only internal bipartitions are shown in the graph, and are given in their binary number notation). The optimal tree is illustrated on the right (see also Fig. 5.18), and bipartitions excluded from the tree are plotted below the x axis. The relatively strong signal supporting the bipartition of *C. aenea*+*L. fallai* (#288) is not in the tree, and *C. aenea* groups with *L. telfairi* (bipartition #544). No fully resolved tree is formed if bipartition #288 is forced to be included in the tree.



1. *L. lin/chl (1)*
2. *L. suteri (2)*
3. *L. n. nigriplantare (4)*
4. *L. microlepis (8)*
5. *La. guichenoti (16)*
6. *C. aenea (32)*
7. *L. smithi (64)*
8. *L. zelandicum (128)*
9. *L. fallai (256)*
10. *L. telfairi (512)*
11. *L. n. polychroma (1024)*
12. *L. infrapunctatum (2048)*
13. *L. moco (4096)*



1. *L. suteri (1)*
2. *L. n. nigriplantare (2)*
3. *L. microlepis (4)*
4. *La. guichenoti (8)*
5. *C. aenea (16)*
6. *L. smithi (32)*
7. *L. zelandicum (64)*
8. *L. fallai (128)*
9. *L. telfairi (256)*
10. *L. n. polychroma (512)*
11. *L. infrapunctatum (1024)*
12. *L. moco (2048)*

Spectral Analysis of Southern New Zealand Skinks

In comparison with the northern taxa (Figs. 5.15 & 5.16) the relationships of the southern taxa (Fig. 5.17) are less certain. All of the internal edges in the latter tree are short. The grouping of *L. grande* with St. Is. Green has the most support. *L. n. polychroma* does not appear to be closely related to other members of the cryptic species complex (*L. maccanni*, *L. inconspicuum*, and *L.notosaurus*) as described by Daugherty *et al.* 1990b. This point will be considered again in Chapter Six.

L. n. polychroma and *L. n. nigriplantare* form a distinct cluster in the tree of northern species (Fig. 5.18a). This is not the case with respect to southern taxa where these two taxa have affinities to *L. lineoocellatum/chloronotum* and *L. acrinasum* (Fig. 5.18b). A division between northern and southern taxa (with *L. n. nigriplantare* and *L. n. polychroma* clustering with southern species) is also apparent in the allozyme data (C.H. Daugherty & G.B. Patterson pers. comm.; and see Chapter Six). *L. n. polychroma* may therefore have spread northwards.

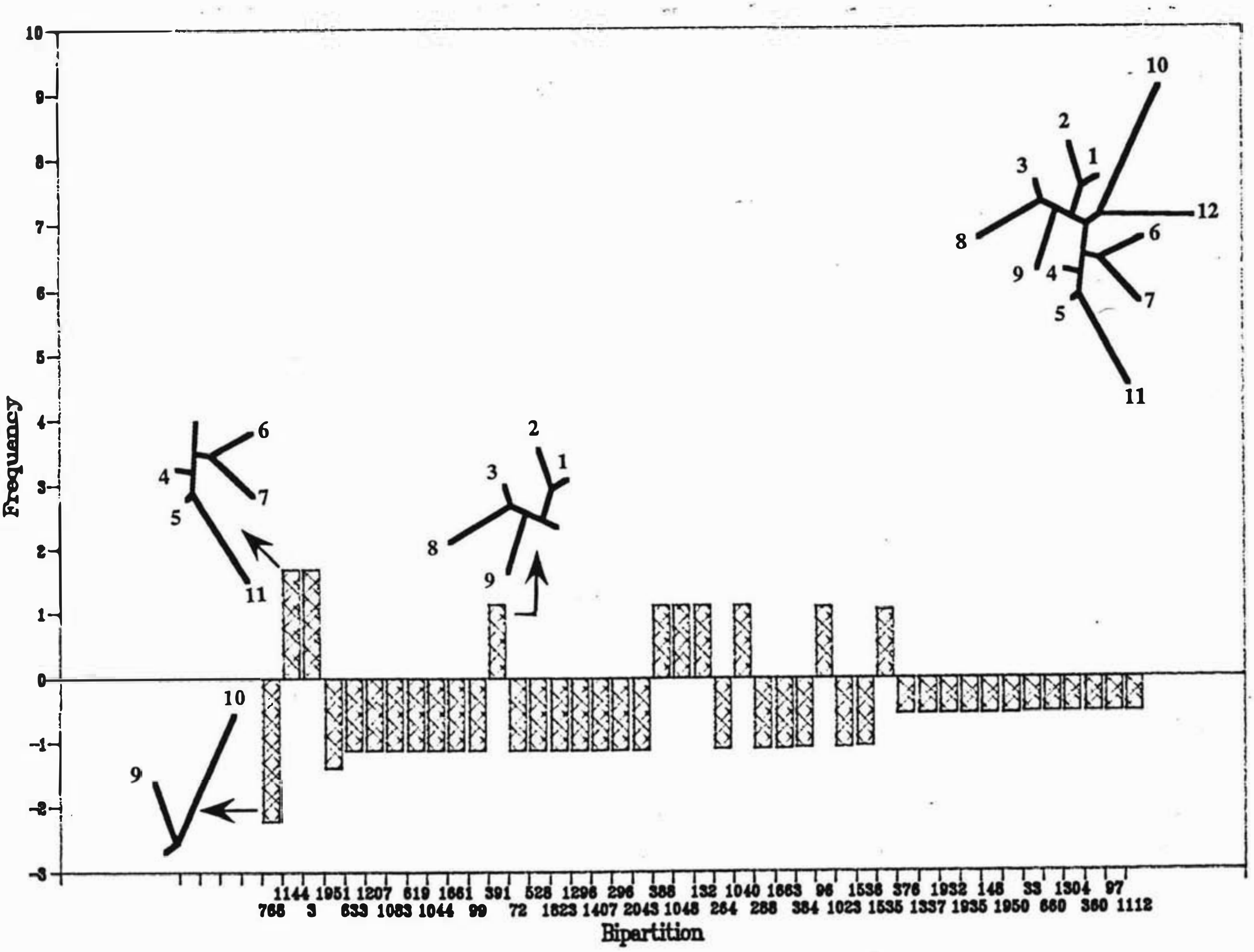
Mitochondrial Lineages of the Skinks

On the basis of both the numbers of differences between taxa (Table 4.1) and the relationships inferred by spectral analysis (Figs. 5.5, 5.15, & 5.18), twelve *Leiolopisma* lineages can be identified (Table 5.3). Taxa which have the strongest spectral signals differ by fewer than 20 substitutions (less than 5% sequence divergence), so I have chosen this value as the criterion for identifying a lineage. As an example, *L. n. nigriplantare* and *L. lin/chl* differ from *L. n. polychroma* by 13 and 19 substitutions respectively (Table 4.1), while 20 or more differences separate *L. n. polychroma* from the other taxa. These three taxa are therefore classed as one lineage, and 11 lineages occur in New Zealand (Table 5.3). Ten of these lineages are no more different from each other than any of them are from *L. telfairi* (Table 5.3), suggesting that New Zealand has several independent skink mitochondrial lineages.

Addition of a New Caledonian Skink

A New Caledonian representative of the *Leiolopisma* group was not obtainable for this study. Sequence information was obtained however from another New Caledonian skink, *Tropidoscincus rohssii*, a member of the *Eugongylus* group to which the *Leiolopisma* skinks are related (Greer 1970). Only 300 bases of 12S rRNA could be reliably determined for *T. rohssii* so this taxon was excluded from the earlier analyses (Appendix 1). It is useful to include it here however. Since HADTREE can currently only handle up to 20 taxa, addition of *T. rohssii* required the removal of another taxon. *L. microlepis* was dropped since it is very similar to *L. smithi*. The first 80 bases, which include the variable helices 26 and 29, as well as the single-stranded region linking domains II and III (Fig. 3.3), are omitted from the data set when *T. rohssii* is included. This reduction appears to remove all phylogenetically informative signals (Fig. 5.19a), but separate analysis of the first 80 bases shows that this region has low resolution on it's own (Fig. 5.19b). This latter point was also shown by the secondary structure comparisons of both closely and more distantly related taxa in Chapter Four. Most of the variable sites occur towards the other end of the molecule (Figs. 3.7 & 4.17). Omission of *L. microlepis* also removes one of the strongest signals (see Fig. 5.1).

Fig. 5.17. Spectrum and optimal tree for 10 southern New Zealand *Leiolopisma*, and the Australian and Mauritian species. In contrast to the spectra for the northern taxa (Figs. 5.15, 5.16), the internal branches of the optimal tree for southern taxa have much less support. Note also that some of the strongest bipartitions included in the tree are for groups of five taxa rather than for pairs of taxa.



1. *St. Is. Green (1)*
2. *L. grande (2)*
3. *L. notosaurus (4)*
4. *L. lin/chl (8)*
5. *L. n. nigriplantare (16)*
6. *La. guichenoti (32)*
7. *L. acrinasum (64)*
8. *L. inconspicuum (128)*
9. *L. maccanni (256)*
10. *L. telfairi (512)*
11. *L. n. polychroma (1024)*
12. *L. otagense (2048)*

Fig. 5.18a. Inferred phylogenetic relationships for *Leiolopisma* skinks found in northern New Zealand. The Australian *Lampropholis guichenoti* and the Mauritian *L. telfairi* are also included. Branch lengths are proportional to the probability of a nucleotide substitution along that branch.

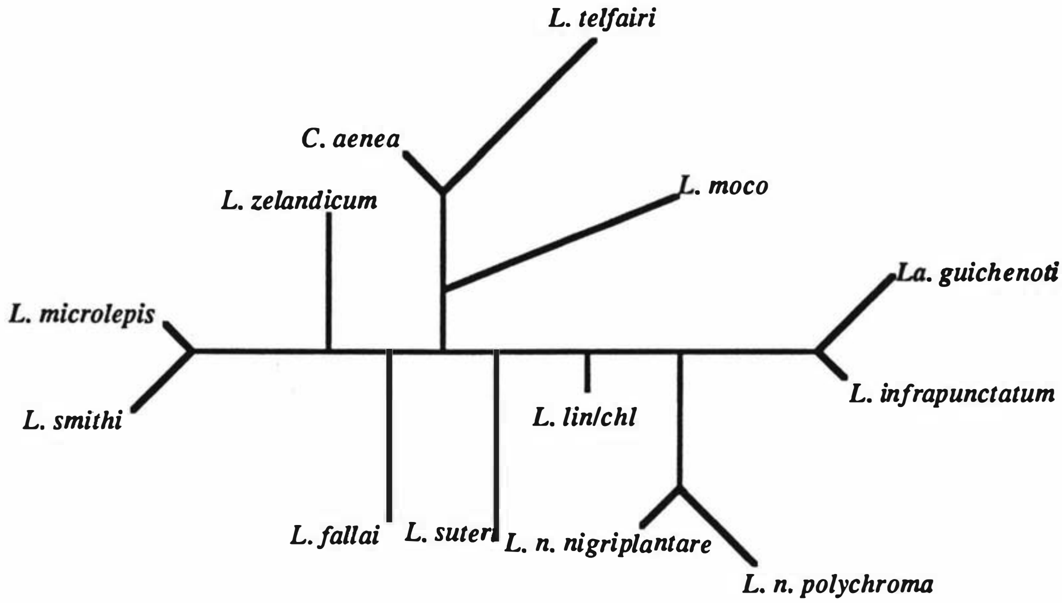


Fig. 5.18b. Inferred phylogenetic relationships for *Leiolopisma* skinks found in southern New Zealand. The Australian *Lampropholis guichenoti* and the Mauritian *L. telfairi* are also included. Branch lengths are proportional to the probability of a nucleotide substitution along that branch. The greater uncertainty about the relationships of the southern taxa compared with the northern group is reflected in the relatively shorter internal edges in the tree for the southern group.

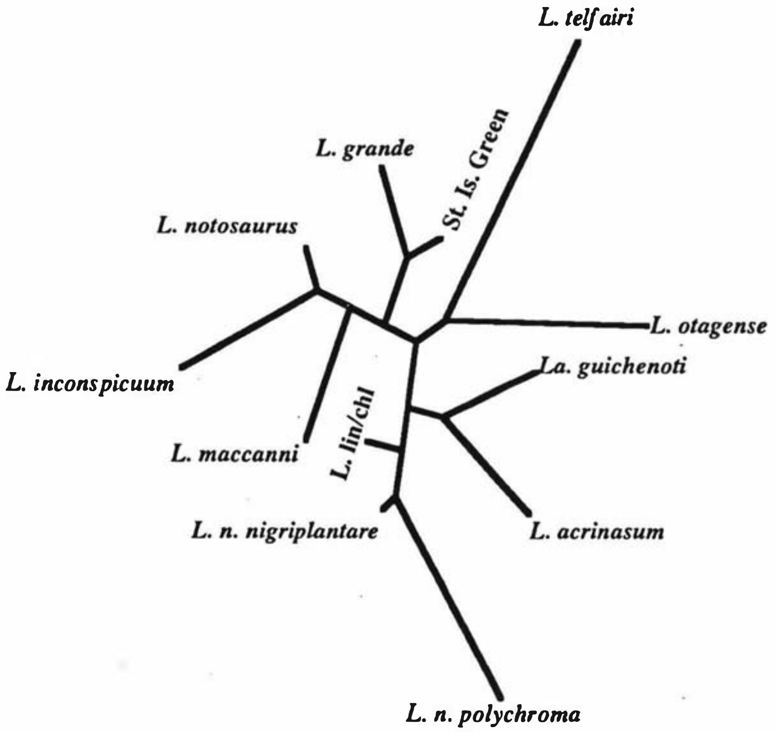


Table 5.3. Mitochondrial lineages in skinks. A lineage is defined as taxa whose 12S rRNA sequences differ by less than 5%. The mean (± standard deviation) numbers of nucleotide differences between the first member of each lineage and the other lineages are presented in the right-hand column. The mean numbers of differences between all lineages are significantly different ($F_{11,120} = 3.25$, $P < 0.01$). However if lineage 1 is excluded, numbers of nucleotide differences bewteen the other lineages are not significantly different from each other ($F_{10,110} = 1.9$, $P > 0.05$), indicating that they may have diverged from each other at about the same time. The same results are obtained when distances are corrected for unobserved changes using the Jukes & Cantor (1969) correction. Using other taxa within a lineage for the comparisons also had little effect.

Lineage & Taxa	Inter-Lineage Differences (mean ± std)
1. *L. grande* St. Is. Green *L. suteri*	22.6 ± 3.3
2. *L. zelandicum*	25.0 ± 3.5
3. *L. inconspicuum* *L. notosaurus*	25.2 ± 4.0
4. *L. acrinasum*	25.5 ± 3.3
5. *L. smithi* *L. microlepis*	26.0 ± 4.0
6. *L. fallai* *C. aenea*	26.4 ± 3.3
7. *L. maccanni*	27.1 ± 3.3
8. *L. otagense*	27.3 ± 3.4
9. *L. telfairi*	27.8 ± 2.8
10. *L. moco*	27.9 ± 2.5
11. *L. infrapunctatum* *La. guichenoti*	28.7 ± 2.9
12. *L. n. polychroma* *L. n. nigriplantare* *L. lin./chl.*	28.7 ± 3.3

Fig. 5.19a. The effect of removing the first 80 sites of the 12S rRNA sequence from the analysis, before adding the New Caledonian *Tropidoscincus rohssii* sequence. The New Zealand taxon *L. microlepis* has also been excluded from the data set (see text). Note the loss of all strong spectral signals (see Figs. 5.1, 5.19b).

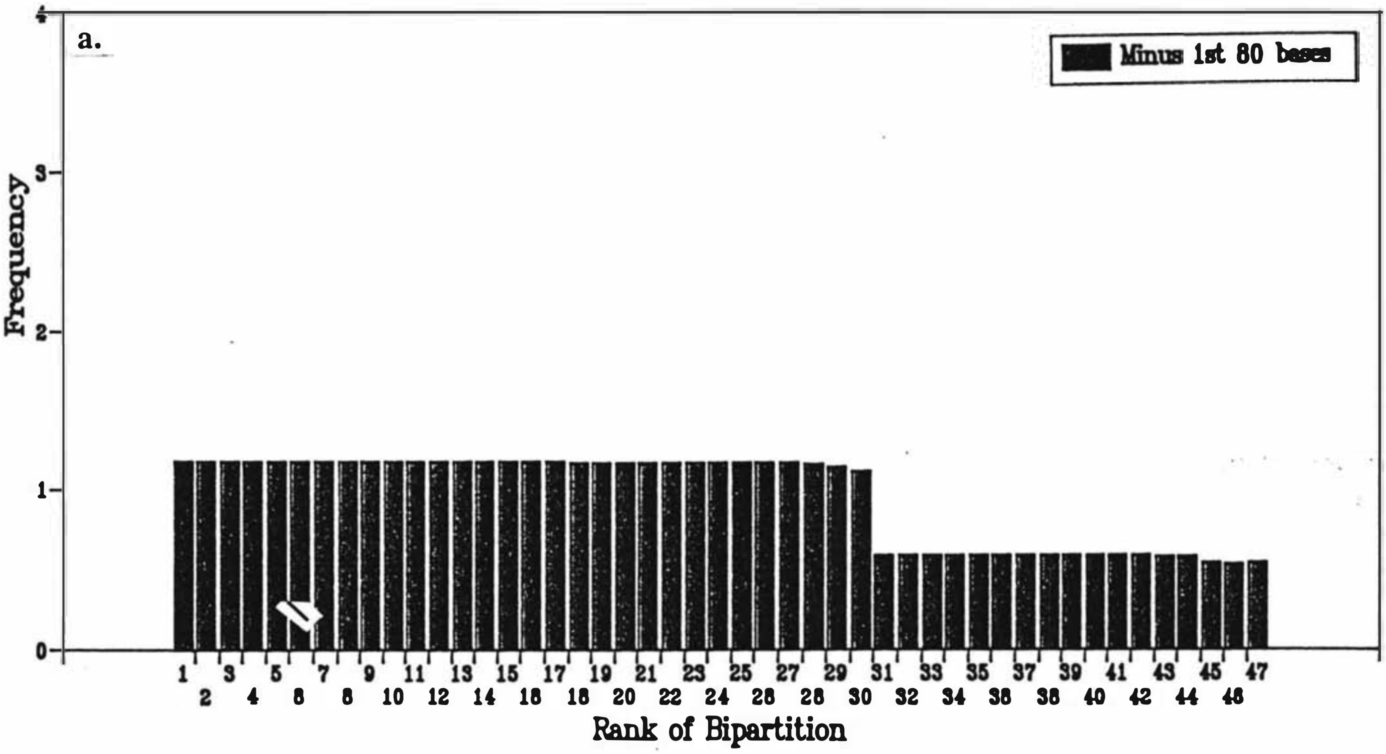
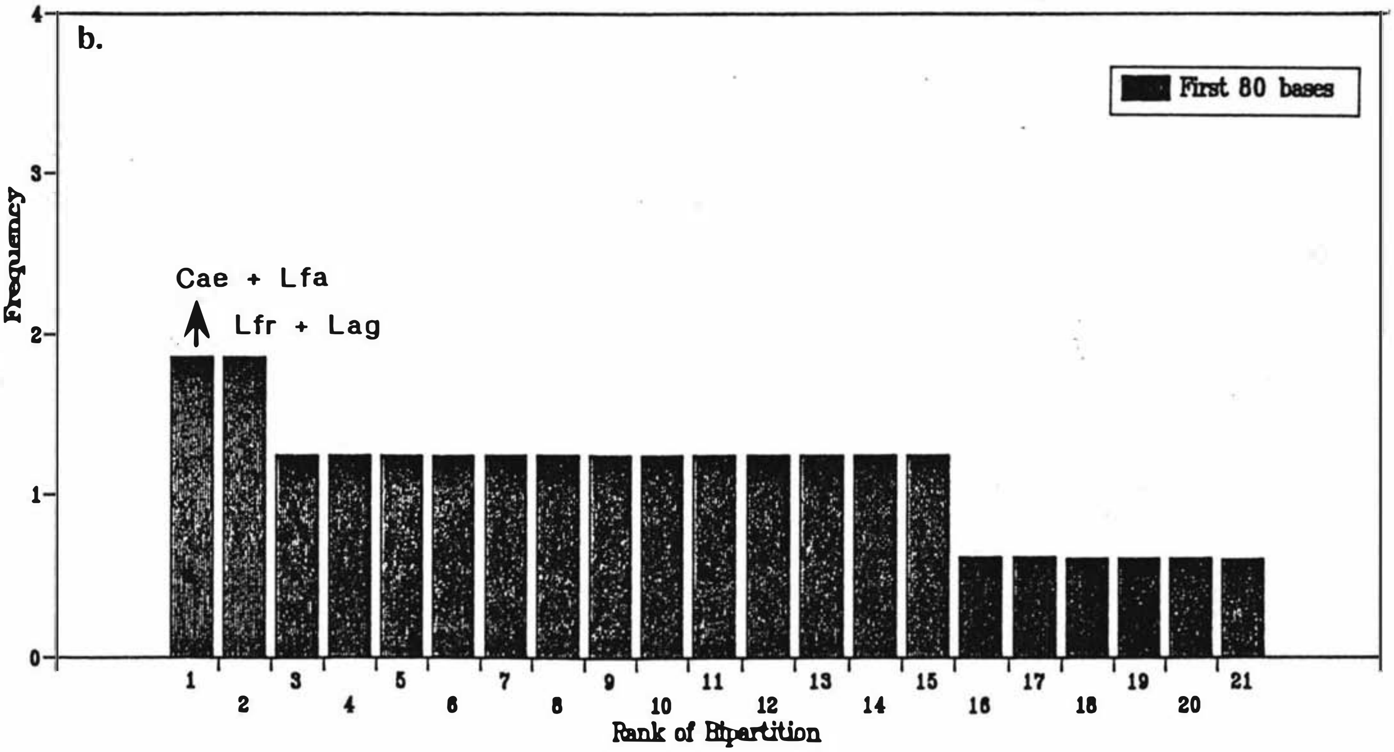


Fig. 5.19b. Spectral analysis of the first 80 sites of the 12S rRNA sequence for 19 taxa. This spectrum indicates that some of the support for the bipartitions *C.aenea+L. fallai* and *L. infrapunctatum+La. guichenoti* is derived from this region of the sequence (see Table 3.1). Use of this region alone however provides less phylogenetic resolution than when the full sequence is analyzed (see Fig. 5.1).



The strongest signal in this comparison links *T. rohssii* with *L. fallai* (Fig. 5.20), but the number of pair-wise differences for the *T. rohssii* sequence, and the location of the substitutions linking it with *L. fallai* suggest that this is not a close phylogenetic relationship. *T. rohssii* has more substitutions (an average of 32) in pair-wise comparisons than do the *Leiolopisma* skinks. For example, *L. fallai* and *L. infrapunctatum* have for the same region of 300 bases on average 18.3 and 20.6 differences, respectively, from other taxa. Furthermore, the bipartition containing *T. rohssii* and *L. fallai* is supported by two observed substitutions, both of which occur in helix 46 (positions 346 & 364), which is one of the most variable regions in the molecule (Fig. 4.17).

Cytochrome *b* Sequence Data

Sequence information (254 nucleotides) from the mitochondrial cytochrome b gene was obtained for four skink taxa. This small data set has 13 informative (parsimony) sites and 40 singleton changes (Appendix 1). Eighty nine percent of the substitutions occur at third codon positions. As noted in Chapter Two, the 12S rRNA sequences of *L. n. polychroma* individuals from Gorge Burn are identical to those of *L. maccanni* from the same location but are quite different from sequences obtained from *L. n. polychroma* at other sites. The Gorge Burn populations of *L. n. polychroma* and *L. maccanni* differ by three transitions in their cytochrome *b* sequences, all at third codon positions, and are quite distinct from the Twizel *L. n. polychroma* sequence (Table 5.4).

The cytochrome *b* sequence has changed more rapidly than the 12S rRNA sequence (Table 5.4). In comparisons between *L. n. polychroma* (Twizel) and *L. maccanni* there are about 50% more substitutions in the cytochrome b than in the 12S rRNA sequence, even though a shorter length of the former was examined. The relative lengths of the branches leading to the four taxa are similar for both the 12S rRNA and cytochrome *b* sequence data (Fig. 5.21), which suggests that there is nothing atypical in the relative rate of change of the 12S rRNA sequence with respect to other skink mitochondrial sequences. Consequently, the more well supported relationships determined from the 12S data (for example *L. microlepis*+*L. smithi*, *La. guichenoti*+*L. infrapunctatum*, St. Is. Green+*L. grande*, and *L. n. polychroma*+*L. n. nigriplantare*; figures 5.15, 5.16 & 5.17) are expected to be confirmed by other mitochondrial sequence data. The very close sequence similarity between the Gorge Burn populations of *L. n. polychroma* and *L. maccanni* will be discussed in the next chapter.

DISCUSSION

The skink 12S rRNA sequence data set has proven to be very useful for demonstrating aspects of the HADTREE algorithm, particularly with respect to the handling of conflicting signals (Figs. 5.5 & 5.16). Spectral analysis is a direct and easy way for assessing conflicts and noise in sequences and for determining alternative sets of relationships. Unlike bootstrapping (Felsenstein 1985) or analysis of the distribution of tree lengths (Fitch 1984, Hillis 1991, Källersjö *et al.* 1992), spectral analysis can directly show where the conflicting signals in the data set come from.

Fig. 5.20. Spectral analysis of 304 base pairs of 12S rRNA for 19 *Leiolopisma* and the New Caledonian species *T. rohssii*. The only strongly supported bipartition is that grouping the New Zealand *L. fallai* with *T. rohssii*, but see the text.

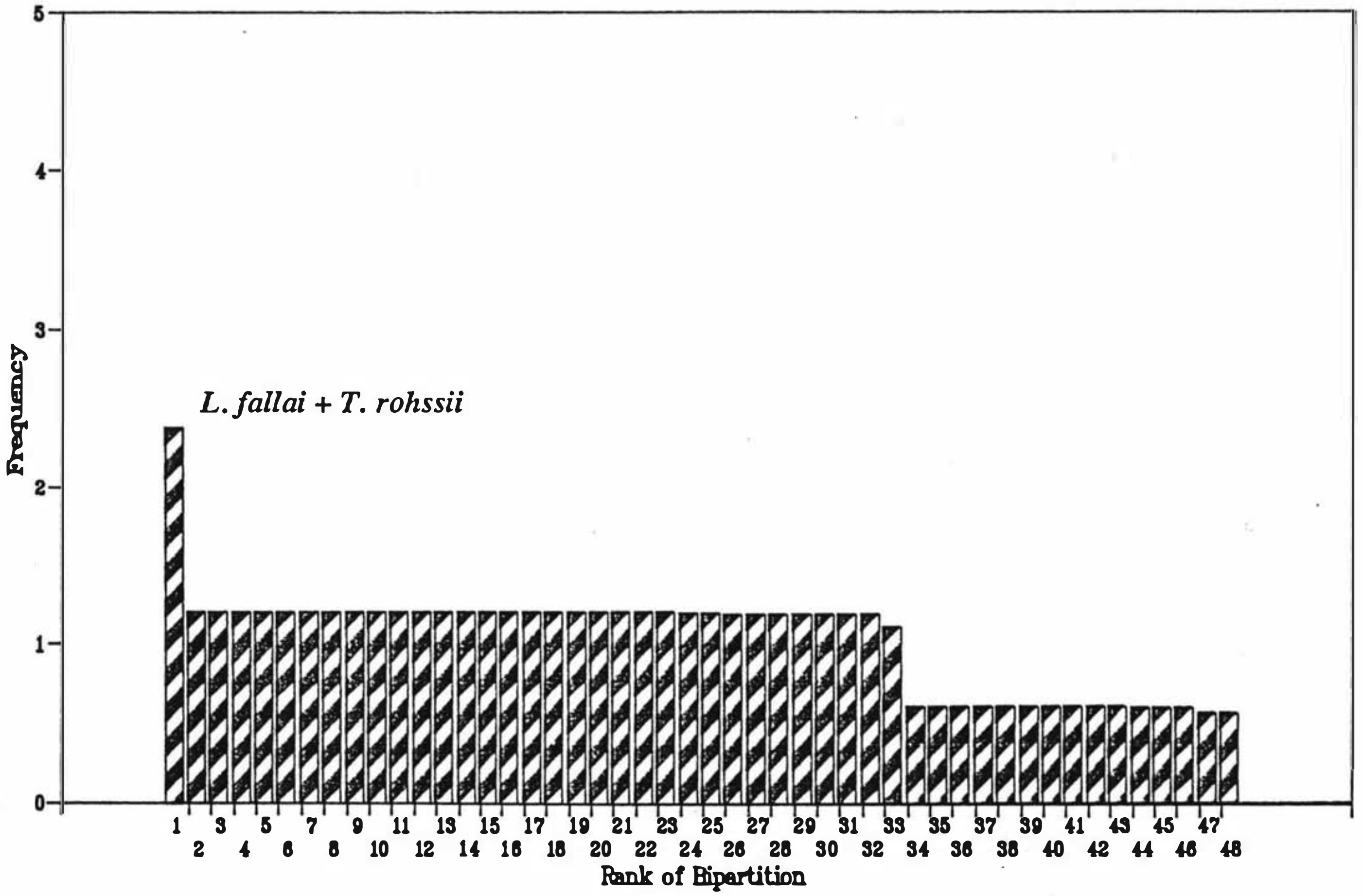


Table 5.4. Numbers of transitions (above diagonal) and transversions (below diagonal) for 254 base pairs of the cytochrome *b* gene. The 12S rRNA distance matrix for the same four taxa (383 bases) is also shown. *L. n. polychroma* (Lnp-GB), *L. maccanni* (Lmac), and *L. inconspicuum* (Linc) are sympatric at Gorge Burn, Southland, while the other *L. n. polychroma* (Lnp-Tw) population is from Twizel, Canterbury.

Cytochrome b

Transversions\Transitions

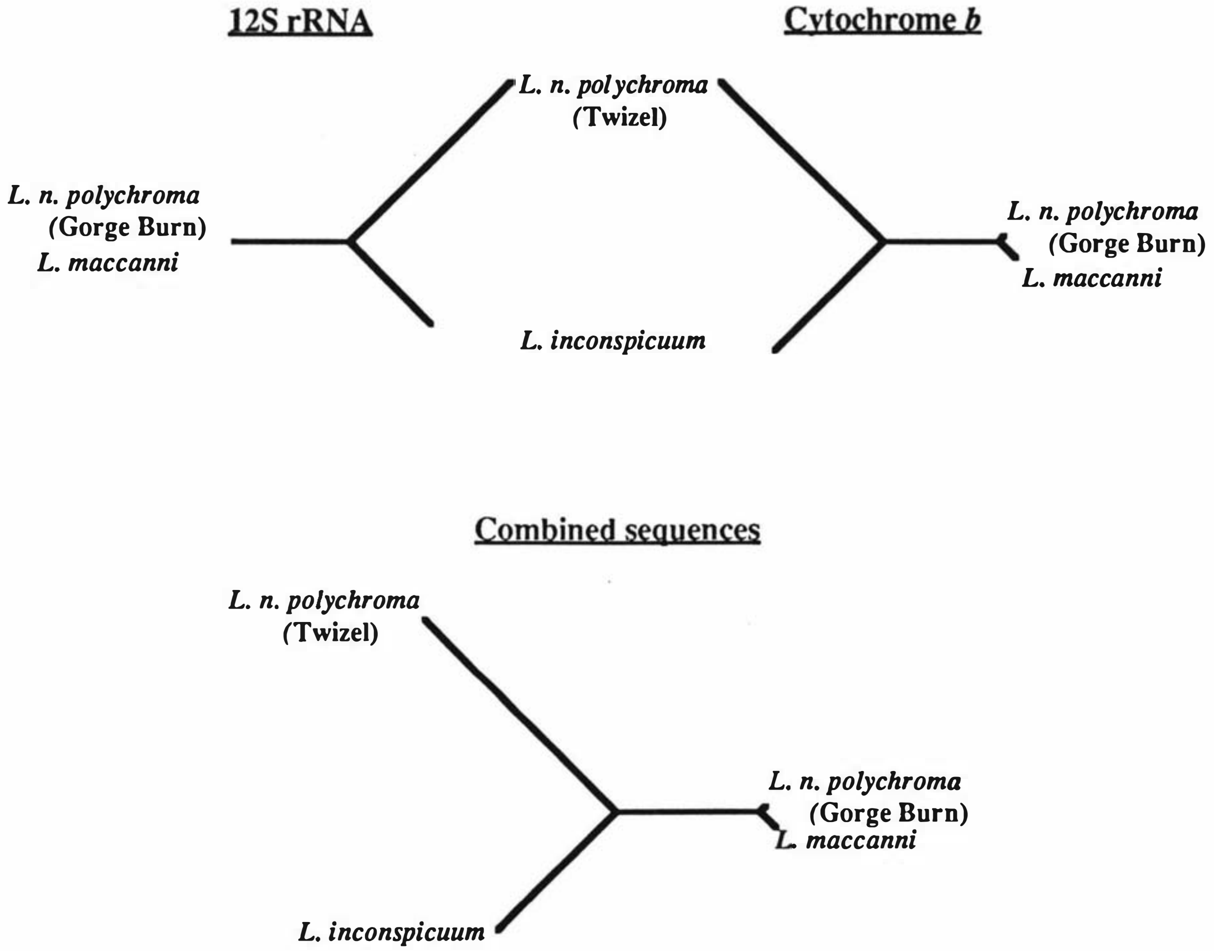
	Lnp-Tw	Lnp-GB	Lmac	Linc
Lnp-Tw	-	22	25	29
Lnp-GB	14	-	3	25
Lmac	14	0	-	24
Linc	12	6	6	-

12S rRNA

Transversions\Transitions

	Lnp-Tw	Lnp-GB	Lmac	Linc
Lnp-Tw	-	17	17	24
Lnp-GB	3	-	0	25
Lmac	3	0	-	25
Linc	5	4	4	-

Fig. 5.21. Inferred phylogenetic relationships of four skink taxa from Southland. Trees derived from 383 nucleotides of 12S rRNA and 254 nucleotides of cytochrome *b* sequence. The *L. n. polychroma* sequences come from two populations: Twizel and Gorge Burn. *L. maccanni* and *L. inconspicuum* are sympatric with the Gorge Burn population of *L. n. polychroma* (Patterson & Daugherty 1990). The resulting tree when both the 12S rRNA and cytochrome *b* sequences are combined for analysis is also shown. Trees are unrooted and branch lengths are proportional to the number of changes expected to occur down that branch. The 12S rRNA sequences of *L. n. polychroma* (Gorge Burn) and *L. maccanni* are identical.



Forcing specific bipartitions to be included in the tree (Fig. 5.16) is one way of determining the likelihood of alternative groupings of taxa. Continued development and refinement of the programs are expanding their utility, enabling more detailed questions to be asked of the data. Of particular use will be the ability to obtain and compare suboptimal trees so that potential phylogenetic relationships can be analyzed in more detail.

Domain III of the skink 12S rRNA is not able to resolve relationships among many of the taxa examined here. This portion of the 12S rRNA gene has been useful for investigating and resolving phylogenetic relationships for a wide range of animal groups (for example, Simon *et al.* 1991, Hedges *et al.* 1991, Hillis & Dixon 1991, Cooper *et al.* 1992) so it was a reasonable premise that it would be suitable for skinks as well. Analysis of the secondary structure (Chapter Three) demonstrated that the skink sequence is evolving in a similar way to other vertebrates. Lack of phylogenetic resolution is therefore not attributable to an unusual pattern of molecular evolution. Nor is it a result of having too few variable sites - the numbers of differences among the skinks are similar to those occurring in other 12S rRNA data sets (Table 4.5a-c).

Removal of constant columns from the HADTREE frequency input file is a preliminary, and in some ways crude, approximation to addressing the problem of invariant (constant) sites. It serves to illustrate however that most of the spectral signals, particularly those included in the optimal tree, are robust and not artifacts of analysis. While it is important to develop more realistic models to take account of unchanging sites, assuming all sites are free to vary does not appear to be cause for poor resolution of the skink relationships.

Four sets of relationships in the skink data set seem robust, in that they have strong spectral signals and/or the taxa consistently grouped together (Figs. 5.5, 5.15, 5.16, & 5.17). These taxa are *L. microlepis*+*L. smithi* (which differ by 5 nucleotide substitutions), *L. grande*+St.Is. Green (8 differences), *L. infrapunctatum*+*La. guichenoti* (12 differences), and *L. n. polychroma*+*L. n. nigriplantare* (13 differences; Figs. 5.5, 5.16, & 5.17). These are all supported by allozyme analyses (C.H. Daugherty & G.B. Patterson pers. comm.; and see Chapter Six). *L. inconspicuum*, *L. notosaurus*, and *L. maccanni* tend to cluster together (Figs. 5.5 & 5.17; though see Chapter Six). As already noted, the relationship between *L. fallai* and *C. aenea* (17 nucleotide differences) is less certain (Figs. 5.15 & 5.16). The number and location of shared nucleotide substitutions for these two taxa suggest however that *C. aenea* may have closer phylogenetic relationships to *L. fallai* than to *L. telfairi*. Additional sequence data is required to investigate this.

The spectral signals and the short internal edge lengths for comparisons among the southern taxa (Fig. 5.18b) suggest that they have had a more complex evolutionary history than the northern species (Figs 5.15, 5.16 & 5.18a). Furthermore, the distinctiveness of *L. n. polychroma* (widely distributed in New Zealand) and *L. n. nigriplantare* (from the Chatham Islands) from the northern groups (Fig. 5.14) suggest a southern origin for them.

"Rapid" Divergence of *Leiolopisma*

The most significant features of the skink data set are that most of the taxa have similar numbers of substitutions (Tables 4.1a, & 5.5), and a resolved phylogeny for all of them is not produced. The model of evolution used in the Hadamard conjuagation has three parts; a tree, a mechanism of change, and edge lengths (probabilities of nucleotide substitutions) for the branches of the tree. The mechanism of change assumed all sites in the sequence were free to vary, but this is incorrect (Fig. 3.3). However removal of constant columns, to simulate the presence of invariant sites, did not change the sequence spectrum (Fig. 5.13) and suggests that the assumption was not misleading analyses of the sequence data. Furthermore, the Hadamard conjugation does produce a resolved tree for the ratite 12S rRNA data set (Fig. 5.2a) under the same assumption of all sites are free to vary. The mechanism of change assumed by the model does not therefore account for incomplete resolution of the skink sequence data.

Analyses of simulated sequence data (Chapter Four and Fig. 5.13) gave very similar results to those obtained for the skinks. The simulated sequences were derived using a star-tree model, whereas the tree assumed by the model in the Hadamard conjugation is a binary, or bifurcating, tree (see Chapter Four). The inability to resolve the skink phylogeny therefore appears related to how the taxa diversified. Rather than a slow diversification over time, the *Leiolopisma* skinks examined here appear to have diversified from each other more rapidly. The bovid data set also has a sequence spectrum (Fig. 5.2b) similar to the skinks (Fig. 5.1), and it also failed to produce a completely resolved phylogeny (see also Kraus & Miyamoto 1991, Allard *et al.* 1992). With support from fossil evidence, Allard *et al.* (1992) suggested rapid diversification of the pecoran bovids over a 5 million year period.

There are no fossils for the skinks to directly place a time on their origin or diversification (Carrol 1969, Molnar 1991). As discussed in Chapter Four, the rate of sequence evolution in the skinks may be slower than that in the bovids, so "rapid" diversification of the skinks may still have occured over millions of years. The fact that *L. telfairi* has similar numbers of differences from New Zealand taxa as these taxa have among themselves (Table 5.3) implies that this Mauritian species diverged from them at about the same time.

L. telfairi has two unique insertion/deletion events, both involving single bases (Table 3.1). Single-base insertion/deletion events do not necessarily indicate greater genetic divergence however since such indels occur between closely related ratites and among the bovids (see Appendix 2). Immunological studies also support approximately equal genetic divergence between the *Leiolopisma* (Hutchinson *et al.* 1990). The New Caledonian *T. rohssii* has a greater number of substitutions relative to all the *Leiolopisma* however, so it probably represents an earlier split in the *Eugongylus* group of skinks.

Estimating Times of Divergence for the Skinks

Analyses of immunological (Baverstock & Donnellan 1990, Hutchinson *et al.* 1990), allozyme (Daugherty *et al.* 1990, C.H. Daugherty & G.B. Patterson pers. comm.; and see Chapter Six), and now sequence data all support an older origin for New Zealand skinks than suggested by morphological studies (five million years or so, Hardy 1977, Towns *et al.* 1985). There is no evidence indicating that the skink sequences are changing more rapidly than other vertebrate groups (Fig. 3.6) so the degree of sequence divergence between the skinks implies periods of separation in excess of 15 million years (Chapter Four, and also Hickson *et al.* 1992). This section examines how more precise estimates for times of separation can be inferred.

Estimates from Immunological Data

As already noted, there are no *Leiolopisma* fossils to indicate when they originated. The immunological and allozyme data could however provide a means for establishing a time frame for *Leiolopisma* evolution. In the immunological studies of Australian *Leiolopisma* one New Zealand species, *L. grande*, was included (Baverstock & Donnellan 1990, Hutchinson *et al.* 1990). Assuming an albumin molecular clock, Baverstock & Donnellan tentatively proposed that *L. grande* may have diverged from Australian species 20 million years ago. *L. grande* and the Australian *La. guichenoti* differ by 21 nucleotide substitutions (Table 4.1a) so, using the immunological clock estimate, this would imply approximately one nucleotide substitution per million years. Applying this rate, most of the New Zealand taxa would have diverged between 12 and 35 million years ago.

The reliability of this immunological data for estimating time is uncertain however. Complete reciprocal matrices were not obtained for the albumin immunological differences (AID; Baverstock & Donnellan 1990) so phylogenetic inferences made from this data may be unreliable (Maxson & Maxson 1990). AID rates must also be calibrated for each taxon (Thorpe 1982), which has not yet been done for skinks. Hutchinson *et al.* (1990), in a more extensive study, did not use the immunological distances to estimate times of separation for *Leiolopisma*.

Estimates from Allozyme Data

Preliminary analyses of the allozyme data for New Zealand skinks do not indicate a rapid divergence of the skinks from each other, and *La. guichenoti* and *L. telfairi* represent much earlier divergences (Daugherty *et al.* 1990b, C.H. Daugherty & G.B. Patterson pers. comm.). Estimates of divergence time based on allozyme data have been made by calibrating genetic distance values (for example Nei's D metric value) against an AID clock. Maxson & Maxson (1979) for instance, used the AID of salamanders to calculate that a Nei's D value of 1.0 represented 14-15 million years separation. Applying this calibration to the skink allozyme data would indicate a diversification of New Zealand *Leiolopisma* commencing 11-12 million years ago (Daugherty *et al.* 1990b, and C.H. Daugherty pers. comm.). If this time frame is used, then if there are about 25 nucleotide differences between New Zealand skink taxa (Tables 4.1a, & 5.5), there have been approximately 2.1-2.3 nucleotide substitutions per million years since divergence. *L. microlepis* and *L. smithi*, with five differences between them, could therefore have diverged just over two million years ago, while the split between *L. n. polychroma* and *L. n. nigriplantare* (13 differences) may have occurred about six million years ago. These dates disagree however with those estimated from the allozyme data (C.H. Daugherty & G.B. Patterson pers. comm., and see Chapter Six).

As with the immunological data, the accuracy and validity of using Maxson & Maxson's (1979) calibration for groups other than their salamanders has not been examined (Thorpe 1982, Nei *et al.* 1983) and there is no evidence for a common multilocus electrophoretic clock (Avise & Aquadro 1982). Furthermore, in the skink allozyme study only 17 presumptive loci provided information for all the taxa (C.H. Daugherty & G.B. Patterson pers. comm.). The accuracy of both the topology and branch lengths of phylogenetic trees determined from allozyme data is low when less than 30 loci are used (Nei *et al.* 1983, Nei 1987), so phylogenetic inferences based on the skink allozyme data set are likely to be unreliable.

Estimates from Biogeographic Information

A third way of estimating divergence times between the skinks is by taking account of their geographic distribution. Australia, New Caledonia, and New Zealand all contain fragments of continental land and were once part of Gondwanaland (Mayes *et al.* 1990, Lawver *et al.* 1991). Fiji is also continental in origin and another *Leiolopisma*, *L. alazon*, occurs there and has morphological similarities to New Zealand taxa, particularly *Cyclodina* (Zug 1985). Pacific members of the *Leiolopisma* group of skinks therefore all occur on continental fragments.

L. telfairi is a biogeographic anomaly, in that it is the only member of the *Leiolopisma* group outside of the Pacific. Sub-fossils of another Mauritian skink *"Didosaurus" mauritianus* have been found, which Arnold (1980) considered to be a sister species of *L. telfairi*. *Leiolopisma* have not been found in Asia or Africa. Africa rifted from Gondwanaland prior to 140 million years ago, while India separated at least 75 million years before present (McKenzie & Sclater 1973, Tarling 1988, Lawver *et al.* 1991). India passed near to the current location of Mauritius and the Mascarene Plateau (McKenzie & Sclater 1973), so ancestors of *L. telfairi* and *L. mauritianus* may have had the potential to be transported there from the Australian region. Fragmentation of Gondwanaland about 80 million years ago (Mayes *et al.* 1990, Lawver *et al.* 1991) and subsequent continental drift could therefore explain the origin and dispersal of *Leiolopisma*.

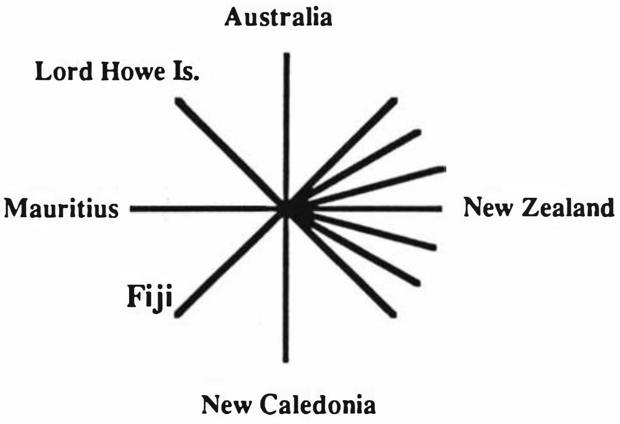
Hypothesis One: A Gondwanan Origin and Diversification of *Leiolopisma*

Both immunological (Hutchinson *et al.* 1990) and sequence data can be interpreted in the framework of a Gondwanan origin and diversification. A Gondwanan divergence for *Leiolopisma* implies a slow rate of sequence evolution (30 nucleotide substitutions over 80 million years yields a rate of 0.38 per million years, or one substitution per 2.7 million years, on average). This figure is not too disimilar from that for the ratites (Table 4.6). Some of the New Zealand *Leiolopisma* are as distinct from each other as they are from *L. telfairi* (Table 5.3, Figs. 5.5 & 5.18). A Gondwanan origin therefore implies that several distinct and old skink lineages inhabit New Zealand, as shown in figure 5.22a.

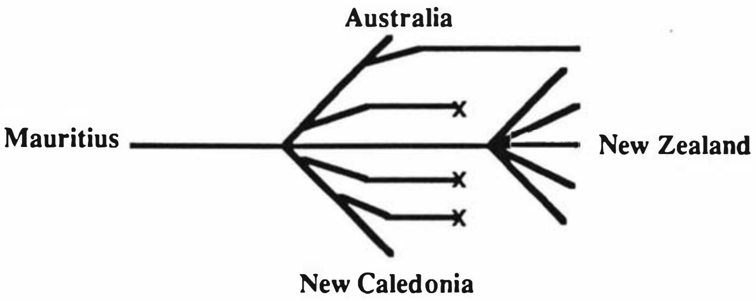
Under this hypothesis 12S rRNA sequence data from New Caledonian *Leiolopisma* and the Fijian *L. alazon* should reveal similar numbers of sequence differences and patterns of change as the New Zealand, Australian and Mauritian taxa. Further investigations of New Caledonian skinks (there are 10

Fig. 5.22. Hypotheses for the origin of *Leiolopisma* skinks. **A)** A Gondwanan origin of the taxa implies that their present distribution is due to the breakup of Gondwanaland during the Creataceous (approximately 80 million years ago). **B)** The "Oligocene drowning" hypothesis suggests that most of New Zealand's extant skink fauna are the result of diversification after the Oligocene (36-25 million years ago), in response to increasing land area and habitat diversity (see the text). C) The "Multiple colonists" scenario indicates that diversification of the skinks occurred sometime after the fragmentation of Gondwanaland, and that several distinct groups of skinks reached New Zealand independently and still survive here. These hypotheses also have relevance to other Pacific and New Zealand biota as discussed in the text.

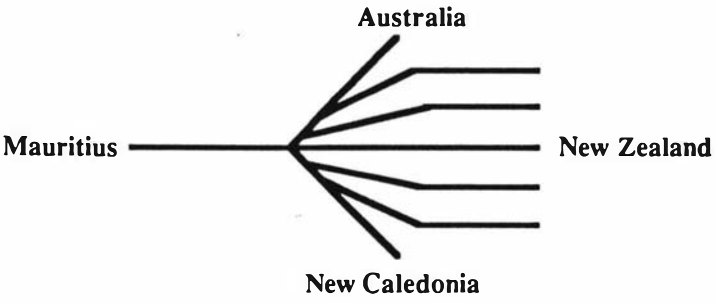
A. Gondwanan diversification



B. Oligocene drowning



C. Multiple colonists



genera; Sadlier 1986) and other members of the *Eugongylus* group of skinks may shed light on the hypothesis of a general radiation of this group about the time that Gondwanaland fragmented. Immunological studies suggest that Australian members of the *Leiolopisma* group are not monophyletic but are derived from a variety of other genera (Baverstock & Donnellan 1990, Hutchinson *et al.* 1990). The morphological taxonomy of the *Leiolopisma* group has been misleading (Towns *et al.* 1985, Daugherty *et al.* 1990b) so *L. telfairi* could be derived from a different source than the Pacific group, possibly an Asian *Mabuya* species, the ancestral group for *Leiolopisma* (Greer 1970). Sequence and immunological data indicate however that there are still close phylogenetic relationships between *L. telfairi* and the Pacific *Leiolopisma*. A close relationship among the *Leiolopisma* group of skinks is also supported by comparison of the relationships of the New Caledonian skink. *T. rohssii* has more sequence differences from any of the *Leiolopisma* than the latter have among themselves, and *T. rohssii*'s sequence similarities to *L. fallai* (Fig. 5.19) are in a variable region so may be coincidental. *L. telfairi* on the other hand shares changes with *C. aenea* and other *Leiolopisma* at more conservative sites in the 12S rRNA molecule (Table 3.1), which suggests that *L. telfairi* does have close phylogenetic relationships to the Pacific *Leiolopisma*.

Other animal groups with representatives in New Zealand have been considered to have had an origin in Gondwanaland. For example, the Leiopelmid frogs (Bull & Whitaker 1975), the tuatara (Crook 1975), and the moa (Cooper *et al.* 1992). The distribution of some of New Zealand's insect fauna may also be accounted for by a Gondwanan origin (Dugdale 1989). A Gondwanan origin for geckos has also been suggested on the basis of karyological evidence (King 1979, 1987, King & Mengden 1990). Bauer (1990), on morphological biogeographical criteria, also advocates a Gondwanan origin for some of the geckos. The *Leiolopisma* skinks may therefore be just one of several groups of old New Zealand inhabitants.

The Mauritian Leiolopisma are Still Enigmatic

The Mascarene islands, to which Mauritius belongs, are volcanic and were formed 7-8 million years ago (McDougall & Chamalaun 1969). No *Leiolopisma*-like skinks have been recorded from India or Madagascar, 700 kilometers west of Mauritius. India may also have separated from Gondwanaland over 100 million years ago (Lawver *et al.* 1991) and *Leiolopisma* may not have been present then. Even under a Gondwanan origin hypothesis, the occurrence of *L. telfairi* on Mauritius is still enigmatic. It is possible that the ancestral group of *L. telfairi* and *L. mauritianus* had a post-Gondwanan origin in the Pacific region and subsequently reached Mauritius by its own dispersal abilities. This is in effect a null hypothesis, to be retained if additional sequence and biochemical data cannot establish an independent and old origin for *L. telfairi*.

Origins of the New Zealand Skinks

Even if there was a Gondwanan origin for the skinks, New Zealand may have been colonized by them after separation from Gondwanaland. If this was the case there are two alternatives for the current diversity of them in New Zealand. New Zealand *Leiolopisma* may be a monophyletic group which diversified in New Zealand from a common ancestor (Fig. 5.22b). Or, several groups of skinks may have reached and colonized New Zealand independently (Fig. 5.22c). Both of these hypotheses have some support from analyses of the sequence or allozyme data.

Diversification Within New Zealand

As the water barrier between New Zealand and Australia and New Caledonia increased following separation from Gondwanaland, the chances of colonization of New Zealand by flightless terrestrial animals would diminish. Many independent colonizations of New Zealand by skinks would therefore seem unlikely. As discussed above, the occurrence of *L. telfairi* on Mauritius may not be due to continental drift. The New Zealand skinks may therefore have a more recent diversification. Analyses of allozyme data for New Zealand *Leiolopisma* by C.H. Daugherty & G.B. Patterson (pers. comm.) suggest that they have diversified in New Zealand, and are not as closely related to *L. telfairi* and *La. guichenoti* as the 12S rRNA sequence analyses indicate. Limitations of both the allozyme and sequence data will be discussed below, but a monophyletic origin for most New Zealand *Leiolopisma* cannot yet be excluded.

Comparison of the 12S rRNA sequence differences among the skinks to those found among other vertebrates indicates that the New Zealand *Leiolopisma* diverged from each other at least 15 million years ago, and probably much earlier (Table 4.6). The timing of this diversification may be related to major changes in New Zealand's geography during and after the Oligocene (36-25 million years ago).

Hypothesis Two: Post-Oligocene Diversification of New Zealand *Leiolopisma*

New Zealand was a low-lying landmass in the Oligocene and rising sea levels created a series of islands. Total land area was 80% less than at present and fluctuating sea levels altered the number and size of these islands (Suggate *et al.* 1978, A.J. Cooper, G.K. Chambers, & R.A. Cooper *in prep.*). Cooper *et al.* proposed that this may have resulted in population "bottlenecks" and extinctions (Fig. 5.22b). Post-Oligocene increases in land area and elevation would have led to the creation of many new habitats and consequent diversification among terrestrial biota.

If the New Zealand lineages (Table 5.3) are the result of Miocene (15-25 million years ago) diversification from one or a few taxa which survived the Oligocene (Fig. 5.22b), then it is expected that they would have fewer differences between themselves than they would to *L. infrapunctatum* and *L. telfairi*. This is not the case (Table 5.3).

A post-Oligocene radiation has been suggested for both the moa and the kiwi (Cooper *et al. in prep.*). The number of observed nucleotide differences between the moa genera and between the kiwi species are about 11 and 15, respectively (Table 4.5a), substantially fewer than the 25 differences between the skink taxa. A Miocene diversification of the skinks would therefore indicate a more rapid rate of sequence evolution in the skinks than in the ratites. Post-Oligocene speciation may account for the diversity of other New Zealand fauna, such as *Leiopelma* frogs (Daugherty *et al.* 1982, and C.H. Daugherty pers. comm.).

Population bottlenecks will decrease the number of mitochondrial lineages, but under the neutral framework of molecular evolution, it should not affect the rate of sequence divergence (Kimura 1983, Wilson *et al.* 1985, Neigel & Avise 1986). Under a "nearly neutral" hypothesis however, population size could affect the rate of molecular evolution (Ohta 1976, 1992). Analyses of *Drosophila* lineages suggested that groups which probably passed through repeated population bottlenecks had a higher rate of molecular evolution than lineages which had more stable population sizes (DeSalle & Templeton 1988). However, the dates of separation of these *Drosophila* species have not yet been reliably established, nor has the fact that some did pass through severe bottlenecks. With respect to the *Leiolopisma*, even if bottlenecks can affect rates of molecular evolution, this does not alter the conclusion from the sequence data that the skinks appear to have diversified relatively rapidly, and that the Australian and Mauritian taxa are not distinct from the New Zealand taxa

Phylogenetic Limitations of the Skink Allozyme Data

The skink allozyme data (Daugherty *et al.* 1990b, C.H. Daugherty & G.B. Patterson pers. comm.) could indicate a monophyletic origin of New Zealand *Leiolopisma* and *Cyclodina*. The number of electrophoretic loci (seventeen) is too few however to reliably infer phylogenetic relationships. Ideally at least 30 loci should be used (Nei *et al.* 1983). More rigourous analyses (such as parsimony analysis, Swofford & Berlocher 1987) of the data have yet to be performed. The measure of genetic distance used by Daugherty *et al.* (1990b), Nei's unbiased D value (see Nei 1987), relies on the assumption of equal rates of substitution at all loci, a condition unlikely to be met (Swofford & Olsen 1990), and this measure may therefore not be an accurate estimate of genetic divergence between taxa. A modified version of Nei's genetic distance (Hillis 1984), or Cavalli-Sforza & Edwards' (1967) distance measure may be more appropriate (Swofford & Olsen 1990), though the limited number of loci must still be borne in mind. Limitations of the sequence data will be discussed in Chapter Six. A post-Oligocene diversification of the New Zealand *Leiolopisma* (Fig. 5.22b) does not account for the 12S rRNA sequence similarity within and between the New Zealand and the Australian and Mauritian taxa.

Hypothesis Three: Multiple *Leiolopisma* Colonizations of New Zealand

Similar genetic distances between the New Zealand skink taxa do not by themselves indicate a Miocene diversification in New Zealand. While the skinks may have diversified from each other relatively rapidly, different groups could have reached New Zealand independently. *L. infrapunctatum* and *La. guichenoti* seem most closely related to each other on the basis of sequence similarity (Table 5.3, Figs. 5.1 & 5.5, 5.15, 5.16). If a Gondwanan origin for the other skink lineages is assumed, then 25-30 nucleotide differences implies a rate of 0.3-0.4 substitutions per million years. (The error associated with these estimates is likely to be high, as discussed in Chapter Four). The 12 differences observed between *L. infrapunctatum* and *La. guichenoti* 12S rRNA sequence could indicate a separation of 20-30 million years. If this is the case then the *L. infrapunctatum* lineage either entered New Zealand more recently than other taxa, or members of it dispersed west to Australia. The most likely route into, or out of, New Zealand would be via New Caledonia and the Norfolk Island ridge, which was once part of the ancestral

New Zealand landmass (Suggate *et al.* 1978, Stevens 1980). Land connections along this route disappeared by the Miocene (approximately 30 million years ago; Stevens 1980), which could place an upper bound on time of entry for many flightless animals. Estimates for the divergence of the ancestors of *L. infrapunctatum* and *La. guichenoti* are compatible with a pre-Miocene entry.

An independent arrival of *L. infrapunctatum* in New Zealand is still compatible with both a Gondwanan diversification of *Leiolopisma* (Fig. 5.22a), as well as diversification of other *Leiolopisma* in New Zealand (Fig. 5.22b). Under the multiple colonists hypothesis the number of different mitochondrial lineages of *Leiolopisma* (Table 5.3) requires many multiple independent colonizations of New Zealand by skinks. Considering the distances and oceans to cross, this is perhaps less likely than the other two hypotheses.

Hypotheses for the testing

The phylogenetic relationships of the New Zealand *Leiolopisma* are largely unresolved by the current sequence data, but they support suggestions (Towns *et al.* 1985, Daugherty *et al.* 1990) of a much longer history in New Zealand than indicated by morphological similarities. Analysis of the sequence data also suggests a rapid divergence of *Leiolopisma* skinks. Whether this divergence reflects a Gondwanan or more recent evolution requires additional sequence information. Strategies to test the three hypotheses are discussed in Chapter Seven. Regardless of which hypothesis receives the most support, the evolutionary history of the skinks, and probably other New Zealand biota, are much more complex, and hence interesting, than previously imagined and offer many opportunities for more detailed investigations of evolutionary processes.

Chapter Six: Comparing Sequence and Allozyme Data

One of the primary reasons for selecting the skinks to address evolutionary questions in New Zealand was that allozyme information was available for virtually all of the described taxa, as well for taxa yet to be formally named (Towns *et al.* 1985, Patterson & Daugherty 1990, C.H. Daugherty & G.B. Patterson pers. comm.). DNA sequence information was obtained from individuals which contributed to the allozyme study so direct comparison bewteen the two data sets is possible. In this chapter the relationships of the New Zealand *Leiolopisma* skinks as inferred from the sequence data are compared with the allozyme divergence data. The data sets do not contain all the same taxa so only the 16 New Zealand *Leiolopisma* common to both are compared.

Preliminary results of the allozyme study have been published (Daugherty *et al.* 1990b), but manuscripts containing the full analyses are in preparation (C. H. Daugherty & G. B. Patterson pers. comm.). Allozyme information could only be obtained from 17 presumptive loci for the skinks, though most taxa were distinguished by fixed allelic differences. The accuracy of both the topology and branch lengths of phylogenetic trees determined from allozyme data is low when less than 30 loci are used (Nei *et al.* 1983), so comparisons between the sequence and allozyme data only address the question of how similar are their estimates of genetic distance.

Conflict Between Allozyme and 12S rRNA Sequence Data

Using Nei's unbiased D (Nei 1972) as a measure of genetic distance Daugherty & Patterson (pers. comm.), identified nine sets of relationships with D values less than 0.4. Some of these groupings are found in the sequence analyses, but D values do not correlate well with numbers of nucleotide differences (Table 6.1). Stewart Island Green and *L. grande*, and *L. maccanni* and *L. inconspicuum* are the most closely related pairs on the basis of allozymes (Table 6.1), but their 12S rRNA sequences differ by 8 and 20 substitutions, respectively. In contrast, only 5 nucleotide differences were found between *L. microlepis* and *L. smithi* but they have a D value of approximately 0.55 (Table 6.1). *L. fallai* and *L. suteri*, and *L. infrapunctatum* and *L. otagense* have no spectral signals linking them together (see Chapter Five) despite having relatively low D values (Table 6.1). Both *L. telfairi* and *La. guichenoti* have D values in excess of 1.0 when compared to each other and to the New Zealand *Leiolopisma* (C.H. Daugherty & G.B. Patterson pers. comm.), in contrast to their sequence similarities (Table 4.1a).

Congruence Between Allozyme and 12S rRNA Sequence Data

The allozyme and sequence data are not in complete disagreement however. Members of the taxon pairs *L. n. polychroma*/*L. n. nigriplantare*, *L. grande*/St. Is. Green, and *L. microlepis*/*L.smithi* are each others closest relatives on the basis of both DNA (Table 4.1a, Fig. 5.5) and allozyme analyses (Table 6.1; C.H. Daugherty & G.B. Patterson pers. comm.).

Table 6.1. Comparisons of genetic distances inferred from 12S rRNA sequence analysis and allozymes (C.H. Daugherty & G.B. Patterson pers. comm.). The relationships of taxon pairs 1, 2, and 4 are congruent in that both allozyme and sequence information identify the members of the pair as most similar to each other. The relative degree of genetic divergence between taxa differ however when the number of nucleotide differences and Nei's unbiased D value (C.H. Daugherty & G.B. Patterson pers. comm.) are compared (Spearman's rank correlation, $r_s = 0.245$, $P < 0.05$).

	Sequences		***Allozymes***	
Taxon Pair	**No. Diff.**	**Rank**	**Unbiased D Value**	**Rank**
1. *L. microlepis/L. smithi*	5	1	0.55	9
2. St.Is.Green/*L. grande*	8	2	0.175	1.5
3. *L. inconspicuum/L.notosaurus*	12	3	0.25	4.5
4. *L.n.nigri./L.n.polychroma*	13	4	0.2	3
5. *L. inconspicuum/L. maccanni*	20	5.5	0.175	1.5
6. *L. maccanni/L.notosaurus*	20	5.5	0.25	4.5
7. *L. fallai/L. moco*	24	7	0.35	7
8. *L. fallai/L. suteri*	26	8	0.275	6
9. *L. infrapunctatum/L.otagense*	32	9	0.4	8

These differences between the sequence and allozyme data may be due to limitations in both the DNA and allozyme data sets. The mitochondrial sequence data reflect maternal relationships only while the allozymes provide information on both maternal and paternal inherited genomes (Wilson *et al.* 1985, Avise *et al.* 1987). Sampling errors due to the short length of DNA sequences and the small number of allozyme loci scored may also contribute to the differing conclusions in each data set. Collection of more mitochondrial and nuclear DNA sequence information is required to determine the accuracy of the phylogenetic relationships. Differing phylogenies based on mitochondrial and nuclear sequence data will indicate whether the mtDNA of *Leiolopisma* is suitable for reconstructing organismal relationships.

The allozyme data has only been analyzed using clustering algorithms (Weighted Pair Group Method using Arithmetic means, Sneath and Sokal 1973) so far (C.H. Daugherty & G.B. Patterson pers. comm.) and further analyses (such as parsimony) are required to determine the robustness of the relationships between the skink taxa (see Swofford and Olsen 1990).

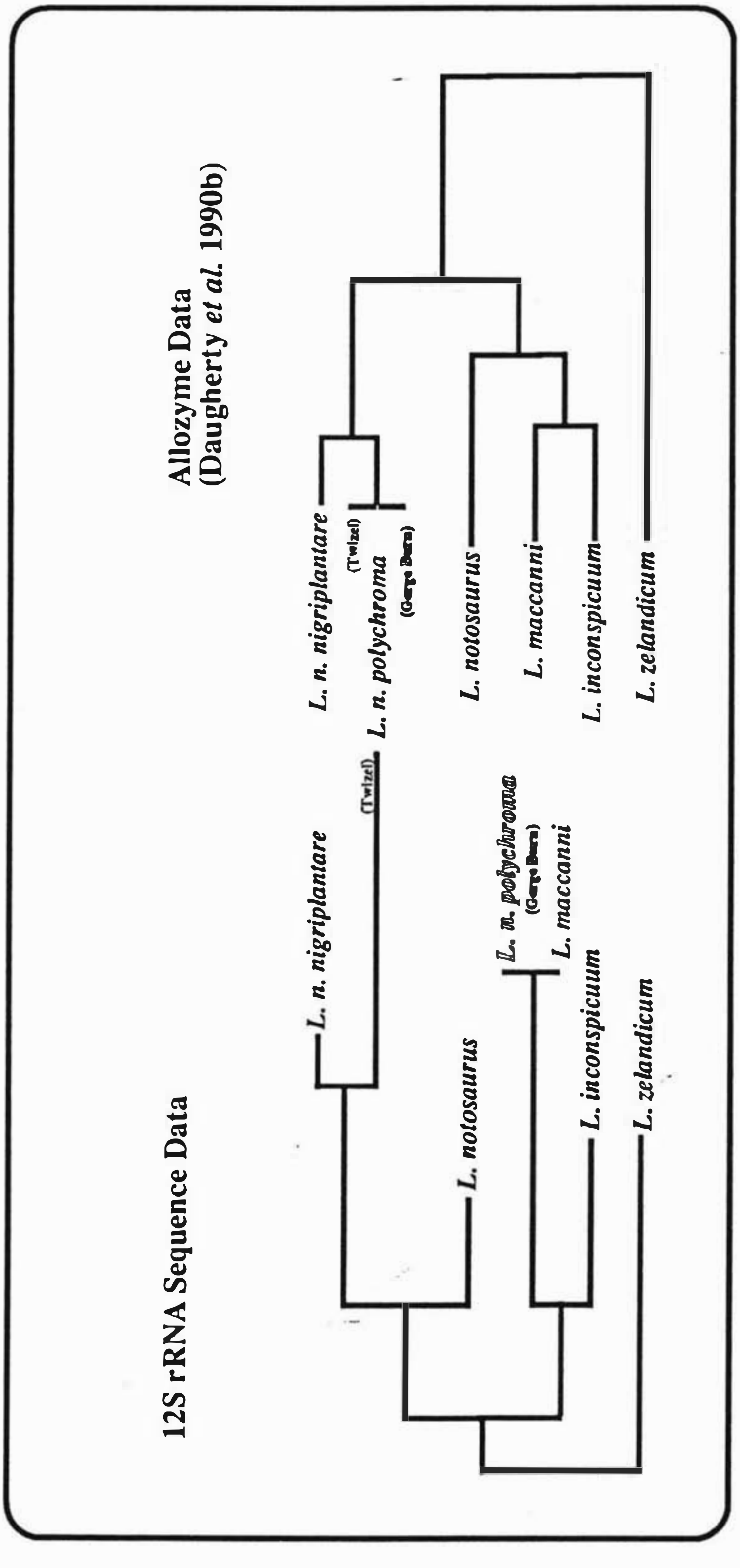
Hybridization

Comparison of the allozyme and the mitochondrial sequence information also illustrate the benefits of a broad approach to evolutionary studies. A difference between the DNA sequences and allozyme profiles of *L. n. polychroma* populations suggests that interspecific hybridization has occured.

The identical 12S rRNA and very similar cytochrome *b* sequences for *L. n. polychroma* and *L. maccanni* at Gorge Burn were presented in the previous chapter (Table 5.4). Several observations show tha this sequence similarity was not due to taxonomic mis-identification nor to mixing of tissue or DNA samples. The allozyme profiles for *L. n. polychroma* individuals at Gorge Burn are consistent with those of *L. n. polychroma* at other locations, and there appears to be nothing incongruous about the *L. maccanni* allozyme data at Gorge Burn either (Daugherty *et al.* 1990b). DNA was extracted from several individuals and from several tissues at different times for both species. Both PCR amplification and DNA sequencing were also performed several times for each sample. Identical DNA sequences were obtained in all cases (Hickson *et al.* 1992). That the cytochrome b sequences for *L. n. polychroma* and *L. maccanni* differ at three positions (Table 5.4) supports the view that the identical 12S rRNA sequences in the Gorge Burn populations is not a result of contamination.

The conflicting relationships for the Gorge Burn population of *L. n. polychroma* are shown in figure 6.1. Note that the relationship of *L. notosaurus* presented in figure 6.1 differs from those in figures 5.5 and 5.18, where more taxa were included. The different placement of *L. notosaurus* is a reflection of the small number of shared sequence signals between the taxa. For example, *L. notosaurus* and *L. inconspicuum* have fewer nucleotide substitutions when compared to each other than either do when compared to *L. maccanni* (Table 4.1a), which may suggest that the latter species is more distantly related to them. In the comparison of six taxa shown in figure 6.1, the bipartition (sites where they both differ from the other taxa) of *L. notosaurus+L. inconspicuum* is supported by two signals, while the *L. inconspicuum+L. maccanni* bipartition has three signals. When more taxa are added *L. maccanni*,

Fig. 6.1. Evidence for sexual hybridization in a New Zealand skink population. Twizel and Gorge Burn populations of *L. n. polychroma* have the same allozyme profile (Daugherty et al. 1990b), but quite different mtDNA sequences. The discrepancy between the allozyme and sequence data can be reconciled by proposing that *L. maccanni* females have mated with *L. n. polychroma* males (see text and figure 6.2).



because of it's larger number of differences, has more conflicting signals to other taxa. This means that the number of signals supporting *L. inconspicuum*+*L. maccanni* to the exclusion of all other taxa decreases, and the relationship between *L. notosaurus* and *L. inconspicuum* consequently strengthens (Figs. 5.5, 5.18). The phylogenetic position of *L. notosaurus* does not however affect the main conclusion shown in figure 6.1; that 12S rRNA sequences from the Gorge Burn population of *L. n. nigriplantare* are identical to those from the sympatric *L. maccanni* population.

Disparity between mitochondrial and nuclear markers is often indicative of sexual hybridization (Wilson *et al.* 1985, Moritz *et al.* 1987, Barton & Hewitt 1989, Arnold 1992) and this seems the simplest explanation for the Gorge Burn population of *L. n. polychroma.* The alternative hypothesis that the Gorge Burn *L. n. polychroma* mitochondrial sequences only reflect a different mitochondrial haplotype (see Avise *et al.* 1987, Moritz *et al.* 1987, Avise 1989a) within the *L. n. polychroma* species appears unlikely. There are 29 differences between the 12S rRNA sequences of the Gorge Burn and the Twizel populations of *L. n. polychroma* and 36 differences in their cytochrome *b* sequences (Table 5.4). This level of inter-population sequence divergence has not been reported even for more rapidly evolving regions of mtDNA (Thomas *et al.* 1990, Smith & Patton 1991). Numbers of differences in the 12S rRNA sequences between species can be as few as three for both kiwi (Table 4.5a) and great apes (Table 4.5d).

The *L. n. polychroma* individuals from Gorge Burn have the *L. maccanni* type mtDNA and either *L.maccanni* or *L. n. polychroma* allozyme profiles. No individuals had electromorphs ("alleles") from both parents. Since mtDNA is primarily maternally inherited (see Wilson *et al.* 1985) this suggests that one or more female *L. maccanni* mated with *L. n. polychroma* males, introducing the *L. maccanni* mtDNA into the *L. n. polychroma* gene pool, as shown in figure 6.2a. Loss of *L. maccanni* alleles in the hybrids can be accounted for by the female hybrids subsequently mating with *L. n. polychroma* males for several generations (Fig. 6.2b). When this hybridization occurred cannot be accurately determined from the sequence data at present. However if we assume a Gondwanan origin for the *L. n. polychroma* and *L. maccanni* lineages (see Chapter Five), then the 39 differences in the cytochrome b sequences of *L. n. polychroma* and *L. maccanni* (Table 6.1) could equate to 0.5 substitutions per million years (39/80). The three differences between the Gorge Burn *L. n. polychroma* and *L. maccanni* populations could therefore indicate 6 million years since hybridization. A more recent separation of *L. n. polychroma* and *L. maccanni*, say 20 million years, would imply hybridization occured about 1.5 million years ago.

DISCUSSION

Lack of congruence between allozyme and sequence data is often attributable to hybridization (see Moritz *et al.* 1987), though it may also suggest variation in rates of change between the two types of data (Dowling & Brown 1989). Allozyme data can be more suitable for examining relationships amongst closely related taxa while sequences may be more informative at greater levels of separation (Buth 1984, Hillis 1987, Hillis & Moritz 1990). The 12S rRNA sequence data indicates that few of the

Fig. 6.2a. Potential offspring from sexual hybridization between two species with different nuclear (allozyme) and mitochondrial markers.The nuclear phenotype is shown by the shading in the large oval while the mitochondrion is represented as the smaller oval. The offspring should have the mitochondrial type of its mother but nuclear markers reflecting both parents (as indicated by cross-hatching).

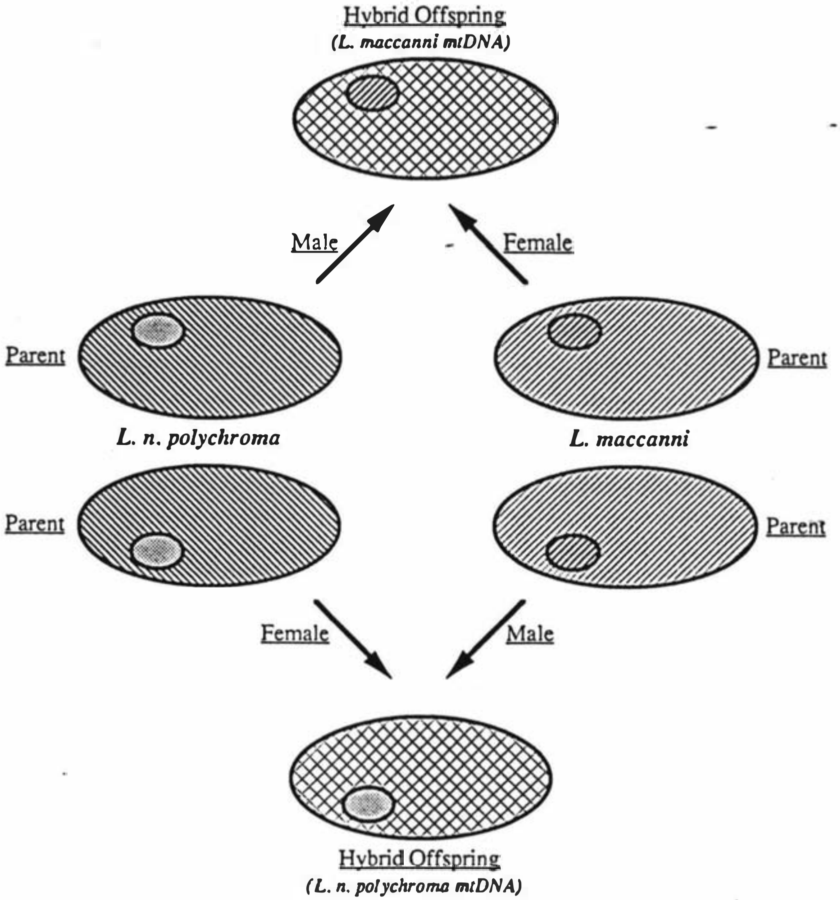
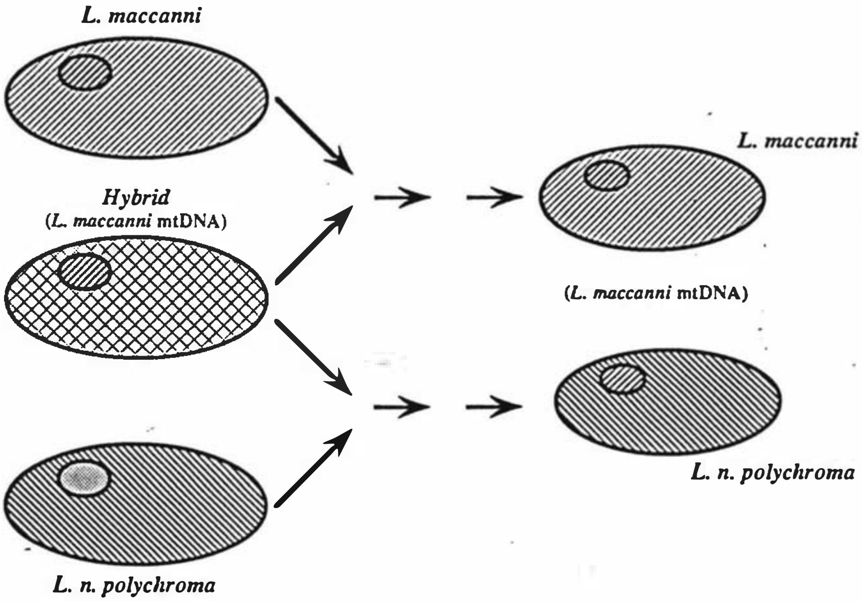


Fig. 6.2b. Effects of subsequent breeding of hybrid with members of the parental populations. Mitochondrial type remains unchanged but allozyme profile should tend towards the non-hybrid parents markers over several generations.



taxa are closely related (Tables 4.1 & 4.2, Fig. 5.5). If, in addition, many of the skinks diverged from each other at about the same time (see Chapter Five), then these two factors may explain why the sequence and allozyme data sets conflict. Nei *et al.* (1983) in simulation experiments showed that relationships derived from fewer than 20 allozyme loci were less likely to be accurate than when more than 30 loci were used. Allozyme information from other presumptive loci could not be obtained for the skinks (C.H. Daugherty pers. comm.), so further investigation of the nuclear genome will require other strategies.

mtDNA provides information about maternal ancestors, and mtDNA sequence divergence may predate speciation so analysis of mtDNA, particularly the conservative 12S rRNA, may fail to identify more recent population subdivisions or hybridizations (Avise *et al.* 1987, Moritz *et al.* 1987, Arnold 1992). This is not the case with the skinks however, the sequence and allozyme data identify differences in all the taxa. The conflict is in estimates of the degree of genetic divergence. Except for *L. microlepis* and *L. smithi*, the sequence data tend to show greater divergence between the taxa than the allozymes (Table 6.1). Nei's unbiased D value is calculated under the assumption of equal rates of change between different loci. This is probably incorrect and a modified version of Nei's genetic distance (Hillis 1984), or Cavalli-Sforza & Edwards' (1967) arc distance measure may be more appropriate (Swofford & Olsen 1990).

As discussed in Chapter Five, interpretations of immunological data from *Leiolopisma* suggest that *L. telfairi* is similarly distant from New Zealand and Australian *Leiolopisma* (Hutchinson *et al.* 1990). This is not apparent in the allozyme analyses (C.H. Daugherty & G.B. Patterson pers. comm.), and may reflect the relatively small number of loci examined. A wider sampling of the mitochondrial genome, either by restriction mapping or the sequencing of other genes, would indicate the accuracy of the 12S rRNA sequence data. Sequencing of nuclear genes, such as the 18S rRNA or intragenic spacer regions in the rRNA cluster (Hillis & Dixon 1991), can be used to test the validity of the mtDNA and allozyme-based phylogenies.

Both the allozyme and mtDNA sequence data suggest however that the genetic diversity of New Zealand *Leiolopisma* is much greater than their morphological diversity, and the skinks are consequently much older than previously (Bull & Whitaker 1975, Hardy 1977) suggested. Similar observations have been made for amphibians (Cherry *et al.* 1978, Roberts & Maxson 1985, Wallis & Arntzen 1991) and East African jackals (Wayne *et al.* 1989).

Hybridization - Questions to Address

The role and significance of hybridization in evolution is not well understood, though it has been suggested to be a major source of genetic diversity and innovation (Huxley 1938, Dobzhansky 1940, Rattenbury 1962, Barton & Hewitt 1989, Arnold 1992, Grant & Grant 1992). Hybrids are often associated with habitat disturbance (see Hair 1966, Arnold 1992). The putative hybridization at Gorge Burn is therefore an important result and requires more detailed studies. There are several questions to address;

1). What is the extent of hybridization? Only a few individuals from Gorge Burn were examined in this study, many more need to be sampled. Do both hybrid and non-hybrid *L. n. polychroma* co-exist at Gorge Burn? *L. inconspicuum* is sympatric with *L. n. polychroma* and *L. maccanni* at several sites (Patterson 1985, Daugherty *et al.* 1990b) and these species and populations should be examined closely as well.

2). The available data implies that there is directional exchange of mtDNA; *L. maccanni* females have mated with *L. n. polychroma* males. This would implicate behavioural and reproductive factors affecting the hybridization (Ferris *et al.* 1983, Lamb & Avise 1986). Examination of more individuals from Gorge Burn is necessary to determine in hybrids with *L. n. polychroma* mtDNA also occur.

3). The large number of sequence differences between *L. n. polychroma* and *L. maccanni* (Table 6.1) suggests that they have been separated for a considerable period of time, perhaps up to 80 million years (Chapter Five), though the allozyme data could reflect a closer relationship between them (C.H. Daugherty & G.B. Patterson pers. comm.). Hybridization between animal species is generally more common among closely related taxa, that is those separated by a few million years (see Barton & Hewitt 1989, Arnold 1992). Frogs however, have a greater ability to hybridize across larger phylogenetic distances (tens of millions of years) than mammals (Wilson *et al.* 1974). The compositional organization of the reptilian nuclear genome may be more similar to amphibians (Bernardi & Bernardi 1991, Olmo 1991) than mammals (Bernardi *et al.* 1988), and this may be reflected in a greater ability to hybridize.

4). The potential for skinks species to interbreed, and the viability and reproductive success of hybrid offspring, can be investigated by captive breeding, as has been done for species of *Drosophila* (Aubert & Solignac 1990). Close study of hybrids may also indicate if there is heterozygote advantage or disadvantage (see for example Paige *et al.* 1991, Grant & Grant 1992). If it is shown that *L. n. polychroma*, *L. maccanni* and *L. inconspicuum* can interbreed and produce viable offspring, despite their large genetic differences, then strong ecological and/or behavioural structuring between the species may have prevented frequent interbreeding. A geological, climatic or other disturbance may therefore have been the cause of hybridization at Gorge Burn. The preliminary calculations in this chapter suggest hybridization may have occurred in the Pliocene, up to six million years ago. The hybridization may have represented a first meeting of *L. maccanni* and *L. n. polychroma* at that site.

Other New Zealand skink species are also taxonomically problematical, for example, *L. waimatense*, and the *L. lineoocellatum*/*L. chloronotum* complex (Pickard & Towns 1988; C.H. Daugherty pers. comm.). These may also provide examples of hybridization.

Hybridization has been suggested for many groups in New Zealand, both plant (Cockayne 1911, Allan 1961, Rattenbury 1962, Fisher 1965, Webby *et al.* 1987, Wardle 1988) and animal (Powell 1949, Bigelow 1965, Batchelar & McLennan 1977, Climo 1978, Solem *et al.* 1981, Hitchmough *et al.* 1990). With the exception of Hitchmough *et al.* (1990), these have not been investigated genetically. Hybrids in kiwi and in shearwaters have also recently been detected by DNA analyses (A.J. Cooper pers. comm.), so hybridization of both plants and animals in New Zealand may be relatively common.

The ability to extract sufficient DNA for PCR analyses from small tissue samples makes it feasible to screen large numbers of individuals without the need to kill them. Removal of a portion of the skinks' tail should not adversely affect it's survival. The development of PCR primers suitable for examination of both conserved and variable regions of nuclear rRNA (Hillis & Dixson 1991) will facilitate comparison of mitochondrial and nuclear derived phylogenies, as well as permit more detailed studies of the nuclear genome and the processes of evolution.

Species Concepts

Does the hybridization between *L. n. polychroma* and *L. maccanni* imply that they are not distinct species? The difficulty in distinguishing these taxa, and *L. inconspicuum* (Patterson & Daugherty 1990), may imply that there is just one variable species. On the basis of allozymes however, they appear to be reproductively isolated (Daugherty *et al.* 1990b). Genetic distance measures cannot be used as the sole criterion for identifying species (see Nei 1987, Coyne 1992), but the fact that *L. n. polychroma*, *L. maccanni*, and *L. inconspicuum* occur sympatrically and have distinct allozyme profiles provides strong evidence that they do not frequently interbreed. They are also ecologically differentiated (Patterson 1985). The number of differences between their 12S rRNA sequences (Tables 4.1a, and 5.3) are also very high (compare to the kiwi (Table 4.5a) and the apes (Table 4.5d), supporting their taxonomic distinctiveness.

Origins of Some Specific New Zealand *Leiolopisma*

L. n. nigriplantare is found only on the Chatham Islands, 800 km east of Christchurch. The Chathams have long been separated from mainland New Zealand, though there is evidence for more extensive land between them in the Eocene (45 MYA; Suggate *et al.* 1978, Hay *et al.* 1970, Craw 1988). The land area of the Chatham Islands has been greatly reduced in the past by rises in sea level (Hay *et al.* 1970) and consequently some of its biota may have arrived there in more recent times (see Fleming 1980, Lloyd 1982, Craw 1988). There are 13 nucleotide differences between *L. n. nigriplantare* and *L. n. polychroma*. If it is assumed that skinks with 25-30 differences diverged during the fragmentation of Gondwanaland (about 80 MYA; see Chapter Five), then the nucleotide substitution rate is 0.31-0.38 substitutions/site/million years. This would place the time of divergence between the lineages leading to *L. n. nigriplantare* and *L. n. polychroma* at between 34 and 42 million years, which may coincide with the Eocene land route. A Miocene origin for New Zealand skinks (Fig. 5.22b), say 20 MYA, could indicate a separation of 9-10 million years between the Chatham Island and mainland skinks.

L. suteri is the only egg-laying skink in New Zealand (Whitaker 1968, Towns 1975b) and the lack of a close relationship between it and *La. guichenoti*, which also lays eggs, indicates that this is a physiological trait which can be gained or lost independently. The predominance of ovovivipary (bearing live young) in New Zealand skinks was considered to be an adaptation to cold periods, such as occurred during the Pleistocene (Whitaker 1968, Towns 1975a). *L. suteri* is now found in the north of the North Island, and it has been suggested (Whitaker 1968, Towns 1975a) that it retained the egg-laying trait because it did not experience the same cold Pleistocene glacial climates as other New Zealand skinks. Many other skinks are found in the far north of New Zealand however (Pickard & Towns 1988) but do not lay eggs. Analyses of both allozyme (C.H. Daugherty & G.B. Patterson pers. comm.) and the 12S rRNA sequence data (Figs. 5.5, 5.18) indicate that *L. suteri* may have relatively close relationships to some of the ovoviviparous New Zealand taxa. Egg-laying by *L. suteri* may (in some as yet unknown way) be associated with it's adaptation to living near the intertidal zone (Towns 1975a). *La. guichenoti* is a widespread and common skink in gardens of south-east Australia (Cogger 1986), and does not inhabit a similar niche as *L. suteri*.

Some skink groups are reported to have good dispersal ability because apparently closely related taxa can be widely separated (Greer 1974), but there is circularity in this argument since the morphological similarities are assumed to imply recent origins. The present study, and those based on allozymes (Daugherty *et al.* 1990b, C.H. Daugherty & G.B. Patterson pers. comm.) and immunological affinities (Baverstock & Donnellan 1990, Hutchinson *et al.* 1990) have demonstrated morphological similarity to be a poor guide to genetic relationships in the *Leiolopisma* group of skinks. Lack of appreciation of the taxonomic status and antiquity of species can lead to inappropriate species management and consequently, extinction (Avise 1989b, Daugherty *et al.* 1990a). Most of the New Zealand skinks have restricted distributions, and are rare or endangered (Pickard & Towns 1988). Many are restricted to offshore island habitats. Rats, cats and habitat destruction have contributed to their decline (Whitaker 1978, Thomas 1982b). Information about skink evolutionary relationships and genetic diversity should be considered in conservation strategies (Vrijenhoek *et al.* 1985, Avise 1989b).

Chapter Seven: Discussion

The New Zealand skinks present a much more interesting and challenging evolutionary problem than previously suspected and there is much potential for further study. In this final chapter the principal results are restated and suggestions for future work discussed.

The seven major features of this thesis are:

1). The demonstration of the utility of DNA sequences for examining evolutionary questions.

2). A secondary structure model for domain III of vertebrate 12S rRNA was developed (Chapter Three), and subsequently used to examine nucleotide substitutions in the context of secondary structure (Chapters Four and Five). One third of this region of the 12S rRNA molecule does not vary among the vertebrate sequences examined.

3). Comparison of vertebrate 12S rRNA sequences also showed that there are similarities in the patterns of variability within the molecule. There may however also be taxon specific regions of variation.

4). The sequence data were analyzed using a new sequence analysis approach termed **Spectral Analysis**. One of the primary advantages of the spectral approach is that it gives a visual and quantitative representation of conflicting patterns of relationships in the data.

5). Most of the skink taxa have similar numbers of nucleotide substitutions when compared to each other and phylogenetic analyses of the 12S rRNA sequences did not produce a completely resolved evolutionary tree.

6). This lack of phylogenetic resolution was interpreted as reflecting old and rapid divergence among the *Leiolopisma* skinks. Three hypotheses were presented to account for the origin of New Zealand skinks.

7). Hybridization between sympatric populations of two New Zealand skink species was inferred from comparisons of allozyme and mitochondrial DNA sequence information.

Secondary Structure and Phylogenetic Analyses

The refinement of the secondary structure model of domain III of vertebrate 12S rRNA is a useful step for phylogenetic analyses of this commonly analyzed region of mtDNA. Comparison of the vertebrate sequences provides information on both the number of sites potentially free to vary and the pattern of variability in the molecule within and between groups.

That not all sites in a gene change at the same rate has long been known (Fitch & Margoliash 1967, Fitch & Markowitz 1970), and models to take account of different rates of change among the codon positions are available (e.g., Holmquist & Pearl 1980, Nei & Gojobori 1987). However, as Fitch & Margoliash (1967) and Shoemaker & Fitch (1989) demonstrated, rates of change between sites can be more complex. Dividing codons into just fast (third codon position) and slower (first+second) classes may not be sufficient to explain the patterns of variability. Methods to take account of different levels of variability within non-protein encoding sequences, such as rRNA, have not attracted the same degree of attention.

While failure to take account of invariant sites can result in underestimates of the numbers of unobserved nucleotide substitutions (Palumbi 1989, Shoemaker & Fitch 1989), this does not appear to mislead phylogenetic inferences for the skinks (Fig. 5.12). Removal of constant columns from the data set (Fig. 5.12) is a simplistic way of investigating this problem however, and mathematical methods for adjusting for variable rates of change among sites in a sequence are required. Manske & Chapman (1987) developed a model for 5S rRNA which assigns probabilities of substitution for each site based on relative nucleotide variance. Palumbi (1989) applied a simple model for corrections to mitochondrial restriction enzyme fragment mapping data. For mammalian D-loop sequences Saccone *et al.* (1991) calculated times of divergence using only those variable sites which fitted a Poisson distribution. Golding (1983) however, showed that gamma and lognormal distributions can be better than a Poisson distribution for describing variable mutation rates among sites in sequences. A gamma distribution model is being investigated for use in the Hadamard conjugation algorithms (P.J. Waddell pers. comm.).

Weighted parsimony, which weights sites on the basis of relative numbers of substitutions, is more likely to give the correct tree than uniform weighting of characters in simulation studies (Williams & Fitch 1989, Fitch & Ye 1991). The method is tree-dependent however and results are sensitive to initial parameters of the tree (Williams & Fitch 1989). Tree-independent methods for taking account of sites of differing variability are currently being considered for implementation in HADTREE.

Evolution of the 12S rRNA Molecule

Knowledge of secondary structure is important for investigating the structural and functional basis of sequence variability between groups. The skink data set presented here is not a particularly useful one for examining variability of regions in relation to increasing taxonomic distance, since most of the taxa are equally divergent from each other and the majority of variable positions have only one or two taxa varying (Table 3.1). Skinks, xantusiids, ratites and mammals each showed similar patterns of variability however(Fig. 3.6), indicating that the skink molecule is not evolving in a different way than other 12S rRNA genes. In general, region S26 has a high proportion of variable sites in the vertebrates. The paired and unpaired regions around helices 43, 45 and 46 also accumulate many substitutions. In addition, it was suggested in Chapter Three that there may be taxon specific regions of variation. Skinks for example, had many variable in region L38' (Fig. 3.6, Table 3.1), whereas ratites, bovids, and the great apes had relatively more variability in SL40 (Figs. 3.6, 4.20). Further comparative sequence analyses for a larger vertebrate data set is required to determine how general these patterns of variability are. If taxon specific regions of variability do exist it may suggest differences in secondary structure and/or tertiary interactions among the vertebrates.

Comparative sequence studies form the basis for experimental investigations of the secondary structure (Woese *et al.* 1983, Noller 1984). Most of the experimental examinations of the small subunit rRNA have been directed at *E. coli* (reviews in Gutell *et al.* 1985, Hill *et al.* 1990), which has the advantages of ease of culturing and amenability to extensive genetic manipulations. The mitochondrial small subunit rRNA lack many of the prokaryote helices (see figure 3.1), and the functional and strutural significance of this is not well understood. The secondary structure of the region bounded by helix 36 requires further study (see Figs. 3.2, 3.3, 3.4), and experimental examinations of the 12S rRNA molecule are important in this respect (see Gutell *et al.* 1985, Hill *et al.* 1990).

In a more general context, the small subunit rRNA offers a good system with which to investigate molecular evolution. Functional rRNA genes occur in both the mitochondrial and nuclear genomes. Sequences of the nuclear small subunit (18S) gene are available for many groups (see de Rijk *et al.* 1992), and so detailed studies of the molecular evolution of the small subunit rRNA are possible. The mitochondrial and nuclear rRNA genes differ in size and evolve at different rates (Gutell *et al.* 1985, Mindell & Honeycutt 1990, Hillis & D~~avis~~ixon 1991). Refinement of secondary structure models for these genes permits patterns of sequence evolution to be related to the structure and function of the molecule. Studies of the evolution of the rRNA genes can also be related to tertiary interactions by investigations of the evolution of the ribosomal binding proteins. While the ribosomal proteins which interact with the nuclear and mitochondrial molecules are all encoded by nuclear genes, the mitochondrial ribosomal proteins are the products of a different set of genes than the nuclear ribosomal proteins. The mitochondrial ribosomal proteins also appear to be evolving more rapidly than their nuclear counterparts (O'Brien *et al.* 1985). Mitochondrial ribosomal proteins, and their genes, however have not been studied in as much detail as nuclear ribosomal proteins, which in turn are less well studied than the equivalent prokaryote proteins (Wool *et al.* 1990, Wittmann-Liebold *et al.* 1990).

Simulation Studies

There were two important results from the simulation study (Chapters Four and Five). The first was that a star tree (as used in the simulations) is a useful null model to investigate the skink diversification. This star model describes the pattern of transitions and transversions among the skinks very well (compare Figs. 4.2, & 4.9). Validity of the model was supported when corrections for invariant sites were made. In the skink data set 41% of the nucleotide substitutions were singletons (occurring on pendant edges). Assuming that all sites were free to vary in the initial simulation model resulted in approximately 60% of substitutions being singletons. When the adjustments to account for invariant sites were made (Chapter Five), 46% of the changes were singletons, agreeing well with the skink data. Further study of the applicability of this model is required however. For instance, the lengths of branches leading to skink taxa vary in size (see figures 5.3, 5.4, 5.18), which is not expected for a star tree model, unless there is variation in the rate of molecular evolution between the lineages.

The second important aspect of the simulation study relates to calculating sequence divergence from transversion substitutions. Saturation of transition nucleotide substitutions occurs with increasing genetic divergence (Brown *et al.* 1982, Wilson *et al.* 1985, Miyamoto & Boyle 1989), so transversions have been used to obtain estimates for the degree of sequence divergence (Miyamoto & Boyle 1989, Kraus & Miyamoto 1991, Milinkovitch *et al.* 1993). The preliminary analyses presented here indicate that such estimates will have a larger variance than estimates derived from total observed substitutions (Figs. 4.14, 4.15, 4.16). Inferences of divergence based on transversion substitutions must therefore be made with caution. Proper statistical analyses of the simulation data are required to investigate this problem in more detail. A binary tree model also needs to be simulated to assess the relevance of the results obtained from the star tree model.

The Hadamard Conjugation and Spectral Analysis

As the skink data set used in this thesis illustrates, molecular sequences contain much information. Development of more detailed mathematical models to examine the information still remains a major challenge. The Hadamard conjugation and spectral analysis provide a powerful tool for examining information within DNA sequences. Data sets such as the skink one have been important and influential in refining the algorithms. As demonstrated here, support for taxonomic associations and the strength of conflicting signals can be directly assessed using spectral analysis. The technique of forcing specific groups of taxa to be included in the tree (Fig. 5.16) is an important means for testing the likelihood of alternative hypotheses. Further refinements to the programs are being directed toward comparison of sub-optimal trees, so that the effects of conflicting signals in the data can be more accurately evaluated.

Incompletely Resolved Skink Phylogeny

The lack of phylogenetic resolution among the skinks was not simply due to there being too few variable or informative sites. Similar numbers of variable sites occur in the ratite data set of Cooper *et al.* 1992 (Table 4.5a), and a resolved tree is produced with this data set (Fig. 5.2a). Nor was the complexity of the skink spectrum (Fig. 5.1) an artifact related to the presence of either invariant or highly variable sites. Weighting or removal of some or all of these sites did not improve resolution (Figs. 5.9, 5.10). Simulation studies (Figs. 4.9, 4.13), as well as the bovid data set (Fig. 4.4b; Kraus & Miyamoto 1991, Allard *et al.* 1992), indicate that a star tree model of divergence may be a good first approximation for the evolution of the skinks. A star-like divergence of the *Leiolopisma* may also be supported by immunological data (Hutchinson *et al.* 1990). With such a model, the many conflicting signals in the skink data set are due to rapid diversification of the taxa, as occured in Pecoran bovids (Kraus & Miyamoto 1991, Allard *et al.* 1992). As noted above however, further testing of this and other models are required.

On the basis of the 12S rRNA sequence information the 17 New Zealand *Leiolopisma* examined in this thesis do not appear to have a recent and monophyletic origin in New Zealand. Strongest support in the sequence data is found for the grouping of five pairs of taxa (Fig. 5.5), implying five more recent speciation events. These taxa are: *L. microlepis* and *L. smithi*; *L. grande* and "Stewart Island Green"; *L. n. polychroma* and *L. n. nigriplantare*; *L. inconspicuum* and *L. notosaurus*; and *L. infrapunctatum* with *Lampropholis guichenoti* (Figs. 5.1, 5.5, & 5.18). A relatively close relationship between *L. fallai* and *C. aenea* was also suggested (Figs. 5.1, 5.16), but sequence similarities between *C. aenea* and the Mauritian *L. telfairi* (Fig. 5.15) indicate that the phylogenetic position of *Cyclodina* is still uncertain.

Hypotheses for the Origins of New Zealand *Leiolopisma*

The occurrence of *Leiolopisma* on islands in both the Indian and Pacific oceans, and their apparent divergence from each other at about the same time (Fig. 5.5) may reflect a Gondwanan origin for this group of skinks (Fig. 5.22a). Continental drift, rather than the skinks own powers of dispersal could account for their present distribution. The suggested close morphological similarity of the Fijian *L. alazon* to New Zealand *Cyclodina* (Zug 1985) is important in this respect since Fiji also appears to be a continental fragment (see Stevens 1980). Australian, New Zealand (*L. grande*, and *Cyclodina oliveri*), and New Caledonian (*L. nigrofasciolatum* and *Caledonescincus autrocaledonicus*) species all have similar immunological distances from *L. telfairi* (Hutchinson *et al.* 1990).

Two other hypotheses for the origin of *Leiolopisma* were presented in Chapter Five. One proposed that the New Zealand *Leiolopisma* are the result of diversification within New Zealand during the Miocene (Fig. 5.22b). Analysis of allozyme data provides support for this hypothesis (C.H. Daugherty & G.B. Patterson pers. comm.), but as discussed in Chapters Five and Six, the small number of electrophoretic loci which could be sampled may make the phylogenetic relationships derived from the skink allozyme data less reliable than the pattern of relationships based on the sequence data. The third hypothesis suggests that many of the New Zealand *Leiolopisma* are derived from several independent colonizations of New Zealand (Fig. 5.22c). Studies of the genetic variation of geckos on Pacific islands indicate that some species have only recently colonized some islands (Moritz *et al.* 1993), indicating that they have good oceanic dispersal abilities. Similar studies are required for the skinks. If New Zealand skinks did colonize New Zealand after the breakup of Gondwanaland, then the degree of differences between the 12S rRNA sequences of the New Zealand *Leiolopisma* indicates that several independent colonizations would have had to have occurred (Fig 5.22c), possibly across large tracts of open ocean. The fact that the sequences from the Australian and Mauritian species are no more distinct from the New Zealand data set than the sequences of New Zealand taxa are from each other (Table 5.3), makes this hypothesis less well supported than the other two hypotheses. The relative closeness of the relationship between the New Zealand *L. infrapunctatum* and the Australian *La. guichenoti* could however imply that there have been at least two colonizations of (or departures from) New Zealand by *Leiolopisma*.

Predictions of the Three Hypotheses

Hypothesis One: Gondwanan Origins

If there was rapid divergence of *Leiolopisma*-like skinks in Gondwanaland, then further immunological and sequencing studies of other *Leiolopisma*-like species are expected to show similar levels of immunological and sequence divergence among the taxa as Hutchinson *et al.* (1990) and this study have found. Analyses of sequence (and immunological) data from other groups of plants and animals with limited powers of dispersal, such as geckos and land molluscs, could also be expected to show that taxa on several different islands of continental origin diverged from each other at about the same time.

Hypothesis Two: Oligocene Drowning

If many of the New Zealand *Leiolopisma* speciated within New Zealand (Fig. 5.22b), then they should be more similar genetically to each other than to New Caledonian or Australian species. This hypothesis is not supported by the available 12S rRNA sequence data. Most New Zealand *Leiolopisma* skinks are as equally distinct from each other as they are from the Australian and Mauritian species (Tables 4.1a, 5.3). More Australian and New Caledonian species need to be examined though.

Initial analyses of allozyme data have been interpreted as indicating a diversification of *Leiolopisma* within New Zealand, with *La. guichenoti* and *L. telfairi* much more distantly related (C.H. Daugherty & G.B. Patterson pers. comm.). The different views of the evolutionary history of New Zealand skinks presented by allozyme data on the one hand and mitochondrial sequence data on the other may be a reflection of the small number of loci and sequence length examined in each case, as discussed in Chapter Six.

If the evolutionary relationships of New Zealand skinks are similar to those indicated by analyses of allozymes and the Oligocene drowning hypothesis (Fig. 5.22b), then both immunological studies and sequence analysis of nuclear genes should group New Zealand *Leiolopisma* together, distinct from other *Leiolopisma*.

Hypothesis Three: Multiple Colonists

The third hypothesis, proposing multiple colonization events in New Zealand, predicts that taxa in New Caledonia and/or Australia will have closer phylogenetic relationships to at least some New Zealand species than the New Zealand species have among themselves. The relatively close relationship between *L. infrapunctatum* and *La. guichenoti* gives support to this hypothesis. The proximity of New Caledonia to New Zealand suggests that New Zealand skinks should show genetic closer similarity to New Caledonian than Australian species, if they arrived by this route. If *Leiolopisma* did diversify in Gondwanaland, then a date of separation between the ancestors of *L. infrapunctatum* and *La. guichenoti* of approximately 40 million years is implied (Chapter Five). Land connections between New Caledonia and New Zealand were more extensive during this period (Suggate *et al.* 1978), so DNA sequence studies of New Caledonian skinks may reveal taxa there with close relationships to both *L. infrapunctatum* and *La. guichenoti*.

Testing the Hypotheses: What Other Skinks to Sample?

More Pacific skinks need to be examined to test all three hypotheses. Several New Zealand skinks also have still to be studied at the DNA level. These include *L. waimatense* (South Island), *L. homalonotum* (Poor Knights Islands), and more members of the South Island *L. lineoocellatum-L. chloronotum* complex, as well as several as yet undescribed species. Sequence studies of *Cyclodina* are already underway. The most critical overseas species to examine are *L. alazon* (Fiji), *L. lichenigerum* (Lord Howe), *Leiolopisma* species from New Caledonia (four are described; Sadlier 1986), and Australian species of the *Leiolopisma* group (which are now being reassigned to several new genera; Hutchinson *et al.* 1990). Other groups of skinks also need to be sampled to determine whether there has been more widespread skink diversification. Australia has about 30 genera of skinks (Cogger 1986), New Caledonia has ten (Sadlier 1986), New Guinea about sixteen (Allison 1982), and many more genera and species are distributed throughout the Pacific and Asia (Greer 1974, 1979). Of particular interest would be examination of other members of the *Eugongylus* group, such as *Emoia* and *Morethia*, and the New Caledonian genera *Tropidoscincus*, *Geoscincus*, *Phoboscincus*, and others (Sadlier 1986). Other genera within Lygosominae, for example *Notoscincus* (Australia), and *Mabuya*, a predominantly Asian group which Greer (1979) regards as the stock for the Lygosominae, could also be investigated. *Emoia* (which is distributed across the Pacific), is not too distant, immunologically, from the *Leiolopisma* (Hutchinson *et al.* 1990).

Testing the Hypotheses: What Other Genes to Sample?

Maternal inheritance, lack of recombination, and the effectively haploid state of mitochondria (Brown 1985, Wilson *et al.* 1985, Moritz *et al.* 1987) make mtDNA a simpler system than the nuclear genome to investigate at the DNA sequence level. It may not be suitable however for investigating the deep branches in the skinks. The 12S rRNA sequence data for *Leiolopisma* is notable for the many conflicting phylogenetic signals it contains (Fig. 5.1). Resolution of the evolutionary relationships of the *Leiolopisma* may therefore require examination of more conservative sequences than domain III of 12S rRNA, as is also required for the Pecoran bovid phylogeny (Kraus & Miyamoto 1991). Analysis of the complete 12S rRNA gene may provide greater taxonomic resolution and, as discussed above, is important for more general studies of molecular evolution. Cloning of the skink mitochondrial genome is underway and sequencing of the 12S rRNA gene is one of the first objectives. The mitochondrial large subunit (16S) rRNA may be another suitable candidate gene for examining the relationships of more distantly related taxa, though alignment of mammalian sequences in the GenBank database (data not shown) suggest that it changes at a similar rate as the mitochondrial small subunit gene, so it may not have greater resolving power for deeper branches in the evolutionary tree.

Other mitochondrial genes, such as cytochrome oxidase I and cytochrome *b*, are widely used, and useful, for phylogenetic comparisons (for example, Kocher *et al.* 1989, Irwin *et al.* 1991, Palumbi *et al.* 1991, Simon *et al.* 1991, Smith & Patton 1991, Pashley & Ke 1992). They tend to have more rapid rates of change than the rRNA genes though (Moritz *et al.* 1987), and so may be inadequate to resolve the deep branching patterns of skink relationships. However, the limited cytochrome *b* data for the skinks (Chapter Five, and Appendix 1), indicates that most of the differences between the skink sequences occur in third codon positions. If there are too many conflicting signals from third codon position sites, analysis of the more conservative first and second codon positions may provide greater phylogenetic resolution. Third codon positions may be useful for studying the relationships between more closely related taxa, such as *L. microlepis* and *L. smithi*. As shown in Chapter Five, the cytochrome *b* sequence did differentiate between the Gorge Burn *L. maccanni* and the putative hybrid (Table 5.4).

Consistent amplification of the cytochrome *b* gene sequence could not be achieved using the cytochrome *b* universal PCR primers of Kocher *et al.* (1989; see Chapter Two). A more efficient, and ultimately more useful, strategy would be to clone and sequence the skink mitochondrial genome, and this is currently being attempted. Suitable PCR primers appropriate for various taxonomic or population comparisons can then be designed. Few molecular sequence studies of reptiles have been undertaken and the organization of the reptilian mitochondrial genome is not known, so such a project has general utility. Mitochondrial gene organization differs between birds, marsupials and placental mammals (Desjardins & Morais 1990, Pääbo *et al.* 1991), and knowledge of gene order in reptile mtDNA may help resolve the relationships among reptiles, birds and mammals (see Chapter Two, and Marshall 1992).

Investigation of Nuclear Gene Sequences

More conserved sequences for studying the branching order of the skinks can be obtained from nuclear genes, and analysis of nuclear rRNA and their intragenic spacer regions may be the most useful. PCR primers for some of these genes (e.g., 18S rRNA), and which work for a range of vertebrates, have already been designed (Hillis & Dixon 1991). As noted above, analysis of the nuclear 18S rRNA gene (the equivalent of the mitochondrial 12S rRNA molecule) would also complement molecular evolutionary studies of the 12S rRNA gene.

Examination of the phylogenetic relationships and evolutionary histories of skinks using sequences from nuclear genes is also important because of the apparent conflicts between the mitochondrial and allozyme studies. Investigation of nuclear protein-encoding genes in this regard would be informative. It would be particularly interesting to obtain sequence data from some of the loci used in the skink allozyme survey. Phosphoglucomutase, for example, had the largest number of alleles among the skinks (ten; C.H. Daugherty & G.B. Patterson pers. comm.), and may contain sufficient variable nucleotide sites to be phylogenetically informative. Specific PCR amplification of introns by using PCR primers which anneal to conserved exon sequences may be the most suitable method to achieve this (Palumbi & Baker 1993, submitted). Glucose-6-phosphate dehydrogenase has received close molecular genetic scrutiny in other studies (Vulliamy *et al.* 1992) and is another potential nuclear gene to be considered for study in the skinks (six alleles were detected for this locus by C.H. Daugherty & G.B. Patterson). If the intron sequences are evolving rapidly then they may only be useful for resolving more recent divergences (for example, the separation of *L. n. polychroma* and *L. n. nigriplantare*, *L. microlepis* and *L. smithi*, and possibly the hybrid and parental populations at Gorge Burn).

Hybridization between *L. n. polychroma* and *L. maccanni*

Hybridization has been considered to have an important role in the evolution of plants (Cockayne 1911, Rattenbury 1962, Grant 1981, Arnold 1992), but it's significance in animals may be underrated (Mayr 1963, Arnold 1992, Grant & Grant 1992, Dowling & DeMarais 1993). Dowling & DeMarais (1993) investigated hybridization among freshwater fish in the genus *Gila*, and suggested that interspecific hybridization has been a major factor in generating morphological and genetic diversity. Interspecific hybridization may also be common among some groups of birds, and hybrids may, temporarily at least, have greater reproductive success (Grant & Grant 1992). Hybridization between *Leiolopisma* therefore requires much closer examination.

The three nucleotide differences between *L. maccanni* and the *L. n. polychroma* cytochrome *b* sequences from Gorge Burn suggest hybridization may have occurred in the Pliocene, over a million years ago (Chapter Six). That hybridization was detected by examination of only a few individuals indicates that hybridization may be relatively common. Whether there is a hybrid zone (Barton & Hewitt 1989) or just a localized introgression requires further examination of individuals from Gorge Burn and adjacent populations (see Patterson 1985, Daugherty *et al.* 1990b). Patterson's (1985) study of the ecology of the Central Otago skinks is the most comprehensive one of the group so far, but the discovery of hybrid skinks emphasises the need for additional detailed ecological studies of both hybrid and non-hybrid populations to determine the behavioural and ecological features which usually keep them from interbreeding.

Of more general significance is the fact that hybridization between the skinks can occur despite tens of millions of years separation from a common ancestor. Reports of naturally occuring hybridization between taxa which diverged more than five million years ago are uncommon in the literature (see Chapter Six), though frogs which diverged up to 35 million years ago have the potential to hybridize (Wilson *et al.* 1974). As discussed above, it is important to obtain more refined estimates for the divergence times of the skinks so that their ability to hybridise can be more accurately related to their degree of divergence.

Ecological studies to investigate the viability and reproductive success of the skink hybrids (see Barton & Hewitt 1989, Grant & Grant 1982) are an essential complement to the genetic analyses. Captive breeding of skinks is a means to examine the potential for interspecific hybridization and the viability and competitive ability of hybrids. Such experimental studies are essential for an understanding of the processes of speciation (Endler 1986, Coyne 1992).

The Potential of *L. n. polychroma* For Population Studies

A strong emphasis of this thesis has been to bring together ecological and evolutionary studies in New Zealand. *L. n. polychroma*, and other New Zealand skinks, offer many opportunities for such research. *L. n. polychroma* is a wide ranging species, encompassing six degrees of latitude (from the central North Island to Southland), and living in a variety of habitats from sea level to 1700 m (Pickard & Towns 1988, Patterson & Daugherty 1990). Wide-ranging and common species are often considered to be more variable (Wallace 1889, Mayr 1963) but genetic evidence in support of this is meagre. Reduced phenotypic variability has also been noted for populations at the geographic or ecological margins of a species' range (Mayr 1963, Lewontin 1974, Brussard 1984). Studies of genetic variation within and between populations of ecologically and geographically distinct *L. n. polychroma* may be informative in this respect.

Analyses of the sequence data (Fig. 5.18) led to the suggestion in Chapter Five that *L. n. polychroma* has an origin in Southern New Zealand. It now inhabits many areas considered to have been glaciated or ecologically depauperate during the Pleistocene (see Fleming 1980, Stevens 1980). C.H. Daugherty (pers. comm.) noted clines in allele frequencies for *L. n. polychroma*, and investigation of sequence differences between populations may permit examination of the origins and the directions and timings of dispersal of *L. n. polychroma*. If current distribution is due in part to post Pleistocene dispersal then sequence analysis of the most variable region of mtDNA, the D-loop (Moritz *et al.* 1987), could be suitable for studying this (see for example, Smith & Patton 1990, Thomas *et al.* 1990, Ward *et al.* 1991). Chromosomal studies (see Hillis & Moritz 1990) and DNA fingerprinting (Jeffreys *et al.* 1991) may also be informative for interpopulation differences.

Rates of Molecular and Morphological Evolution in Skinks

A Gondwanan origin for *Leiolopisma* skinks implies a low rate of sequence evolution (25 to 30 substitutions (Table 4.1a) per 80 MY, or 0.3 to 0.375 substitutions per MY). The ratites have approximately 0.5 substitutions/MY, and bovids a rate of 1.2/MY (Table 4.6). A slower rate of molecular evolution in lizards than in birds or mammals may be related to a lower metabolic rate in reptiles (Martin & Palumbi submitted), though the xantusiid lizards may have a high rate of mutation (Table 4.6; Hedges *et al.* 1991). A low metabolic rate may result in both less oxidative damage to DNA (Richter *et al.* 1988, Shigenaga *et al.* 1989, Martin & Palumbi submitted) and slower turnover of DNA, and consequently slower rates of mtDNA change (Martin & Palumbi submitted). Non-invasive analysis of oxidative damage is now possible using HPLC (Shigenaga *et al.* 1992), so more detailed examinations of the relationship between metabolic rate and degree of DNA damage may be feasible.

A Gondwanan, or even Miocene (15-25 MYA), origin of the skinks also suggests a low rate of morphological change in the skinks. Marked differences in rates of morphological and genetic change are well documented (Wilson *et al.* 1974, King & Wilson 1975, Cherry *et al.* 1978, Wayne *et al.* 1989). The lack of morphological diversification in New Zealand skinks is unusual however both with respect to their ecological and latitudinal ranges (Bull & Whitaker 1975, Pickard & Towns 1988, Daugherty *et* *al.* 1990b), and their suggested period of separation from each other. ~~Their morphological uniformity may however be related to ecological features.~~ Australian skinks are morphologically very diverse (Ricklefs *et al.* 1981), and this may be due to a combination of factors, such as habitation in infertile and arid environments, which have few avian or mammalian predators, and the abundance of termites as a food source (Morton & James 1988). New Zealand geckos are morphologically more varied than skinks, though the degree of genetic divergence among geckos appears to be smaller than for skinks (R. Hitchmough pers. comm.). A combination of exploitation of forest habitats and adoption of nocturnal lifestyles by some New Zealand geckos may have been a contributory factor in their greater morphological diversity (R. Hitchmough pers. comm.).

Perhaps, as in the case of African jackals (Wayne *et al.* 1989), an open and fertile grassland habitat, and potential abundance of prey may reduce both interspecific competition and development of morphological divergence among the New Zealand skinks. The existence of sympatric species of skinks (Patterson 1985, Daugherty *et al.* 1990b) however suggest that subtle microhabitat differentiation and behavioural features must be important for maintaining separate gene pools. Some of the sequence data presented here indicates that such differentiation may break down occasionally.

Evolutionary Investigations of New Zealand Biota

Many of the problems which perplexed early biologists in New Zealand are still unresolved. The chief difficulty has been accurate determination of evolutionary relationships among groups. Biochemical and molecular approaches, as demonstrated here and elsewhere (Daugherty *et al.* 1982, Green *et al.* 1989, Daugherty *et al.* 1990a,b, Cooper *et al.* 1992) are essential for evolutionary investigations of New Zealand's biota.

As aspects of the present study, and those of Patterson (1985) and Daugherty *et al.* (1990b) have shown, evolutionary and ecological questions are linked. The coordination of ecological and evolutionary studies of New Zealand skinks provide general insights into both the biological and geological history of New Zealand, as well as processes of molecular and organismal evolution.

Application of molecular techniques to evolutionary questions has contributed much to an understanding of phylogeny (Wilson *et al.* 1985, Hillis 1987, Patterson 1987, Wilson *et al.* 1989, Moritz & Hillis 1990). They have been helpful not only in resolving relationships which have been difficult to address by other means, but can also provide a more reliable indicator of the degree of phylogenetic divergence (for example, King & Wilson 1975, Roberts & Maxson 1985, Wayne *et al.* 1989, Meyer *et al.* 1990, Martin *et al.* 1993, see also Hillis 1987, Moritz & Hillis 1990). Molecular systematics has lead to a renaissance in morphological taxonomy (Patterson 1987), and satisfyingly, molecular and morphological phylogenies are more often in agreement than conflict (Hillis 1987, Moritz & Hillis 1990). Where morphological and molecular data are in apparent conflict (for example with the mammals; Wyss *et al.* 1987, Gauthier *et al.* 1988, Hedges *et al.* 1991, Marshall 1992), emphasis should be on examination of the advantages and limitations of each data set, rather than which data set is 'right' (Moritz & Hillis 1990).

Phylogenetic trees should be regarded as hypotheses, and steps along the way to understanding evolutionary processes. Mayr's observation (1963, page 9) that ...

> "... *even today there still are some zoologists to whom the term 'evolution' signifies little more than the determination of homologies, common ancestors, and phylogenetic trees.*"

should not be forgotten.

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