Genetic and Phenotypic Lineages in Neogastropod Molluscs: A Journey Through Time and Morphospace

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For Arlo and Lillian

Tree space

We enter at night and the moon which was round and falls in thin
oblongs now.

Not a forest but the forest and the infinite which is another word for
indefinite.

In the dark birds are heavier and we can hear the small valleys of
their foot falls.

It's true that death and life smell the same here but all we have to
do is find the most likely tree of all possible trees.

We must compare our tree to the tree next to it and, if necessary,
leap like a sugar glider to the more likely tree.

Or we swap branches with it and then decide.

We do this a million times, a million more. The membrane
between our legs is almost a wing, almost flight.

It is possible to get stranded on small hillocks of quite likelihood
and birds may follow you and disturb the mud and in damp places.

Minute slime moulds may congregate together and become a single
slug that rises up on its tail and stands as a flower.

In brilliant colour. We see things illuminated where they should
not be and lie and stare into the small grotto.

Made by the arch of an ancient tree and cannot figure.

To understand tree space you must search all tree space which is
impossible.

There you go. You can climb hills and holler all you like. You and
your Nearest Neighbour Interchange

What are the chances I say and you say we never did but when we
stood up we too were glowing.

Maria McMillan

Abstract

Extant and fossil snail shells provide important insight into the morphological evolution of species allowing assessment of evolutionary mode within lineages. Recognising biologically distinct groups is problematic when only shell characters are available on which to base decisions, as is the case with fossil material. Therefore, the concordance of evolutionary lineages inferred by different methods need to be confirmed before evolutionary patterns and models can be tested. Recent advances in techniques and technology enabling more detailed investigations into both morphologic and genetic variation is helping to recognise taxonomic groupings that reflect evolutionary relationships. Genetic tools may provide the most accurate description of evolutionary relationships among taxa and morphologic tools can be used to investigate evolutionary patterns extending into the fossil record. These two methods have the ability to both complement and challenge one another. In this thesis I have generated genetic data using high throughput DNA sequencing and amplicon sequences. This provides a range of data from whole mitochondrial genomes through to nuclear single nucleotide polymorphisms to help clarify genetic clusters and build phylogenies. The morphologic data was generated using two dimensional landmark morphometric tools allowing empirical comparison of complex shell shapes without the confounding effects of size. By separating the variation among snail shells into uncorrelated variables (principal components) model-based Bayesian assignment analyses provide an unbiased tool to cluster specimens without priori hypotheses. This combination of new methods to both describe shape variation and determine the most efficient model to group specimens greatly improves studies of natural phenotypic variation and identification of evolutionary lineages. I have used these approaches to examine

morphologic and genetic variation in three genera of Neogastropod molluscs from New Zealand to clarify species boundaries and test evolutionary theory.

Variation in snail shell shape has provided evolutionary biologists with excellent material for the study of local adaptation to local environments. However, treating shell shape variation as evidence of isolated lineages (species) may have led to taxonomic inflation within some gastropod lineages. Here I compare shell shape variation and genetic structure of rocky shore whelks, *Buccinulum*, from the coast of New Zealand. The species *B. vittatum* is split into two subspecies, *B. vittatum vittatum* and *B. vittatum littorinoides*, and is interposed geographically by the species *B. colensoi.* The current taxonomy based primarily on shell morphology, is in conflict with results obtained from genetic data from both amplicon sequencing and next generation sequencing. I found that *B. colensoi* and *B. vittatum littorinoides* form a genetic cluster distinct from *B. vittatum.* I conclude that the shell shape variation associated with *B. colensoi* would be better viewed as an ecotype of *B. v. littorinoides,* and the northern taxa as a separate genotypic cluster. As the ecotype *B. colensoi* is not genetically isolated from *B. v. littorinoides,* this suggests selection is stronger than gene flow.

Punctuated equilibrium encompasses both morphological stasis and rapid morphological change. The period of rapid morphological change is linked to the process of lineages splitting to increase taxon diversity (speciation). In order to go some way towards testing the theory of punctuated equilibrium a number of elements within a taxon should be resolved: (1) the biological reality of fossil "species" which can be assessed via examination of traits in living relatives (2) the elimination of biological invasion that might be mistaken for speciation,

which can be achieved if monophyly of the group is established. (3) a period of morphological stasis separate from speciation events. The olive shells (*Amalda*) are a speciose genus of Neogastropod mollusc with an extensive distribution. In New Zealand they have an excellent fossil record and have been presented as support for biologically distinct extant and fossil lineages, and morphological stasis in the fossil record, two prerequisites for punctuated equilibrium.

I tested the hypothesis of New Zealand monophyly of *Amalda* using long DNA sequences from both mitochondrial (whole mitogenome) and nuclear (45S rDNA) markers from 14 *Amalda* species collected from New Zealand and around the world. Genomic data was generated with high throughput sequencing and whole mitogenome and 45S alignments were assembled from short reads. The reconstructed phylogenies from both the mitochondrial and nuclear DNA sequences support the hypothesis that the New Zealand *Amalda* are a monophyletic group and the extant species evolved in New Zealand waters in isolation.

Within the olive shells (*Amalda)* in New Zealand there is strong concordance between the accepted taxonomy in the extant taxa and genetic data generated with amplicon and next generation sequencing. The recognised species show distinct genetic clustering associated with morphologic clustering based on two dimensional morphometrics. There is some overlap of morphologic clusters as is to be expected within such a morphologically homogeneous genus. There is a comprehensive fossil record for *Amalda* in New Zealand providing the means to test fundamental evolutionary theories and recent improvements in dating fossil horizons allows incorporation of better data. By using a morphometric data set including fossil representatives of three extant species I show that the species in the fossil record represent lineages with the modern fauna at the tips enabling the testing of evolutionary models. Three candidate evolutionary explanations were represented as statistical models, which were then evaluated using likelihood-based inference. Mixed models showing both stasis and unbiased random walk were best fit for phenotypic traits in all three species. Having shown that stasis in the fossil record is temporally separated from speciation events in distinct lineages some of the prerequisite conditions for the evolutionary theory of punctuated equilibrium have been met.

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Chapter 1

General Introduction

Evolutionary units

Our understanding of how evolution works is constantly changing. New theories are formulated and new methods for testing them are developed, each refining and providing more detail to the story of how the diversity of life on earth arose (Eldredge and Gould, 1972; Fisher, 1930; Mallet, 2008). At the centre of this is the species, widely viewed as one of the fundamental units in evolutionary biology and considered central to evolutionary thinking, yet how species are defined is highly contentious (de Queiroz, 2007; Hausdorf, 2011). Clarity of taxonomy is vitally important when testing theories of biodiversity evolution. Recognising a species is often intuitive; “the opinion of naturalists having sound judgment and wide experience seems the only guide to follow” (Darwin, 1859). However, as more sophisticated tools become available and investigations involve larger data sets a more empirical approach seems possible and necessary. Numerous species concepts rely on interpretations of a wide range of data including behavioural, geographic, morphological, phylogenetic, ecological, and genetic (Hey, 2006; Sites and Marshall, 2003). All species delimitation methods rely on identifying independent clusters of individuals that have some measure of affinity with the underlying expectation of a “separately evolving metapopulation” (de Queiroz, 2007). Compatible with the meta-population approach to species is that they can be recognised as clusters of individuals that are in some way separate from other such clusters, with few intermediates (Mallet, 1995). In practice, this often means genetic clusters as recognised using phylogenetic tools but can also apply to other data clusters such as morphologic traits from fossils.

Species can be considered parts continuous lineages that extend into the deep past. Modern species representing the tips of the lineages and fossil species temporal sections of these lineages. How lineages are defined remains contentious because the process of evolution will blur the boundaries between lineages (e.g. by divergence, extinction, and hybridisation). This is perhaps most problematic when investigating the fossil record in which the temporal dimension is added to the species boundaries. With fossil species the boundary in time is often chosen arbitrarily, maybe influenced by breaks in the fossil record. As one tries to delineate taxa among closely related lineages the boundaries become less clear as the mechanics of evolution including hybridisation run counter to species definitions (Vaux et al., 2016a). When defining species in the fossil record the character set is restricted to body parts that are preserved most consistently and often there is a scarcity of samples on which to base species delimitation decisions. In some cases as little as a single tooth or femur can be the only evidence (e.g. Worthy et al., 2006), but perhaps here it is finding difference between taxa rather than similarities that is important. Fossil species need to be defined within a morphologic species concept as little if any information is likely to remain regarding their soft tissue, sexual dimorphism, colour, smell, behaviour, or palaeoenvironment that could be informative about species composition.

Inferring the relationships among lineages being studied is important when trying to understand fundamental aspects of their evolution. In this thesis I use the approach advocated by Mallet (1995) that species names are hypotheses that can be tested but species boundaries should be identified independently of the data used to understand how they arose (speciation). So I recognize species as distinct genetic clusters with few intermediates and that the genetic clusters are associated with clusters of biological traits. In the context of this study is the biological trait of intrest is morphology of shell shape. My analysis of evolutionary trends in the palaeontological record is less concerned with identification of species rather, it is more important that what is being regarded as a continuous lineage is indeed so. In this study identification of species is helpful because I am starting with the hypothesis that the species in the modern fauna are clusters representing the tips of lineages that extend deep into the fossil record. The use of genetic tools and independent traits for different aspects of the study ensure that there is separation of the information used to identify lineages and those used to draw inferences about their evolution through time and morphospace.

Recognising genetic lineages

If taxonomic units do not reflect evolutionary lineages our inferences based on those units are unreliable. Taxonomists studying extant taxa have at their disposal a greater array of characters on which to base their classification than do palaeontologists. Understanding species diversity through time is an integral part in the understanding of evolution and how species concepts are applied can greatly impact estimates of diversity (Aze et al., 2013; Barrowclough et al., 2016). Molecular techniques go some way to providing definitive taxonomic relationships among extant taxa, but evolution is a process in which divergence and gene flow are not mutually exclusive (e.g. Dowle et al., 2014; Stankowski, 2013). How clearly lineages are partitioned genetically, can be explored through multi-locus techniques providing some robustness in inferring species trees despite potentially conflicting gene trees (Spinks et al., 2014). Techniques for generating data that can be used to identify genetic clusters have advanced rapidly from allozymes through DNA restriction site cutting, DNA sequencing and now high throughput sequencing. These tools provide resources to infer evolutionary relationships with increasing detail on many scales. Many studies of extant species provide examples in which established taxonomies, generally based on morphology, are overturned by robust molecular analysis. A goal of systematics is determining monophyletic clusters. It is expected that nested sets of genotypic clusters result in monophyletic groups which are best for explaining evolutionary relationships, but there is contention about the treatment of paraphyletic and polyphyletic groups, regarding whether they can be helpful when examining biodiversity despite not being complete evolutionary units (e.g. Brummitt, 2002; Ebach et al., 2006).

Recognising lineages using morphology

The role of morphology in taxonomy is changing from being the primary source of information regarding evolution to a role more commonly associated with species identification only. Morphology can often separate species but phylogenetic relationships are not so easily resolved (Hills et al., 2012; Michaux, 1989). Morphological differentiation between species can be problematic in cases of cryptic or polymorphic species. However, new methods and increased computational power have enabled more intensive analysis of morphological data allowing the inclusion of continuous features rather than reliance on discrete characters (Felsenstein, 2002). Two-dimensional landmark based geometric morphometric techniques have improved, allowing empirical comparison of complex shell shapes (Bookstein, 1996). By separating the variation within a dataset into linearly uncorrelated variables (principal components), model-based Bayesian cluster analyses provide an unbiased tool to classify specimens without a priori hypotheses. This combination of new methods to both describe shape variation and determine the most efficient model to group specimens greatly improves studies of natural phenotypic variation. Extracting accurate and detailed measures of morphological variation within species is important for 1. resolving taxonomic differences to define species boundaries and if possible find diagnostic characteristics, 2. Describing the material on which evolution operates and 3. Reconciling different inferences from genetic and morphological data.

Environmental conditions play an important role in the evolution of morphology. In marine systems natural selection drives phenotypic adaption to particular conditions often leading to similarity among taxa. For example, the streamlined body shape of tuna, penguins, sharks and dolphins are considered to be convergent adaptations for efficient movement through water. Closely related specis may be morphologically criptic, which is a source of difficulty when defining species boundaries and identifying diagnostic traits. Convergence of shell shape is a common feature of marine gastropods. (Allmon and Smith, 2011).

If species inhabit a range of ecological conditions this can drive polymorphism and development of ecotypes (Baker et al., 2004; Palmer, 1990) leading to erroneous taxonomic splitting of a lineage (Aze et al., 2013; Hills et al., 2012; Olabarria and Thurston, 2004). It is also possible that ecotypes could represent incipient speciation too early for complete lineage sorting to have occurred. Disparities between taxonomic identification and evolutionary relationships have become more apparent as molecular data are used to test taxonomic hypotheses. Although species recognised in the fossil record are treated as proxies for evolutionary lineages it is acknowledged that morphology is not always concordant with genotypic clusters or biological species in the extant fauna (Hills et al., 2012; Jablonski, 2000; Morlon et al., 2011). The use of a combined molecular and morphometric approach can help resolve both crypsis and intraspecific polymorphism leading to more reliable taxonomic and evolutionary inferences.

Speciation: rates, duration and dates

Speciation is usually not a discrete event (exceptions being polyploidy and hybrid speciation) but an accumulation of differences over time, which at some point, is sufficient to separate a single lineage into two or more lineages (de Queiroz, 2005; Hills et al., 2012). Speciation may take thousands of generations (Barluenga et al., 2006; Hunt, 2008; McKinnon and Rundle, 2002) but reproductive isolation may occur much more quickly than that (Hendry et al., 2007). Gould and Eldredge (1977) supposed that speciation took 5,000–50,000 years and that species' duration was around 10 million years. Allmon and Smith (2011) calculated that on average a marine gastropod lineage would exist for around 5–15 million years and the process of speciation may occur in less than 100,000 years. Determining speciation rates is generally a palaeontological exercise needing well-dated fossil beds that provide examples of form change. Molecular genetic tools are also capable of providing estimates of speciation rates and timing (Cranston and Rannala, 2005; Heath, 2012; Morlon, 2014) but cannot include data from extinct species. There have been advances in combining the two approaches to provide better estimates of diversification over time (Heath et al., 2014; Hunt, 2008; Hunt and Slater, 2016; Slater et al., 2012). Accurate estimates of genetic mutation and substitution rates, and fossil sampling probabilities, which temporally constrain species lineage durations, are vital for dating speciation. The validity of molecular dating methods needs verification to ensure independence of calibration points and dates of speciation inferred and to avoid circularity.

Darwin (1859) recognised the paucity of the fossil record and the limitations this posed on investigations. As the fossil record has been increasingly explored, species histories have become clearer with improved dating of fossils and depositional environments. This has contributed to a clearer picture of the tempo and mode of evolution. Simpson (1944) introduced the idea of tempo and mode, the rate and descriptions of the way evolutionary changes occur, into the consciousness of evolutionary biologists. Ideas about genetic processes that were understood in modern organisims were integrated into theories about the fossil record as a way to explain the processes influencing the changes seen. Still with more detailed understanding of evolutionary theory and better fossil collections the lack of transitional forms remained apparent. This led to the genesis of the idea that stable species form was the normal condition and this was incorporated into the theory of punctuated equilibrium (PE) (Eldredge and Gould, 1972). The concept, a description of evolutionary patterns, primarily interprets the fossil record as mostly consisting of long periods of little morphological change without lineage splitting. Interspersed within this were short periods of rapid morphological change associated with speciation. Eldredge & Gould (1972) suggested that transitional forms were only present for short geologic duration in the lifetime of a species and therefore largely absent from the fossil record. Most of the time, species were morphologically stable (stasis). This theory sent ripples through the evolutionary community that are still felt today. This idea of a series of (geologically) abrupt evolutionary steps ran counter to the long held presumption that evolution was a gradual process with small changes accumulating over time.

Models of morphological evolution

In trying to explain models of evolution, approaches to describing pattern gradually coalesced around three ideas; random walk, gradualism and stasis. These models broadly correspond to ideas of how selection pressures act on species and therefore form a good basis for understanding evolutionary processes. How well the evolution of a species in the fossil record was to fit one of the models was initially a subjective measure leaving the potential for different interpretations. Statistical models have recently been incorporated into analyses to explicitly test how well morphological data fit each of these three models of evolution (Adams and Otárola-Castillo, 2013; Hunt, 2007, 2006; Hunt et al., 2015). For a comprehensive review of evolutionary models see Hunt and Rabosky (2014).

Phyletic gradualism

The classical understanding of morphological evolution, consisting of slow accumulation of changes leading to a progression of shape change and recognisably different forms through time. Here the evolutionary steps are not random and are weighted in a particular phenotypic direction. Over time these steps lead to a phenotypic mean shifting in the direction of the weighting. This is the expected outcome of directional selection. Evolutionary pressure acting continuously on a trait leads to the accumulation of change. Having once been thought of as a common mode of evolution the notion of phyletic gradualism (or directional evolution) is now increasingly recognised as relatively rare in the fossil record (Benton and Pearson, 2001; Hallam, 1997; Hopkins and Lidgard, 2012; Hunt, 2007; Hunt et al., 2015; Jackson and Cheetham, 1999).

Random walk

The process of random walk in evolutionary terms involves each generation taking a phenotypic evolutionary step from the previous generation with random directionality. The size of the step is constrained by what is biologically possible but there are no bounds on the direction. This is an extension of Brownian motion. Evolutionary form can wander through morphospace, with shape changes accumulating and/or being lost through time. The simplest evolutionary explanation is an absence of constraining selection or drift (Hunt, 2006).

Stasis

Stasis implies no change, but in real world situations some amount of variation occurs. When this change is distributed around a constant mean over time then statistically it is considered to equate to morphological stasis. Within this model evolutionary change can occur but as steps are taken away from the ideal or optimal phenotype then it becomes increasingly likely that subsequent steps will be back towards the mean. These steps approximate a Gaussian distribution around a constant mean. (Hunt and Rabosky, 2014). The pattern of stasis is easily accounted for by stabilising selection. When traits are well adapted to stable conditions, movement away has negative fitness consequences (Charlesworth et al., 1982; Estes and Arnold, 2007; Haller and Hendry, 2014; Lieberman and Dudgeon, 1996). But there is also the contention that species inhabiting dynamic environments should show stasis, a reflection of the adaptability to be able to cope with changing environments (Grey et al., 2012; Sheldon, 1996) in contrast to the idea that stasis should arise only in stable environments (Lieberman and Dudgeon, 1996). Gene flow between populations is another likely explanation for stasis because it could help maintain a static mean across a species by preventing divergence between populations facing different selective pressures (Eldredge et al., 2005; Hansen and Houle, 2004; Lieberman and Dudgeon, 1996). Another possibility is the contest as evolutionary pressures come up against biological limits and directional evolution cannot continue. Stasis in the fossil record is the foundation of the theory of punctuated equilibrium, and the predominance of morphological stasis in the fossil record is predicted and explained by the theory.

Distinguishig between changes occurring within a lineage and those between divergent lineages is important for inferences about evolutionary modes (Ezard et al., 2013; Hunt, 2013; Ingram, 2011; Mattila and Bokma, 2008). Conceptually differentiating between anagenesis and cladogensis is fraught with problems. There are many factors that are open to interpretation and so a clear separation is not always easy to determine (Aze et al., 2013; Cheetham et al., 1994; Strotz and Allen, 2013; Vaux et al., 2016b, 2016a). Are changes more closely associated with time or with speciation events? In the case of punctuated equilibrium, we are largely interested in the divergence of lineages but it may be possible to explain multiple changes within a lineage as part of a PE speciation event.

Thesis outline

Variation in snail shell shape has provided evolutionary biologists with excellent material for the study of local adaptation to local environments and broader theories on evolutionary processes. However, treating shell shape variation as evidence of isolated lineages (species) may have led to incorrect taxonomic assignments within some gastropod lineages which can undermine inferences based on assumptions of morphospecies reflecting true evolutionary relationships. Being able to examine morphologic and genetic variation together down to population level provides a way of clarifying evolutionary relationships highlighting any disassociation between the two. Once species have been confirmed a better understanding of the processes influencing species' phenotype can be made. The role of adaptation on phenotype can be examined along with evolutionary theories based on morphologic changes in lineages through time.

There were two primary goals of this research project; firstly, to examine the genetic diversity and the phenotypic diversity of marine snails around New Zealand, in particular two groups of species in the genera *Buccinulum* (chapter 2) and the *Amalda* (chapter 3) , secondly, to explore models of evolution within a punctuated equilibrium framework in the *Amalda*, making use of its extensive fossil record (chapter 5).

Punctuated equilibrium encompasses both morphological stasis and rapid morphological change. The period of rapid morphological change is linked to the process of lineages splitting to increase taxon diversity (speciation). In order to go some way towards testing the theory of punctuated equilibrium a number of elements within a taxon should be resolved: (1) the biological reality of fossil "species" which can be assessed via examination of traits in living relatives (2) the elimination of biological invasion that might be mistaken for speciation, which can be achieved if monophyly of the group is established. (3) a period of morphological stasis separate from speciation events.

Chapter One

In this present chapter I examine the broader concepts that are important in exploring the evolution of species. Species concepts are discussed with reference to the methods used in the study. Aspects of evolutionary theory are then discussed with particular focus on punctuated equilibrium, and how the fossil record can be used to help infer evolutionary models which best explain the patterns seen

Chapter two

I examine species in the genus *Buccinulum.* A group of species in this genus has an unusual distribution with one species split into two subspecies and dividing them geographically is a second species. I use genetic and morphometric tools to see whether there are genetic clusters that correlate with the phenotypic clusters and whether or not these are in concordance with the recognised taxonomy. I include analysis of another whelk with the same geographic range as the *Buccinulum* examined here to act as a comparison to help discover any geographic patterns

Chapter three

I use next generation sequencing to build mitochondrial genomes and 45s cassettes alongside mitochondrial *cox1* amplicons to test the monophyly of the New Zealand *Amalda.* Monophyly has not been previously explicitly tested. Samples were accessed from around the world especially the Pacific, to build phylogenies. Samples from some unidentified specimens are included in some analysis to attempt to clarify the taxonomy of the New Zealand *Amalda.*

Chapter four

I examine the New Zealand *Amalda* to test for concordance between genetic and phenotypic clusters. I primarily utilised *cox1* amplicons to determine genetic clusters and identified phenotypic clustering using shell shape and a geometric morphometric approach. A subset of samples with both genetic and morphometric data are analysed, then the expanded morphometcic dataset is tested.

Chapter five

Three lineages of *Amalda* species that have extensive fossil records and modern representatives are used to bring together the fossil dataset with the genetic dataset. I examine the morphological delimitation of the three phenotypically similar species in the fossil record and then investigate how well the evolution of the three species conforms to one of three models that is directional evolution, unbiased random walk or stasis. The testing of evolutionary model fit is done with a view to accessing the suitability of using *Amalda* as exemplars of punctuated equilibrium as has been done in the past.

Chapter six

A discussion of the merits of using a combined approach in investigating species delimitation both in the modern fauna and the fossil record.

The chapters are intended for publication and have been formatted as such with references presented at the end of each chapter.

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Chapter 2

Contrasting Geographically Aligned Phenotypic and Genetic Patterns in Coastal Whelks

Introduction

Local adaptation among animal populations has been inferred from phenotypic traits, but determining the role of natural selection in shaping geographically partitioned variation is not simple (Merilä and Hendry, 2014). Phenotypic variation among geographic populations might result from genetic drift in isolation or selection on functional characters, and/or represent ecophenotypic plasticity. Patterns of local phenotypic adaptation are expected to reflect patterns of environmental variation (Dowle et al., 2015), but population clusters of neutral genetic variation might not coincide with this. Where population differences are purely the result of divergence due to drift then phenotype and neutral genetic markers are both expected to be correlated with a degree of isolation. Habitat-specific convergent evolution provides a framework for testing adaptive hypotheses (Minards et al., 2014) as similar responses in separate lineages indicate that traits may be adaptations resulting from local selection (Butlin et al., 2014). In this study I compare phenotypic variation of two independent, but co-occurring, lineages of rock-shore gastropods, collected from the same coastline. I contrast their patterns of phenotypic variation with genetic structure within each lineage to understand the role of natural selection in creating the spatially aligned phenotypic variation.

Some of the best examples of ecological speciation in which local adaptation results in the formation of ecotypes are found in studies of snail shells (e.g. Butlin et al., 2014; Quesada et al., 2007; Stankowski, 2013; Wada et al., 2013). The phenotype of a snail shell is a response to a wide variety of genetic and environmental influences. In marine environments factors such as water depth, temperature, currents, wave energy, and predation affect shell shape (Avaca et al., 2013; Baker et al., 2004; Butlin et al., 2014; Haig et al., 2015; Hills et al., 2012; Hollander and Butlin, 2010; Olabarria and Thurston, 2004; Palmer, 1990; Shelmerdine et al., 2007); reviewed by (Bourdeau et al., 2015). Different ecological conditions across a species' range can drive polymorphism that might lead to erroneous taxonomic splitting of a lineage (e.g., subspecies of the turritellid *Maoricolpus roseus* in New Zealand (Donald and Spencer, 2015). Conversely, constraining selection that maintains traits associated with certain conditions can lead to similarities among taxa in separate lineages. if such species are closely related they may be cryptic, another cause of problems in taxonomy (see Allmon and Smith (2011) for a summary of cryptic gastropod species).

In New Zealand the marine gastropod genus *Buccinulum* Deshayes, (1830) comprises species that are broadly morphologically similar and, in some cases, difficult to distinguish (Ponder, 1971). *Buccinulum vittatum* (Quoy & Gaimard, 1833) is a common rocky shore whelk with an unusual distribution; it occurs almost the full length of New Zealand, but is absent from an approximately 400 km stretch of eastern coast (Fig. 2.1). Where *B. vittatum* is absent the congeneric *Buccinulum colensoi* (Suter, 1908) is common. The exclusion of one species of rocky shore whelk by another might be due to spatially abrupt differences in environmental conditions facilitating interspecific competitive exclusion by the other species. Alternatively, the local environment might select for an ecotype of *B. vittatum* that has mistakenly been assigned separate taxonomic status. Here I have analysed genetic and morphological data to investigate these possible competing explanations. An ecotype would not be expected to be strongly differentiated from other populations at neutral genetic loci, whereas, a separate species would show concordance between shell shape and genetic markers. To determine whether the local environment selects for a particular shell phenotype I examined an independent lineage in the same environment.

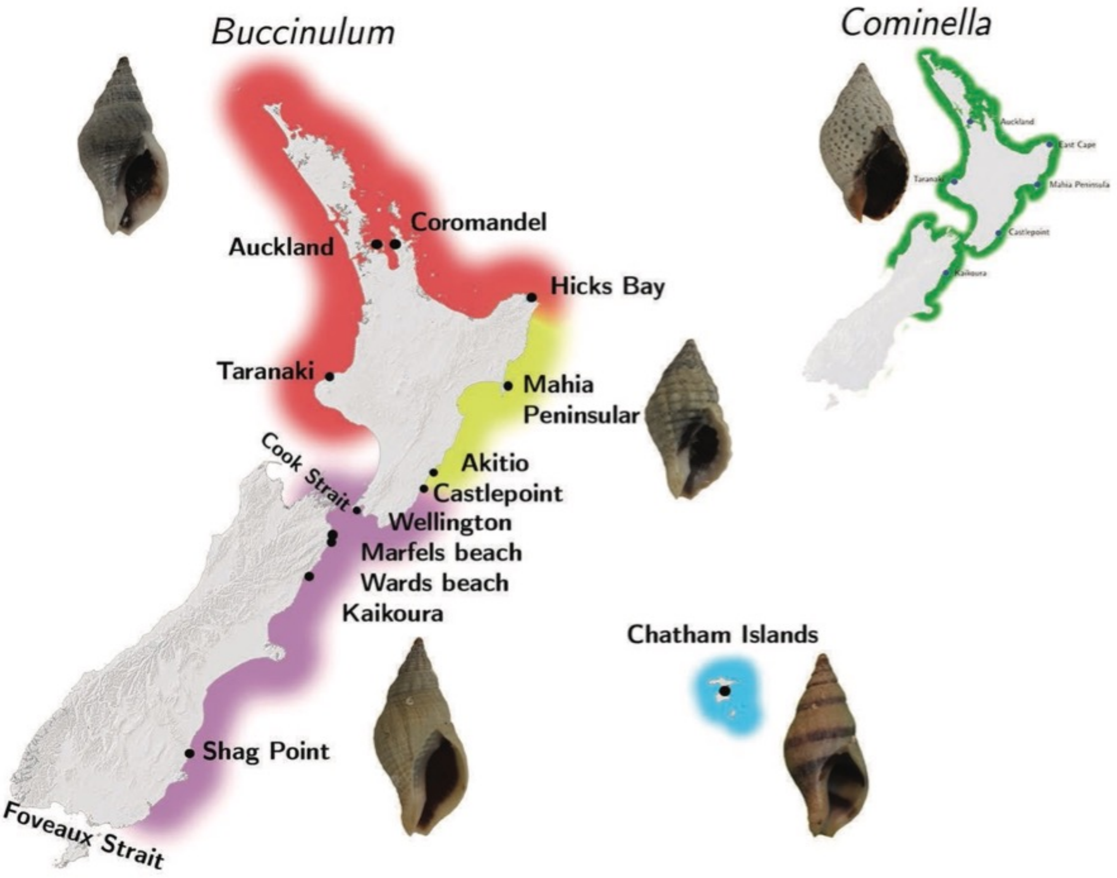

Figure 2.1 The distribution of four small, coastal marine snails in the genus *Buccinulum* around New Zealand. Named sampling locations are indicated (black circles). Red, *B. v. vittatum*, northern group (Taranaki, Auckland, Coromandel, Hicks Bay); Yellow, *B. colensoi*, eastern group (Mahia, Akitio, Castlepoint); Purple, *B. v.littorinoides*, southern group (Wellington, Marfels Beach, Ward Beach, Kaikoura, Shag Point); Blue, *B. v. bicinctum*, Chatham Islands. Insert shows approximate distribution of *Cominella maculosa* in green.

The rocky shore snail *Cominella maculosa* (Martyn, 1784), although superficially similar in appearance is not closely related to *Buccinulum* (Donald et al., 2015; Vaux et al., 2017). Both lay eggs that hatch as small snails (Carrasco et al., 2016; Dohner, 2016; Donald et al., 2015; Morley et al., 2006;

Ponder, 1971) with no pelagic larval stage and they are therefore are expected to have similar dispersal rates. Both species are intertidal predatory whelks that mostly feed on polychaete worms, and co-occur in the same rock pools along the same coastline (Ponder, 1971). Matching spatial distribution of shell shape variation in *C. maculosa* mstching the *B. colensoi* distribution, would indicate a convergent adaptive response to local environmental conditions. Alternatively, if *B. colensoi* is a separate evolutionary lineage from *Buccinulum vittatum* we would expect to see concordance of genetic markers and genetic structure indicating low (or absence of) gene flow.

Materials and Methods

Taxonomic background

Buccinulum and *Cominella* are small (10–30 mm shell length) members of the globally distributed gastropod family Buccinidae (Bouchet et al., 2005), that in New Zealand also includes *Aeneator* Finlay, 1926, *Antarctodomus* A. Adams, 1863, *Austrofusus* Kobelt, 1879, *Euthrenopsis* Powell, 1929, *Pareuthria* Stebel, 1905, and *Penion* Fischer, 1884 (Powell, 1979; Spencer et al., 2016). Most of the 14 currently recognised species of *Buccinulum* are readily distinguished from each other, but a group of taxa are referred to as the *B. vittatum* complex. This complex includes taxa that have all at some time been considered subspecies of *Buccinulum vittatum*, currently include *B. v. vittatum*, *B. v. littorinoides* (Reeve, 1846), *B. v. bicinctum* (Hutton, 1873), and *B. colensoi* (Spencer et al., 2016). These taxa are separated primarily on the basis that they form distinct geographic clusters (Fig. 2.1), but, the *B. colensoi* shell morphotype is smaller and more robust than the other members of the complex and has rugose external texturing (Ponder (1971). *Buccinulum v. vittatum* is found north of *B. colensoi* whereas *B. v. littorinoides* is found south of *B. colensoi*, on the east coast of New Zealand's North and South Island to the Foveaux Strait (Fig. 2.1). *Buccinulum v. bicintum* is restricted to the Chatham Islands (Fig. 2.1). Nine *Cominella* species are found in New Zealand waters (Donald et al., 2015; Powell, 1979; Spencer et al., 2016). *Cominella maculosa* is sympatric with the *B. vittatum* complex though not extending as far south (Fig. 2.1) and recent mtDNA sequencing has revealed fine scale population structure on the eastern coastline of North Island suggesting mixing of northern and southern haplotypes of this species (Fleming et al., 2017).

Sample collection

Specimens of *Buccinulum* and *Cominella* were collected by hand from rocky shore locations around New Zealand. Locations were chosen to provide widespread sampling within the species' distribution as possible within substrate type and accessibility constraints. Sampling numbers were intended to provide enough specimens from each location for population level variation to be investigated. Freshly caught live snails were frozen and transferred to the laboratory where they were thawed, extracted from their shells and preserved in 95% ethanol. Shells were retained for morphometric analysis (Table 2.1). Specimens were identified using gross morphology and collection location (Powell, 1979)

Table 2.1. Sampling location and regional designation of samples from the *B. vittatum* complex and *C. maculosa* used in morphometric and genetic analysis. Region boundaries are based on distribution boundaries of *Buccinulum* taxa. In total 92 *Buccinulum* and 22 *Cominella* specimens were used in mtDNA analysis. SNPdata was from 28 *Buccinulum* individuals. Digital images were taken of 88 *Buccinulum* and 46 *Cominella* specimens.

Buccinulum				*Cominella*		
Location	*cox1*	SNP	morphometic	Locaton	*cox1*	morphometic
Northern Region						
Taranaki	8	3	8	Taranaki		5
Auckland	8	4	13	Auckland	6	11
Coromandal	1		2	East Cape	6	10
Hicks Bay	4	3	4			
Eastern Region						
Mahia	15	8	17	Mahia		3
Akitio	4		3	Castle Point	5	8
Castle Point	10	3	4			
Southern Region						
Wellington	3		1	Kaikoura	5	9
Marfells Beach	3					
Ward Beach	1		8			
Kiakoura	15	7	14			
Shag Point	6					
Chatham Islands	14		14			
Total	92	28	88		22	46

Geometric morphometric data

Digital images of the shells were obtained following the approach of in Dowle et. al. (2015) and the recommendations of Collins & Gazley (2017). Shells were placed in a bed of contrasting coloured sand with ventral surface upwards and positioned so the aperture was horizontal. Photographs were taken using a Canon EOS 600d with EF100 mm f2.8 USM macro lens mounted on a high-precision Kaiser stand. Two digital 'combs' were positioned over the image of each shell using Adobe Photoshop cs6. (Supplementary Figure 2.13). Shell morphology was analysed using a two-dimensional, landmark-based geometric morphometric approach with a combination of fixed landmarks and semi-landmarks. Biologically homologous points at the tip of the spire, either side of intersection of the body whorl and the next whorl, top of the arpeture and base of the columella provided positions for fixed landmarks, and sliding semi-landmarks were positioned along aperture and outline curves (supplementary figure 2.13) and slid to minimise bending energy, effectively shifting them along the curve to enable more effective shape comparison (Zelditch et al., 2004). All landmarks are interpreted as Type I landmarks (Bookstein, 1991; Gunz et al., 2005). Shell phenotype analysis using this method is effective in discovering variation among ecotypes and species (Collins et al., 2013; Dowle et al., 2015; Hills et al., 2012; Smith and Hendricks, 2013). Digitizing was undertaken in tpsdig2 2.17 (Rohlf, 2013) on a Wacom Cintiq 22HD digitizing tablet. Digitized semi-landmarks were slid using SEMILAND, part of the imp714 package, implementing the Procrustes distance method. Landmark X–Y coordinates were then imported into MorphoJ 1.05f (Klingenberg, 2011) for nonparametric statistical analysis. An initial set of five fixed and 42 semi-landmarks was digitised on the curves of the shell and subjected to preliminary analysis. To reduce degrees of freedom (a problem resulting from having more landmarks than samples) the number of landmarks was reduced to five fixed and 21 sliding landmarks, after testing for effect on group separation was found to be small, for further analysis. This reduces the possibility of Type I errors, which are more likely with datasets with high numbers of landmarks.

To quantify the amount of error introduced into analysis from the digitising process, a disparity test was performed in Geomorph v 3.0.3 (Adams and Otárola-Castillo, 2013) implemented in R programing environment (R Development Core Team, 2014). To test placement, the shell was placed and photographed and removed, this was repeated five times and each photograph digitised for analysis. To test digitisation error alone a single photo was duplicated five times and the digitisation process performed on each duplicate. The effect of other photographic variables, camera height and position of shell within frame, have been tested and found to be small (Collins and Gazley, 2017) and were not tested in this study.

Shell shape variation was examined using the Java based package MorphoJ (Klingenberg, 2011). Shape was assessed using principal component analysis (PCA) across all individuals and all landmarks. A broken stick test was implemented using the Vegan (Oksanen et al., 2015) package in the R programing environment, to determine how many statistically significant, informative principal components were present, using Eigenvalues produced in MorphoJ. Canonical variates analysis (CVA) was used to test the discrimination of four groups based on the current taxonomic treatment. Discrimination success was estimated using leave one out cross-validation scores (number of individuals correctly assigned to each group with 1000 permutations).

A model-based clustering approach was used to explore the distribution of variation and to test for natural clusters without *a priori* classification, using the Mclust package (Fraley and Raftery, 2002) in R. The Mclust v5.0.2 algorithm (Fraley et al., 2012) uses Gaussian modelling in which the total data set is considered as a mixture of multivariate normal data sets, with a selection of covariance structures and vectors of expectation (Fraley et al., 2016; Nanova, 2014). Unlike discriminant analysis, Mclust analysis does not require prior information about specimen identity to classify sample data (Fraley and Raftery, 2003, 2002, 1999). The best model and optimal number of clusters in the data are selected based on Bayesian Information Criteria (BIC), using the value of the maximized log likelihood, with a penalty for the number of parameters in the model (Cordeiro-Estrela et al., 2008; Fraley and Raftery, 2003, 2002, 1999; Nanova, 2014). In Mclust, BIC scores are multiplied by -1, so that higher BIC scores indicate lower global average and median classification uncertainty, and better model fitting (Cordeiro-Estrela et al., 2008; Fraley and Raftery, 1999).

Data were partitioned into four groups based on geographic location which corresponds to species distribution for comparisons to avoid issues of identification.

Mitochondrial DNA sequence data

DNA was isolated from snail foot tissue using either the Geneaid™ column extraction kit or a modified CTAB extraction protocol involving the removal of unwanted organic compounds with Chloroform isoamyl (Doyle and Doyle, 1990; Trewick et al., 2009). In most cases the CTAB method produced higher yields of genomic DNA than kit extraction. DNA quantity and quality were assessed using agarose gel electrophoresis and with Qubit™ broad-range DNA assay.

Partial sequences of the mitochondrial Cytochrome Oxidase subunit I (*coxI)*was amplified using the primers HCO2198 and LCO1490 (Folmer et al., 1994). Polymerase chain reactions were performed in 20 μL volumes containing: 200 μM dNTPs, 2.5 μL NEB thermopol 10x buffer with 1.5 mM MgCl, 1 μM primers, 0.2 μL of NEB taq DNA polymerase and 1–10 ng of template DNA. Standard thermal cycling conditions were followed, with 50°C annealing temperature and 35 cycles, in a Biometra T1 thermocycler. PCR products were sequenced using primer HCO2198 using BigDye Terminator v3.1 on an ABI 3730 genetic analyser. DNA sequences were visualized, checked for errors and ambiguities, and aligned in Geneious v.7 (Kearse et al., 2012).

Mitochondrial *cox1* fragments for the two whelk lineages, the *Buccinulum group* and *C. maculosa*, were separately aligned using the Geneious v.7 alignment tool with default settings. Alignments and protein translations were checked by eye for anomalies. Phylogenetic analysis of the *Buccinulum* used MrBayes implemented in Geneious with 10,000,000 iterations and a burn-in of 1,000,000 (10%) using a GTR inv-gamma model (Tavaré, 1986). Out group species were *Buccinulum linea* and *Buccinulum palladium poweli.*

Minimum spanning networks (Bandelt et al., 1999) were inferred for mtDNA sequences from both the *Buccinulum* complex and *Cominella maculosa* datasets in PopART (Leigh and Bryant, 2015) . Haplotypes were coloured by taxon localities.

SNP genotypes

Anonymous single nucleotide polymorphic nuclear markers (SNPs) were generated following the protocol outlined by Peterson et al (2012) with minor modifications. The enzymes used for digestion of genomic DNA were Nsil HF and MboI (New England Biolabs), which were selected after a trial of potential enzymes to determine the best cutting efficiency. Whole genomic DNA of 78 *Buccinulum* specimens was digested. Short barcode sequences were ligated to fragments using Invitrogen T4 Ligase to enable identification of individuals after pooling of samples for sequencing. High throughput sequencing was performed using an Illumina Hi-seq (NZGL) and resulting data processed using the stacks pipeline (Catchen et al., 2013).

STACKS pipeline

Selection of nuclear markers was undertaken so analyses would be performed on loci likely to be single copy and for which the maximum number of individuals could be genotyped (Harvey et al., 2015). In STACKS(Catchen et al., 2013), a range of parameter settings relating to read coverage, individual number and population coverage were implemented. Initial exploration of the data suggested coverage varied for the 73 individuals. Recommended read coverage settings vary in the literature (Buerkle and Gompert, 2013; Peterson et al., 2012), so I experimented with parameter optimisation. After initial tests using the STACKS pipeline to assess information content, samples with file sizes of less than 2mb were excluded from further analysis. These had low read numbers and inclusion reduced analytical power downstream. Low coverage combined with high error rate has the potential to reduce the number of true loci detected by a substantial amount (up to 51%); (Catchen et al., 2011).

Parameter optimisation

The number of mismatches allowed between alleles when processing a single individual (-M) was explored. If -M is too low, some real loci are not being formed, and subsequent alleles will be treated as different loci (undermerging). If -M is too high, repetitive sequences and paralogs will form large nonsensical loci (over merging). I tested –M= 5–25, and at high values (-M=25) catalogue construction failed. At –M 5–15 some variation in F_{ST} (used here as a proxy for identifying population/species differentiation) was recorded but the relationships among population samples remained the same.

The number of mismatches allowed between alleles within a locus (amongst individuals) when building the catalogue -n was also varied. For -n = 0, there would be loci represented independently across individuals that are actually alleles of the same locus. If n > 0, the consensus sequence from each locus is used to attempt to merge loci. This is important for population studies where monomorphic or fixed loci may exist in different individuals. Merging fixed alleles as a single locus can increase the probability of assembling real loci and therefore decrease the allele error rate. However, erroneous loci will be created if -n is too high. It is recommended that the setting of -n should consider the genetic similarity expected among samples within each study (Peterson et al., 2012). I tested values of –n = 2-25. When –n = 25 was used catalogue construction again failed. When values of –n = 5-15 were used some variation in in F_{ST} (used here as a proxy for identifying population/species differentiation) was recorded but the relationships among population samples remained the same.

SNP identification

To create the dataset used to examine genetic structuring I used the optimised parameters from above. I used a minimum of 5 reads per individuals (-m) as providing a reliable set of markers for downstream analysis and excluding all stacks with a lower coverage. Potentially spurious highly repetitive stacks were removed. Within an individual, I allowed a maximum of 3 mismatches between alleles (-M) and 5 mismatches between primary and secondary reads (-N=M+2). Ten mismatches were allowed between alleles in different individuals (-n) when generating the SNP set. The final parameter settings were as follows: -m = 5, -N = 5, -M = 3, -n = 10.

The *de novo* map function in STACKS was used to build a catalogue using 28 individuals from Taranaki, Auckland, East Cape, Mahia, Castlepoint and Kaikoura. These settings enabled comparison of two species for which reasonable sequence variation might be expected. Analysis was restricted to a single SNP site per putative locus (always the first) avoiding potential problems of data nonindependence.

Sample representation per population

A range of values for the number of populations a SNP marker was required to be present in before being recorded was tested using POPULATIONS which is part of the STACKS pipeline allowing computation of population-level summary statistics, and the output of site level SNP calls for subsequent analysis in Structure (Evanno et al., 2005). Large differences in the resulting number of loci were found when tightening or relaxing the stringency at which the presence of a SNP was required in a number of putative populations or proportion of individuals in a population. The data were initially analysed as a single population to ameliorate the possibility of misidentification of samples. Population data was reintroduced downstream for comparisons. The dataset required that each putative locus included was genotyped in individuals from 1 or more of the 6 populations (-p 1) and genotyped in at least 40% of individuals within those populations (-r 0.4). This resulted in allelic data for at least 12 of the 28 specimens for each locus. Values above this (-r >4) gave little usable data and having some missing data is preferential to the large number of lost loci. The minor allele frequency was set at 0.1 (-min_maf 0.1) removing rare alleles from the dataset. Prior to including this setting there were many rare alleles that added noise to a small data set. Only the first SNP from each locus was used. This resulted in the following final setting [-p 1 -r 0.4 -f p_value --write_single_snp --fstats --min_maf 0.1-k].

The final SNP data set was analysed using Bayesian clustering in STRUCTURE (Hubisz et al., 2009; Pritchard et al., 2000) to identify population differentiation. Ten replications of the admixture model with independent allele frequencies with a burn in of 200,000 and 1,000,000 generations were used. Potential populations (K) was set from 1—6. To determine the optimal number of clusters, I examined estimates of the posterior probability of the data for a given K (Pritchard et al., 2000) and ΔK, the rate of change in log probability of the data (Evanno et al., 2005) implemented in Structure Harvester 0.6.8 (Earl and vonHoldt, 2012).

Results

Geometric morphometrics

Buccinulum

Shell shape variation was studied with fixed and sliding landmarks on 88 *Buccinulum* snail specimens from 11 locations. A disparity test indicated that errors introduced from the digitisation process, comb and landmark placement, (0.3% of total shape variation), and total variation and variation from shell placement and digitisation (1.4%) were negligible and were ignored in subsequent analyses.

The first five principal components (determined by broken stick test) obtained from a principal component analysis (PCA) across all *Buccinulum* specimens explained 84.4% of shell shape variation and provided statistically significant information (Table 2.2). When the first two principal components (63.8% variation) were used to visualise shape variation there was some clustering of specimens (Fig. 2.2) with samples from the eastern region being most distinct (the range of *B. colensoi*, Fig. 2.1). Regional structuring of morphology in other regions was also apparent with non-overlapping 95% confidence means but there was no clear cluster separation among these groups. A model with a single morphologic cluster was inferred as optimal fit to the data for *Buccinulum* using the Bayesian approach in Mclust utilising the first five principal components.

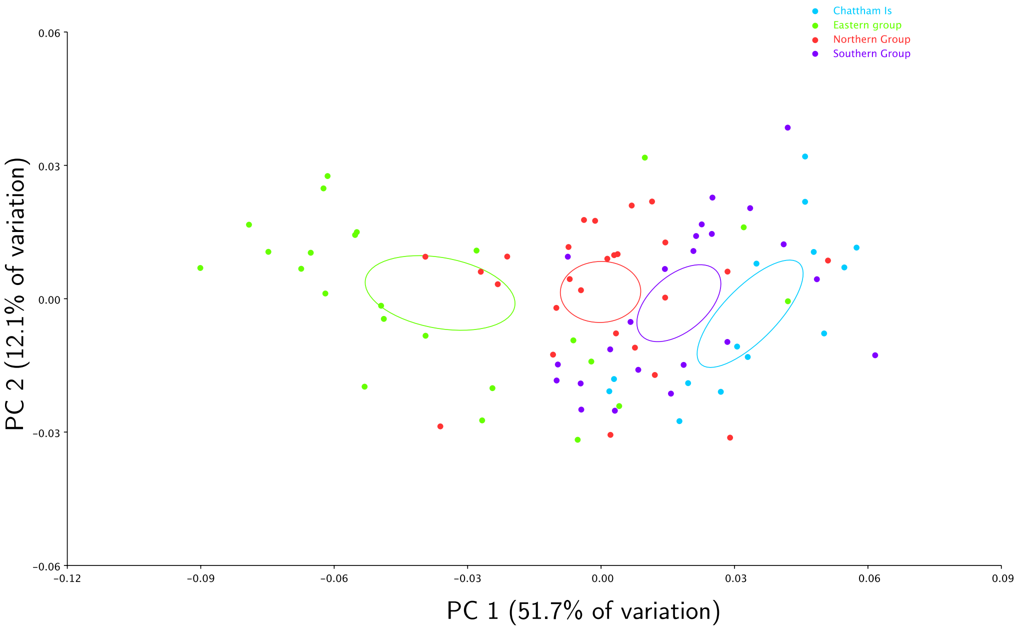

Figure 2.2 Principal component analysis of *Buccinulum* shell shape coloured by sample collection region based on recognised taxa distributions. Ellipses are 95% confidence means and indicate some regional partitioning of morphological variation.

Table 2.2. Shell shape variation within the *B. vittatum* complex whelks. Principal Component Analyses scores with % variance and cumulative % variation.

PC	Eigenvalues	% Variance	Cumulative %
1	0.00123315	51.734	51.734
2	0.00028736	12.056	63.790
3	0.0002087	8.756	72.545
4	0.00014793	6.206	78.751
5	0.00013428	5.634	84.385

Canonical variates analysis showed some regionally aligned separation although incomplete. The Chatham Island group was the most clearly separated (Fig. 2.3). Not all individuals were able to be allocated to their original sample groupings in cross validation tests (p values for difference between means ranging between 0.37 and 0.97) (Table 2.3)

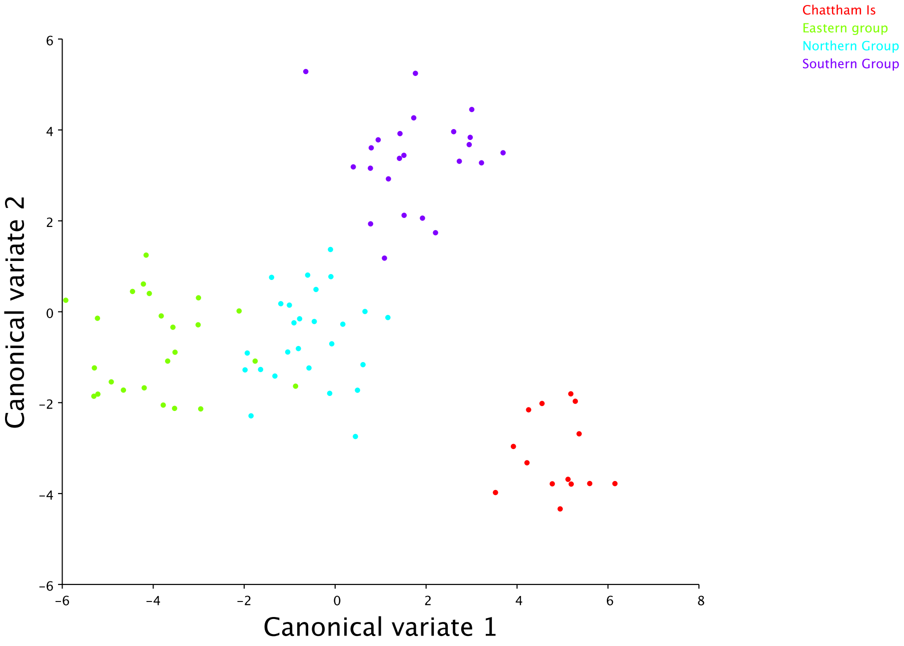

Figure 2.3 Canonical variate analysis of *Buccinulum* identified by region. Regional groupings are apparent. The Chatham Is group is the most easily distinguished while other clusters are close to one another reflecting the homogeneity of the *B. vittatum* complex.

Table 2.3. Cross validation scores from canonical discriminant analysis for shell shape variation of *B. bicintum*, *B. v. vittatum*, *B vittatum littorinoides* and *B. colensoi* separated into geographic groupings. Proportion of individuals assigned to incorrect populations are shown based on sampling locations Chatham, Eastern, Northern, and Southern.

	Northern	Eastern	Southern	Chatham
Northern		0.12	0.35	0.15
Eastern	0.25		0.37	0.25
Southern	0.43	0.48		0.30
Chatham	0.14	0.00	0.15	

Since most of the shell shape variation was explained by the first principal component (PC1; 51.7%) (Fig. 2.4), and because PC1 suggests regional differentiation (Fig. 2.2), further model based clustering of specimens was performed using just PC1. Bayesian assignment of PC1 revealed that data best fitted to a model with two morphological clusters (Fig. 2.5). One cluster contained 15 specimens collected from within the known range of *B. colensoi* (eastern region) and two specimens from the northern region (Taranaki). Seven specimens from the eastern region (Mahia, Akitio & Castlepoint) were in the alternate cluster along with samples from both the northern southern and Chatham groups (assignment probabilities are shown in Fig. 2.6).

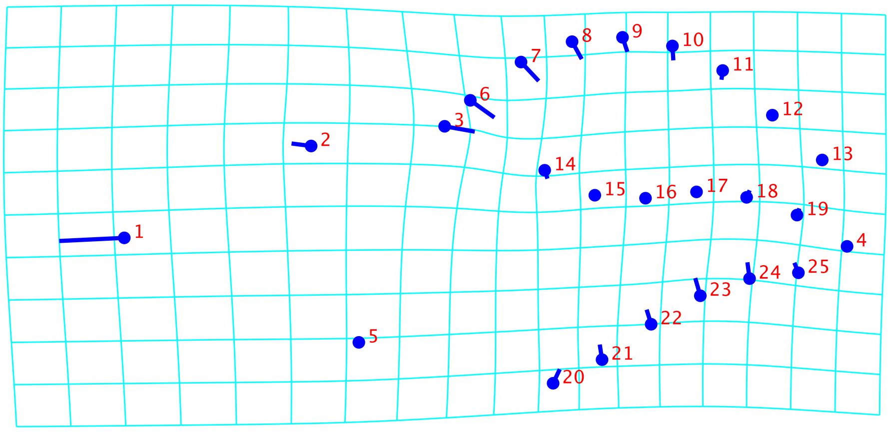

Figure 2.4 Thin plate spline warp grid showing the shape variation captured from *Buccinulum* shells by PC1. Variation was mostly based on the length of the spire as indicated by the length of the Landmark 1 lollipop, and the shape of the top of the aperture as indicated by the lollypops at landmarks 3–10

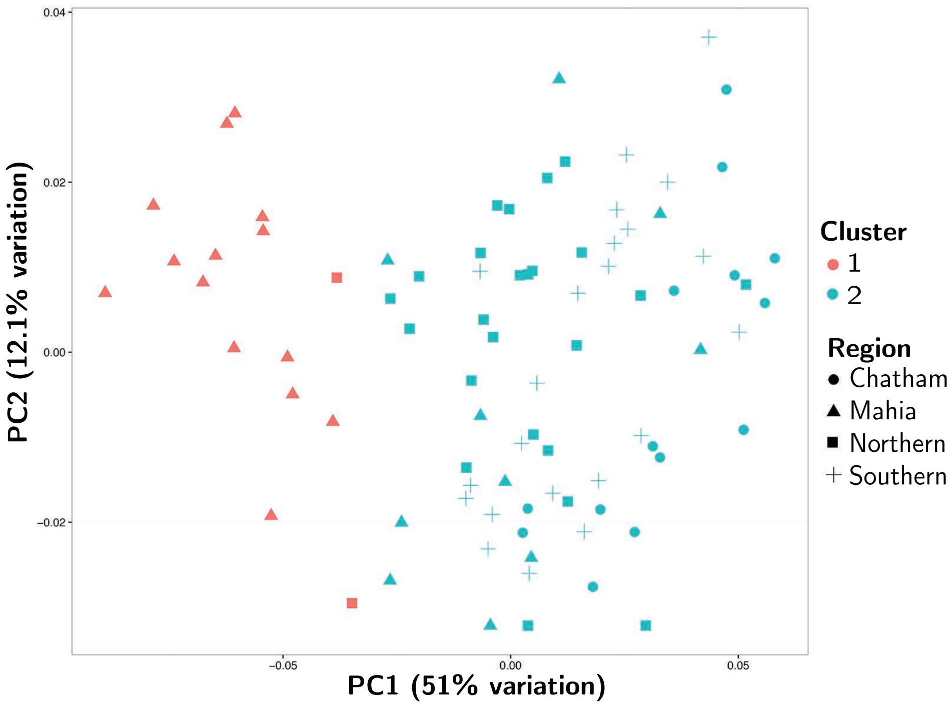

Figure 2.5 Principal component analysis chart showing the two clusters formed by Mclust using the first principal component. Cluster 1 consists mostly of specimens from the eastern region with two specimens from the northern region. Cluster 2 is all other specimens and includes eight specimens from the eastern region

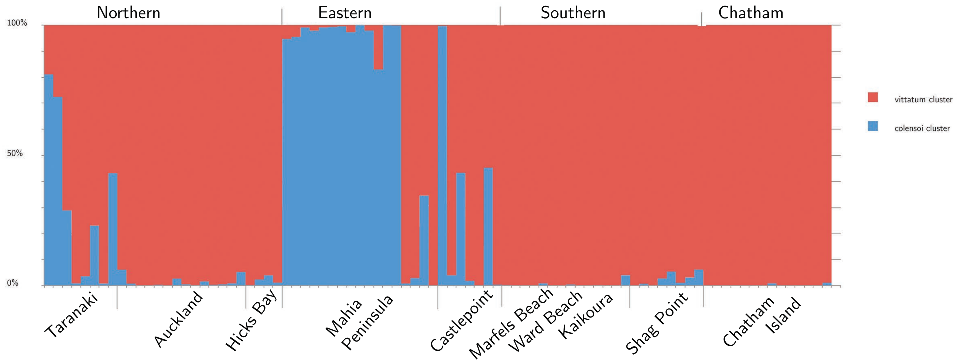

Figure 2.6 Assignment probabilities of individual *Buccinulum* to two clusters based on the first Principal component of shell shape variation. Samples arranged by sample location

Cominella

Shell shape variation within *Cominella maculosa* was examined using 46 shells from six sampling locations. The first three principal components from all *Cominella* specimens explained 73.74% of shell shape variation and provided statistically significant information (broken stick test; Table 2.4). When the first two principal components (65.26% of variation) were used to visualise shape variation little regionally aligned clustering of morphological variation was evident. There was morphological overlap of regional samples in morphospace although eastern region samples have a 90% confidence mean ellipse that does not overlap the ellipses of the other two regions (Fig. 2.7). Nevertheless, a single morphological cluster was identified by model based clustering of PC1 and also from the first three principal components using Bayesian clustering in Mclust.

Table 2.4 Shell shape variation in a sample of *C. maculosa* shells from around New Zealand PCA % variance accounted for by principal components with significant variation (selected by broken stick test).

PC	Eigenvalues	% Variance	Cumulative %
1	0.00084272	47.813	47.813
2	0.00030747	17.445	65.258
3	0.00014948	8.481	73.739

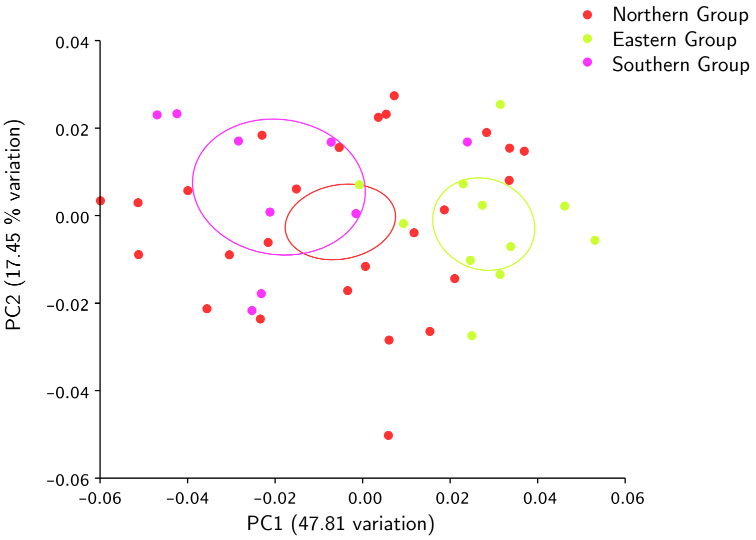

Figure 2.7 Principal component analysis of shell shape variation within the whelk *Cominella maculosa*, specimens coloured by sample collection region corresponding to *Buccinulum* taxa distributions.

Genetic structure

Buccinulum

An initial alignment of a short section of mitochondrial Cytochrome Oxidase I (285 bp) was compiled for 81 individuals from the *Buccinulum vittatum*-complex and used to create a haplotype network to show the relationships among the specimens. The 285 bp haplotype network, including haplotypes from Chatham Island *B. vittatum bicinctum*, reveals four clusters. Haplotypes from *B. v bicinctum* form two clusters of specimens. Haplotypes from the northern group (*B. v. vittatum*) form a third cluster, and haplotypes from the eastern ans southern regions (*B. colensoi* and *B. v. littorinoides*) together form the fourth cluster. At this mtDNA locus there was as much sequence diversity among Chatham Island samples as seen between *B. v. vittatum* and *B. v. littorinoides*/*B. colensoi* haplotypes (Fig. 2.8).

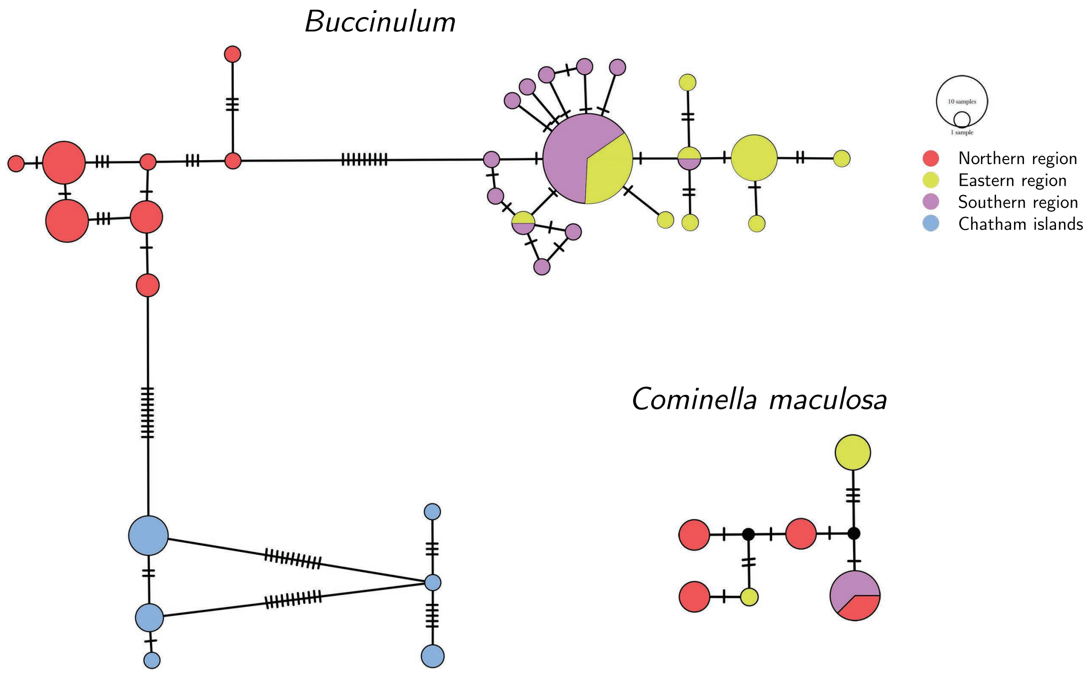

Figure 2.8 Minimum spanning network of mtDNA haplotypes from the whelk *B. vittatum* complex (*cox1*; 285 bp) from four regions and *C. maculosa* (*cox1*; 573 bp). Colours indicate sampling region. Within *Buccinullum* three distinct haplotype clusters associated with geographic regions are apparent. No regional based clustering is apparent in the *Comminella* network. Hash marks between haplotypes represent base substitutions.

A longer 585 bp fragment of *cox1* sequence was used for phylogenetic inference of *B. v. vittatum*, *B. v. littoronides* and *B. colensoi*. The outgroup comprised *Buccinulum linea* and *Buccinulum pallidum powelli*. Within the *Buccinulum vittatum* complex two mtDNA clades were resolved (Fig. 2.9), one representing the northern subspecies *B. v. vittatum* and the other made up of individuals of the southern subspecies *B. v. littorinoides* and *B. colensoi.*

Cominella

A fragment of *cox1* was sequenced from 22 *Cominella maculosa* individuals from the same geographic range as *Buccinulum vittatum* complex. These mtDNA sequences differed by a maximum of 8 bases (1.4%). The haplotype network based on 573 bp of *cox1* sequences resolved just six haplotypes. No clear geographic pattern was resolved and the same haplotype was found in northern and southern populations (Fig. 2.8 and 2.9).

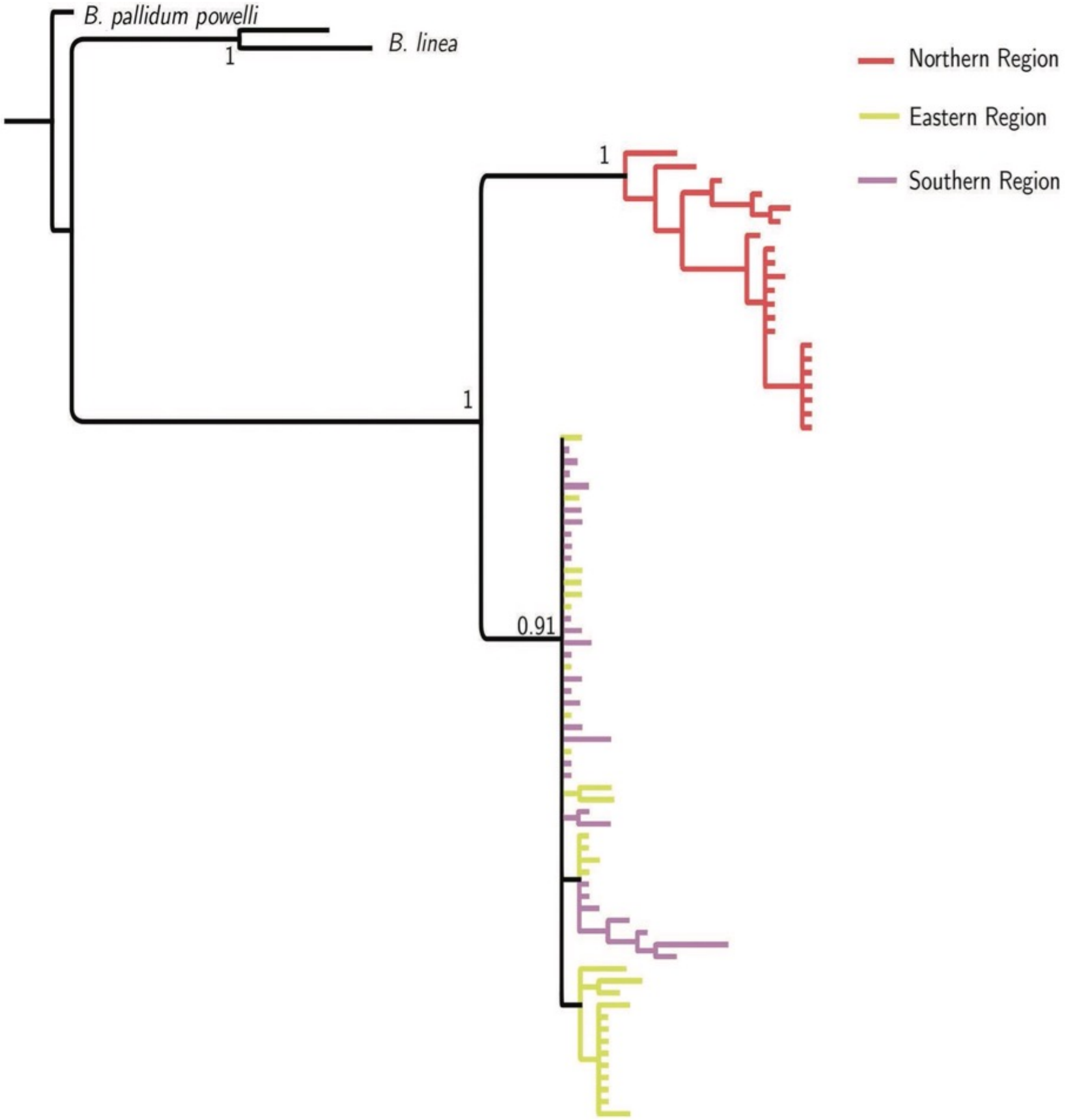

Figure 2.9 Rooted Bayesian Phylogenetic inference for the whelk *B. vittatum* complex based on mtDNA sequence (*cox1*, 553 bp) from *Buccinulum* species. A GTR invgamma model was run with 10000000 iterations after a 500000 burn in. The northern clade includes all samples from Auckland Coromandel, Taranaki, and Hicks Bay, The eastern/southern clade includes samples from Mahia Peninsula, Castlepoint, Wellington, Marfells beach, Wards Beach, Kaikoura and Shag Point. Outgroup *Buccinulum pallidum powelli* and *Buccinulum linea.*

Genetic structure of nuclear markers: SNP data

A set of 700 anonymous nuclear loci (Single Nucleotide Polymorphisms) were produced for 28 specimens within the *Buccinulum vittatum* complex, from six locations (Table 2.1). STRUCTURE harvester indicated that optimal K was two, dividing the *Buccinulum* specimens into two clusters (Fig. 2.10). Ten specimens (from Taranaki, Auckland and Hicks Bay) grouped with assignment probabilities between 99 and 69%, 18 specimens (from eastern and southern regions) grouped together with assignment probabilities between 99 and 79%. This grouping of specimens was consistent with the mtDNA results that indicate that the specimens from the northern region form a distinct genetic clade from eastern and southern populations. However, significant allele sharing was apparent between samples from Hicks Bay and the southern group.

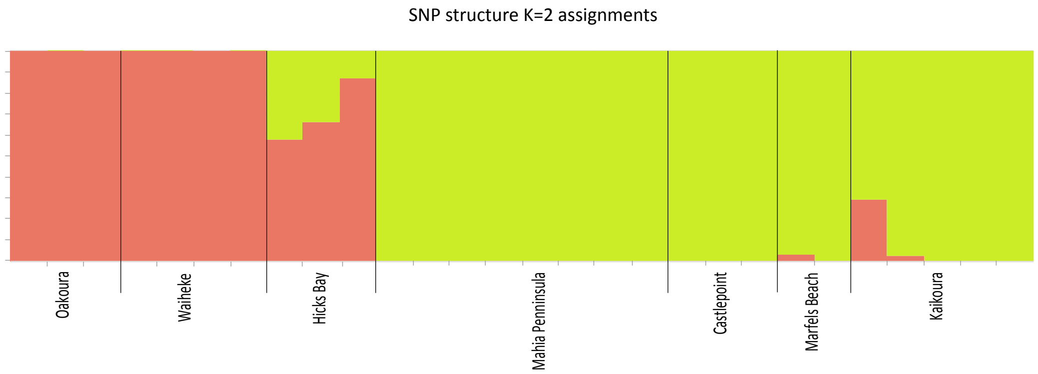

Figure 2.10 Assignment probabilities of whelks of the *Buccinulum vittatum* complex genotypes into clusters from Structure output from 849 SNP alleles. K=2 as indicated by structure harvester. Sample location North group Oakoura Taranaki, Waiheke island Auckland, Hicks bay; Eastern group Mahia Peninsula, Castlepoint; Southern group Marfells beach, Kaikoura.

Discussion

The distribution of taxa within the *B. vittatum* complex suggests that phenotype might be misleading us about evolutionary relationships. Distinct shell characteristics restricted to *Buccinulum* specimens from the East Coast of North Island might result from plastic developmental responses, selection on ecotypes, divergence in isolation, or distinct evolutionary lineages. Despite the current taxonomy recognising distinct species and subspecies within the *B. vittatum*-complex, initial geometric morphometric data did not resolve distinct morphological clusters. *Buccinulum colensoi* has the most distinct shell shape, but the most identifiable characteristic of the shell of *B. colensoi* is the strong surface sculpturing (Ponder, 1971) that was not examined by the landmark morphometric analysis. I noticed within my samples that even these shell surface traits were graduated over the range of *B. colensoi.* Using principal components generated from my two dimensional, landmark-based morphometric data, a single morphologic cluster fitted the Bayesian model better than a set of discrete clusters. However, when only PC1 was analysed using Bayesian assignment, a cluster predominantly from the eastern region closely corresponding to the species *B. colensoi*, was distinguished. Members of this cluster are not exclusively from this region, two of the specimens came from Taranaki, and four specimens from the eastern region are not part of the cluster. These results do not show taxonomically diagnostic characteristics of shell shape that would be consistent with recognising a separate species from the eastern North Island (*i.e.*, *B. colensoi*). Within the *B. vittatum* complex (excluding *B. colensoi*) shell shape (PC1) varies with moderate geographic correlation, possibly indicating some clinal variation (Fig. 2.2). One possible explanation for the recognition of the East Coast species *B. colensoi* morphotype is that the local environment is distinctive and selects for a particular shell shape. For example, the eastern region where the geology is dominated by sedimentary rocks more so than surrounding regions which have more metamorphic rocks possibly affecting water conditions, sediment loads or substrate types (Fig. 2.11 (GNS Science, 2016)). For this reason, I examined the shell shape of a sympatric whelk that occurs in the same habitat over the same geographic range, *Cominella maculosa*. Species in these two genera have different behavioural traits and habitat preferences which may limit their direct comparison but their close sympatry means some inferences about general geographic trends can investigated. Regional phenotypic partitioning was not strongly supported by study of *C. maculosa*. Morphometric analysis of the shell shape variation of *C. maculosa* collected over the same coastline showed some regional variation but did not resolve distinct clusters. *C maculosa* does not exhibit the shell structuring shape differences observed in *Buccinulum*.

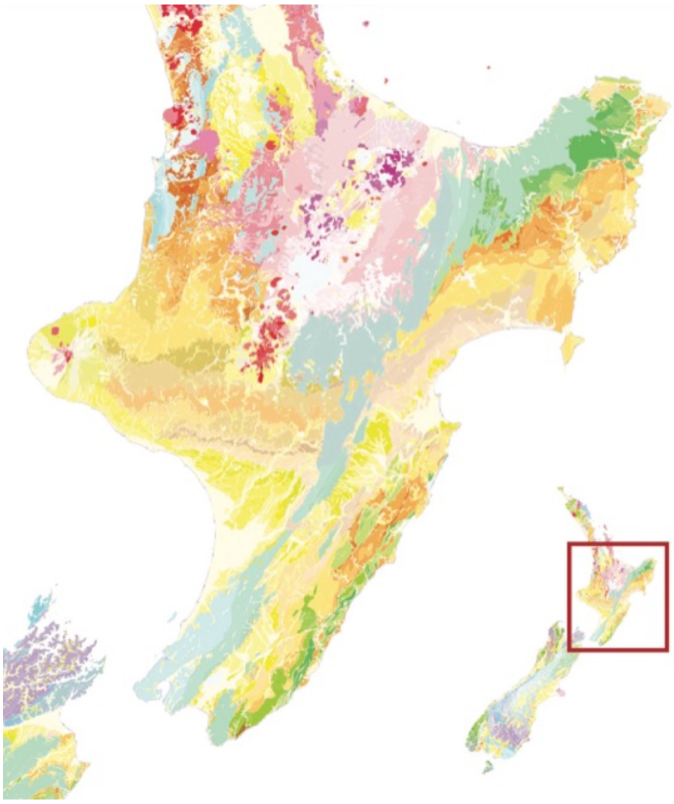

Figure 2.11 Geology of New Zealand. The area where *B. colensoi* is distributed is dominated by sedimentary rocks (browns). Surrounding areas are dominated by metamorphic or volcanic rocks (greens and pinks). Image was created using data from the LINZ NZMS 1:250 000 series and, data from GNS Science (2016)

Determining the process that results in local phenotypic differentiation is not simple. Shell shape variation of snails can result from a combination of drift, selection, plastic responses and historic phylogeographic effects. If variation results from divergence due to drift, then phenotype and neutral genetic markers are expected to show similar patterns. This was not what was seen in *Buccinulum.* Phenotypic variation within a single species maintained by selection (ecotype) would explain the pattern I observed. However, I did not detect the habitat-specific convergent evolution expected in *C. maculosa* if the traits of *B. colensoi* are adaptations resulting from local selection (Butlin et al., 2014). The factors that distinguish the niche of these two similar whelks might be key to understanding why the *B. vittatum complex* has a distinct East Coast ecotype but *C. maculosa* does not.

In contrast to the single morphometric cluster identified with five principal shape components, mitochondrial DNA sequence (*cox1* gene) resolved distinct partitioning within the *B. vittatum* complex. Individuals from the Chatham Islands (identified as *B. bicinctum* from their location) formed a distinct genetic cluster splitting into two subgroups. Individuals from within the range of *B. v. vittatum* (Northern) formed a second distinct genetic cluster. The third cluster was composed of individuals from within the range of *B. v. littorinoides* (Southern) and *B. colensoi* (Eastern). Analysis of 700 anonymous nuclear loci also showed this same pattern, however the nuclear markers also suggest that gene flow between *B. v. vittatum* (Northern) and *B. colensoi* (Eastern) is ongoing. The three snails from Hicks Bay, East Cape share alleles with both genetic clusters. Sampling from the area between Hicks Bay and Mahia has been undertaken but material was not suitable for analysis. I have found the genetic partitioning of the mitochondrial and nuclear markers is in conflict with the recognised taxonomy and phenotypic patterns.

Studies of marine species have previously recognized a biogeographical break on the North Island at East Cape (Gardner et al., 2010). The pattern of genetic structuring found in *B. vittatum* complex is similar to that found in *Haliotis iris* (pāua) (Will et al., 2015, 2011), in which specimens from the north and west coast of the North Island form a genetic cluster and samples from the east coast of the North Island and South Island form a second cluster, with a third separate Chatham Island cluster suggestion isolation by distance with a genetic break near East Cape. This genetic break at the East Cape may be facilitated by ocean currents in the area (Fig. 2.12). Here the southerly moving east Auckland current becomes the east coast current and moves slightly offshore. South of the East Cape a branch of the southern current moves northward close to the coast.

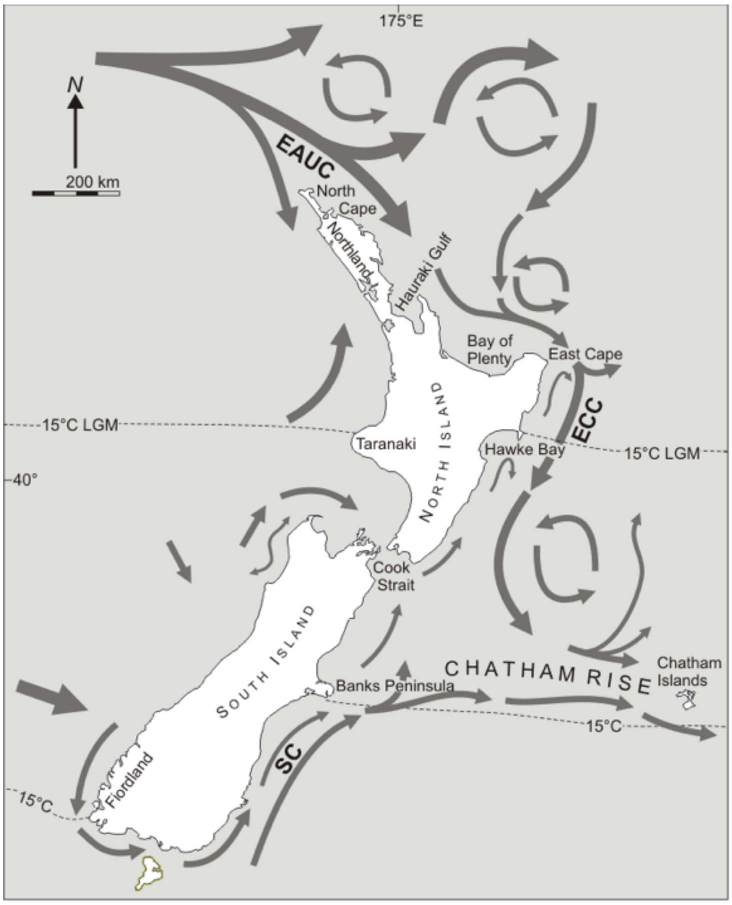

Figure 2.12 currents around New Zealand. Two major currents dominate the East Auckland Current (EAUC) becoming East Cape Current (ECC) moving in a southerly direction and the Southern Current (SC). These two currents meet at the East Cape potentially providing the means to explain a genetic break seen in *Buccinulum* in the area. Image reproduced with permission care of Joe Buchanan (Buchanan and Zuccarello, 2012)

A detailed sampling of *C. maculosa* populations found mtDNA haplotype variation over the same coast line, and detected abrupt changes in haplotype frequencies at both the southern and northern limits of the range of *B. colensoi* (Fleming et al., 2017). However, the mtDNA diversity within *C. maculosa* over the same geographic range was an order of magnitude smaller than that seen within the *B. vittatum* complex. These two whelk lineages are ecologically similar and sympatric and occur over much of the same coastline, however, they do not share similar shell shape variation. Lack of evidence of convergent evolution helps exclude the possibility that a gross environmental effect is in action in a restricted area causing the phenotypic differences between *B. colensoi* and *B. vittatum.*

Conclusions

There is a morphotype of *Buccinulum* currently identified as *B. colensoi* present on the east coast of the North Island, but the shell traits are not diagnostic and variation within populations suggest the *B. colensoi* form might be better considered an ecotype. There is no clear indication of any environmental conditions that might be influencing phenotype. Preliminary results (Donald et al., 2015) indicate the possibility of some disparity between mitochondrial and nuclear markers in determining phylogenetic relationships. I found strong genetic clustering in *Buccinulum* in contrast to the shell shape clustering, revealing the northern samples of *B.* v. *vittatum* as genetically differentiated from those south of East Cape. The two genetic clusters found suggest a taxonomic revision is necessary. my data indicate *B. colensoi* and *B. v. littorinoides* are probably a single polymorphic genetic cluster and that there is enough genetic differentiation to consider treating this as a separate genotypic cluster (species) from *B. v. vittatum.* In this example shell shape and sculpture can be misleading when identifying evolutionary lineages. Three clades of *Buccinulum* appear morphologically conservative yet the similarity between two of the clades seems to be a shared ancestral trait hiding divergence while two morphotypes of one genetic clade highlight polymorphisms.

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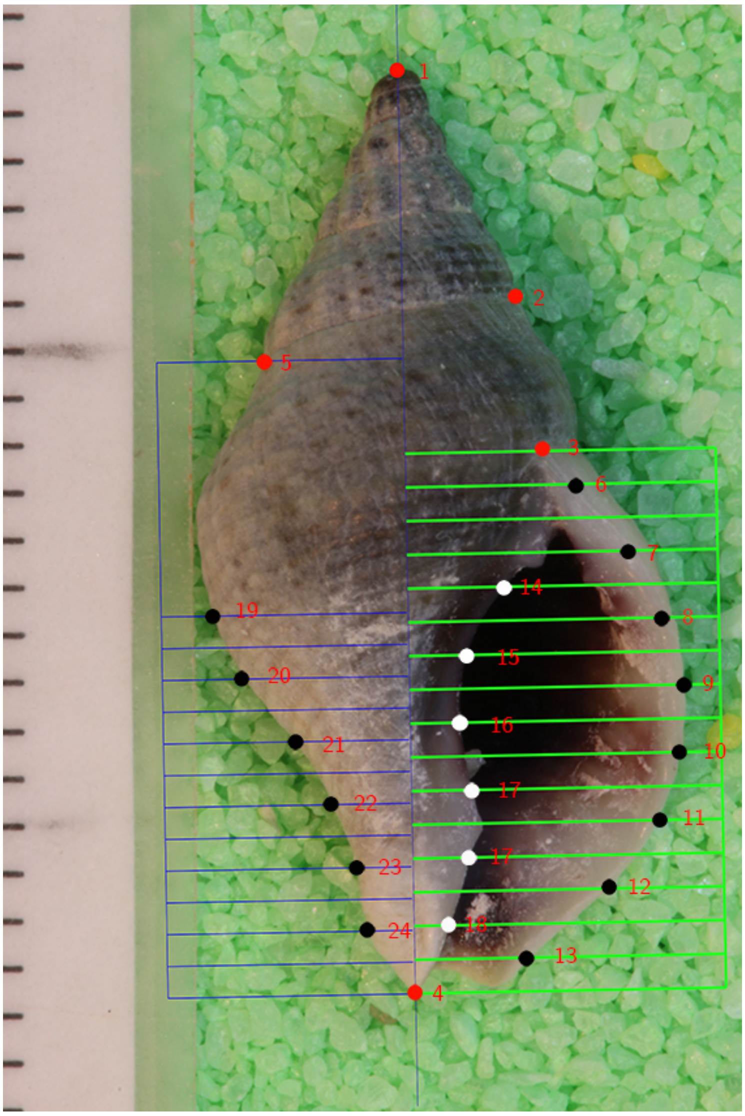
Supplementary figure 2.13

A *Buccinulum vittatum vittatum* shell showing digital combs used for landmark placement and landmarks used in analysis. Red circles indicate fixed landmarks (1–5) and black and white indicate semilandmarks (6–24). The same landmark process and landmarks were used digitising *Comminella maculosa* shells.



Chapter 3

Testing the Monophyly of New Zealand Olive Shells Using an Integrated Genomic and Amplicon Approach

Introduction

Amalda H. & A. Adams, 1853, the olive shells belong to the marine gastropod family Olividae, which is mostly confined to temperate regions (Olson, 1956) with at least 100 species throughout the Pacific and Atlantic oceans. Species of *Amalda* generally live in sandy near-shore environments where they are predators, primarily feeding on bivalves (Powell, 1979). The benthic habitat of *Amalda* lends itself to high levels of fossilisation, and in New Zealand *Amalda* has a continuous fossil record dating back to around 45 million years to Eocene fossil beds containing *Amalda morgani* (Beu and Maxwell, 1990). Seven extant species are recognised from New Zealand in two subgenera *Baryspira* Fischer 1883, (*A. australis* Sowerby I, 1830, *A. bathame* Dell, 1956, *A. depressa* Sowerby II, 1859 and *A. mucronata* Sowerby I, 1830), *Gracilispira* Olsen 1956, (*A. benthicola* Dell 1956, *A. northlandica* Hart 1995, and *A. novaezelandiae* Sowerby II, 1859). Type species are *A. australis* and *A. novaezelandiae* respectively (Olson, 1956). The *Gracilispira* are primarily recognised on the basis of shell size; the *Gracilispira* group are generally smaller than the *Baryspira* group. Difference in the radula of *A. novaezelandiae* from the radula of the representatives of the *Baryspira* group is cited as support for the categorisations (Powell, 1979). Of the seven extant species, four have lineages that extend into the fossil record: *A. mucronata*, *A. australis*, *A. depressa* and *A. novaezelandiae* (Beu and Maxwell, 1990; Olson, 1956).

Amalda feature in debate about models of morphological change since they were presented as examples in support of punctuated equilibrium (Gould, 2009, 1991). New Zealand *Amalda* appear to show both good evidence of fossil morphological stasis (Michaux, 1989) and reproductive isolation of recognised morphospecies (Michaux, 1987).

The premise of punctuated equilibrium derives from interpretation of a continuous fossil record that represents all forms that have existed in a lineage (Eldredge and Gould, 1972). The concept interprets the fossil record as representing long periods with little morphologic change and without lineage splitting. Largely missing from the fossil record are transitional forms because morphological change associated with speciation occurred in very short geologic periods relative to the lifetime of a species (Eldredge and Gould, 1972). Most of the time, species are morphologically stable; in stasis. This theory sent ripples through the evolutionary community that are still felt today, because the idea of a series of (geologically) abrupt evolutionary steps ran counter to the long held presumption that evolution was a gradual process with small changes accumulating over time. Palaeontologists argue that inferences about the pattern and model of morphological evolution can be made by following fossil lineages through time (Hunt, 2006).

Michaux (1987, 1989) used New Zealand *Amalda* were used to demonstrate the biological reality of fossil forms identified as “species” and provided strong evidence of morphological stasis, both important elements of the punctuated equilibrium theory. Paralogous sampling of separate evolutionary lineages could, however, result in misinterpretation of the rate and model at which shell morphology changes. Geologically abrupt morphological evolution associated with lineage splitting needs to be distinguished from alternative ways a new species can appear in a location (Fig. 3.1). Biological invasion is an alternative explanation for the first appearance of a species that could be misidentified as rapid *in situ* change in morphology. This is especially vexing in the fossil record if there are no other lines of evidence (Van Bocxlaer et al., 2008). Biological invasion is expected to result in a local fauna that is not monophyletic. Thus, for interpretations of *in situ* speciation to be valid monophyly of the set of related taxa under scrutiny must be established. The inference of discrete but continuous lineages in a region can be supported if alternative explanations of abrupt change are excluded. Appearance of a novel form within a lineage is usually inferred to be the product of speciation, although other processes could be involved. Hybridisation, which can introduce morphological changes that are not part of a speciation event (Morgan-Richards et al., 2009; Perrie and Shepherd, 2009), may be apparent in phylogenies differing between genes, but is not being explicitly tested here. Also morphologic change within a lineage might be decoupled from speciation (Charlesworth et al., 1982) which will be tested for in Chapter Five.

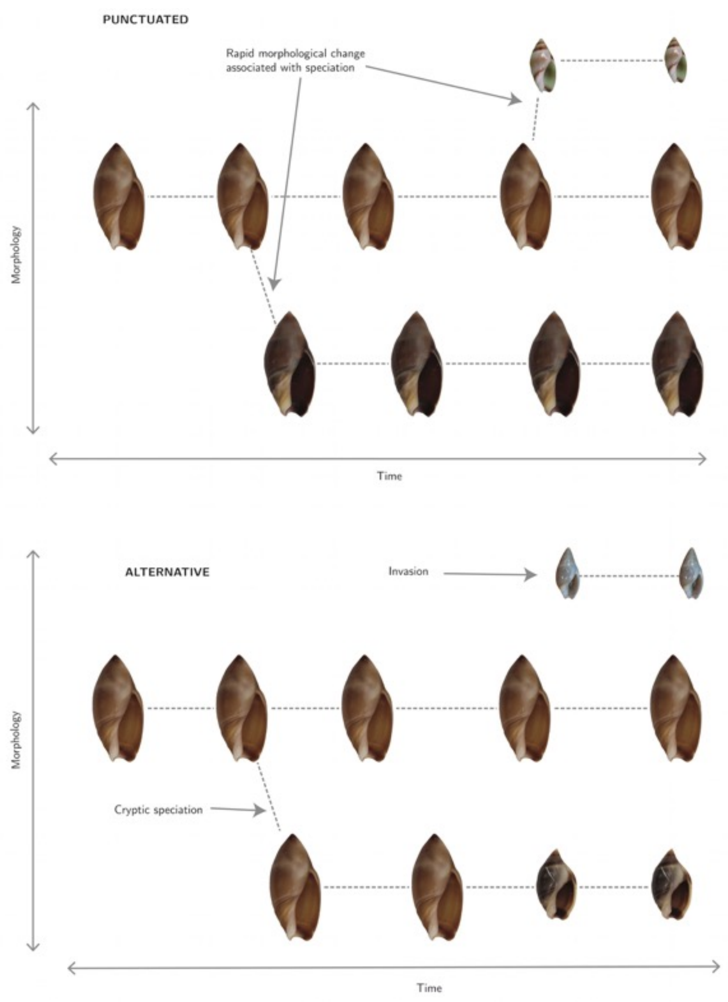


Figure 3.1. Change associated with either punctuated equilibrium (top) or alternative explanations for the appearance of forms in the fossil record. The punctuated hypothesis suggests rapid morphologic change is associated with speciation whereas alternative hypotheses include biological invasions or cryptic speciation and morphologic change not associated with lineage divergence

In previous work, monophyly of the New Zealand *Amalda* was not tested but was assumed on the basis of the perceived insular nature of New Zealand's fauna. For some time, the New Zealand biota has been regarded as a closed system (Bellamy et al. 1990) with the New Zealand land mass having been isolated from other fragments of Gondwana for close to 80 million years, which is reflected in the number of endemic Cenozoic fossil species and the endemicity rates in molluscs (Cooper and Millener, 1993; Crampton et al., 2006; Spencer et al., 2009). Recent evidence suggests that dispersal is a major contributor to New Zealand biodiversity (e.g. Donald et al., 2015, 2005, Trewick et al., 2008, 2007; Trewick and Gibb, 2010; Wallis and Trewick, 2009; Waters et al., 2000). In marine systems the westerly circumpolar current is recognised as a dispersal vector to New Zealand (Beu et al., 1997; Fleming, 1979). The New Zealand *Amalda* species do not occur outside of New Zealand waters. However, there are many other olive shell species recorded around the Pacific and Indian oceans (Kilburn and Bouchet, 1988; Pastorino, 2003), indicating potential for biological exchange over geological time.

Interpretation of the diversity of the Mollusca has been aided by phylogenetic analysis (*e.g.* Dunn et al., 2008; Kocot et al., 2011; Ponder and Lindberg, 1997; Sigwart and Lindberg, 2015; Stöger and Schrödl, 2013). Resolution of some clades within the Gastropoda has progressed through use of multigene trees (Barco et al., 2010; Couto et al., 2016; Fedosov et al., 2015; Puillandre et al., 2014, 2009) and analysis of mitochondrial genomes (Gaitán-Espitia et al., 2013; Grande et al., 2008). The gradual increase in the quantity and quality of data is integral to the resolution of deeper relationships leading to more biologically meaningful understanding of molluscan systematics. One recent study included data from *Amalda* as an out-group (Fedosov et al., 2015) but no focussed analysis of the genus has been done. With larger genetic datasets there is the opportunity to test the monophyly of the New Zealand *Amalda* with confidence in the broader context of worldwide *Amalda* and at the same time infer the evolutionary relationships among New Zealand *Amalda* species.

Having data from a number of genes and non-coding regions helps ameliorate some problems in phylogenetic inference caused by analysis of fragmentary gene data and too few loci. For example concerted evolution (Hillis and Dixon, 1991; Stage and Eickbush, 2007) can lead to conflicting interpretations of phylogeny based on different genes. Two sources of genetic markers are used in this study, whole mitochondrial genomes and 45S ribosomal DNA. DNA sequences of both these markers have been used extensively to infer phylogenies (Avise, 1994; Whiting et al., 1997). MtDNA is the most commonly used source of data for animal phylogenetics because it has several characteristics that make it useful: high number of copies, maternal inheritance (usually e.g. Gusman et al., 2016; Passamonti et al., 2011) and lack of recombination. A fragment of the mitochondrial Cytochrome Oxidase subunit I has been used as a DNA barcode (Meusnier et al., 2008) and sequence data for a diverse range of organisms are publically accessible. There is some uncertainty about how well the assumption of neutrality is being met (Galtier et al., 2009; MacRae and Anderson, 1988) but still it remains a cheap and popular marker. The nuclear ribosomal-cassette 45s rDNA is inherited biparentally, but evolves via concerted evolution (Liao, 1999; Naidoo et al., 2013). The use of multi locus trees in a Bayesian framework is seen as being more robust and multi gene trees may provide more reliable phylogenies than concatenation of genes (Cronin et al., 2014; Heled and Drummond, 2010). Here I infer the phylogeny of representative *Amalda* species to test the hypothesis that the New Zealand taxa form a monophyletic group.

Materials and Methods

Taxonomic sampling

Amalda specimens were collected from coastal marine habitat around New Zealand using dredging or hand sampling, directly by my team or via contributors (Fig. 3.2). Locations were chosen to provide sampling from within the species' distribution providing sampling from distinct geographic regions intended to cover any geographic variation. Sampling numbers were intended to provide enough specimens from each location for population level variation to be investigated. Live *Amalda* specimens were collected from Nelson Harbour during biosecurity monitoring by The National Institute of Water and Atmospheric Research (NIWA). *Amalda* by-catch from Cloudy Bay in the Marlborough Sounds were provided by Cloudy Bay Clams. Specimens collected from Sprits Bay Northland and near the Three Kings Islands were included as they were considered to be putative new or uncertain species. Additional material came from Te Papa Tongawera National Museum of New Zealand. In total all New Zealand species except *A. bathamae* had genetic material available for study.. The Muséum National d'Histoire Naturelle (MNHN) France, provided specimens of *Amalda* and *cox1* sequences from the Pacific and Africa selected on the basis of its species own unpublished genetic data. Every effort was taken to sample as comprehensively as possible but we recognise that complete taxonomic sampling is not always possible. This analysis includes five species from New Caledonia where seven species are recognised and two species from Australia where 36 species are recognised, 10 of which are present in southern Australia the closest proximity to New Zealand. Available relevant gene sequences were accessed via Genbank, including the full mtDNA of *A. northlandica* (McComish et al., 2010) and mitochondrial genomes of Neogastropod molluscs used as an outgroup (see supplementary table 3.3).

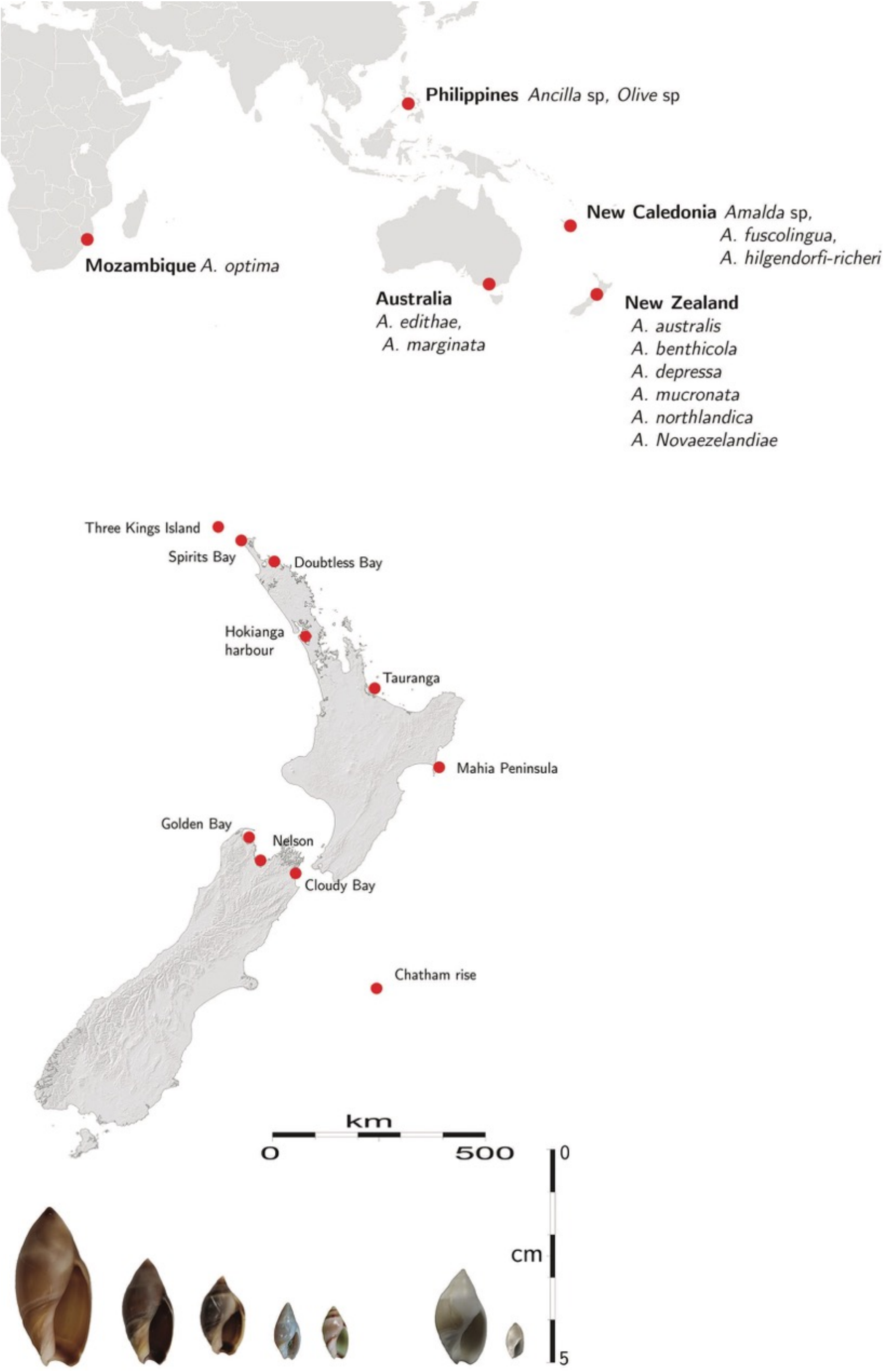


Figure 3.2. Collection locations of marine gastropods in the genus *Amalda* and Olividae outgroups in the western Pacific and Indian oceans. The shells are the New Zealand taxa from left *A. mucronata*, *A. australis*, *A. depressa*, *A. novaezelandiae A. northlandica*, *A. bathamae* and *A. benthicola*

DNA isolation

Fresh caught live snails were frozen after collection and transferred to the laboratory. Specimens were thawed, soft parts removed from the shells and stored in 95% ethanol until required. DNA was isolated from snail foot tissue using either the Geneaid™ column extraction kit or a modified CTAB extraction protocol involving clean up with Ampure™ microbeads as necessary (Doyle and Doyle, 1990). In most cases the CTAB method produced higher yields of genomic DNA. DNA quantity and quality were assessed using agarose gel electrophoresis and with Qubit™ broad-range DNA assay.

Short DNA sequences

An initial survey of New Zealand *Amalda* was undertaken using analysis of a short fragment of the mitochondrial cytochrome oxidase (*cox1*) to clarify lineage assignment. This assisted the selection of samples for high throughput (next-generation) DNA sequencing. As phylogenetic inference could be misled by inaccurate taxon identification and because single individuals are used to represent a species I ensured that I selected specimens that were genetically confirmed as members of their identified taxa for large scale DNA sequencing. If possible, data were obtained using samples sourced from more than one location within the spatial range of each species.

Primers LCO1490 and HCO2198 (Folmer et al., 1994) were used. Polymerase chain reactions (PCR) were performed in 20 µL volumes containing: 200 µM dNTPs, 2.5µL NEB thermopol 10x buffer, 1 µM primers, 0.20 U of NEB taq DNA polymerase and 1–10 ng of template DNA. Standard thermal cycling conditions were followed, with 50°C annealing temperature and 35 cycles, in a Biometra^{tm} T1 thermocycler. PCR products were sequenced with from the HCO2198 end using BigDye Terminator v3.1 on an ABI 3730 by the Massey

University Genome Service. DNA sequences were visualized, checked for errors and ambiguities in Geneious v.7 (Kearse et al., 2012).

The *cox1* sequences were aligned and variation was visualized using minimum spanning networks (Bandelt et al., 1999) generated using POPART (Population Analysis with Reticulate Trees; (Leigh and Bryant, 2015)). A phylogenetic analysis was separately inferred from an alignment of a region of *cox1*.

Next generation sequencing

Approximately 1000 ng of DNA from each of nine samples representing five New Zealand species (*A. australis*, *A. depressa*, *A. mucronata A. novaezelandiae*, and an uncertain species) and three outgroup species from New Caledonia (*A. bellonarum A. fuscolingua* and *A. hilgendorfi richeri*), and Mozambique (*A. optima*) were selected for high throughput sequencing. Neither A. benthicola and A. bathamae had DNA of sufficient quality to be included in the high-throughput sequencing. Genome libraries were generated and indexed by service providers and submitted for Illumina high throughput sequencing. A library of tagged DNA fragments was constructed for each species. Library construction involved random fragmentation of DNA, size selecting, ligation indexing and pooling into an equimolar mix for running on BigDye terminator. Paired reads were trimmed to remove adapter and index tags using the cutadapt code implemented in Python. Short DNA reads were mapped to a published *Amalda northlandica* mitochondrial genome (Genbank accession GU196685.1), using the Geneious mapping algorithm in Geneious v. 7 (Kearse et al., 2012) at medium-low sensitivity and iterated up to five times for each library. Iterations involved remapping reads to the previous consensus sequence until coverage did not increase further. A consensus sequence was then generated from the mapped reads for each sample. Gene annotations were assigned from the *A. northlandica* reference genome (McComish et al., 2010) and verified with start stop codons and protein translation.

The 45S ribosomal DNA cassette of each specimen was extracted from the short DNA reads using the same techniques as the mitochondrial genomes. A reference 45S sequence for the RNA cassette (28S, ITS1, 5.8S, ITS2, 18S) was obtained from unpublished data and short DNA reads mapped iteratively.

Phylogenetic reconstruction

Phylogenetic analysis of Neogastropoda was inferred to provide a broader context using protein coding genes acquired from representative mitochondrial genomes accessed from Genbank and our data (supplementary Table 3.3). Mitochondrial genomes, and the 45S ribosomal DNA cassette sequences were aligned using Geneious using the default settings on the Geneious alignment tool. Alignments were checked by eye for anomalies and translated where appropriate to ensure genes were in agreement with the reading frame.

Complete mitochondrial genome and 45S sequence alignments were used to infer phylogenetic trees and these were compared to trees constructed from an alignment of protein coding regions and 16S and 12S (mtgenome) or 18S 5.8S and 28S (45S). No topologic differences were noted and so the full mtgenome and 45S were used in all downstream analyses. Phylogenetic trees were also inferred from data sets from which sequence regions with missing data (no coverage for some taxa) had been omitted. Partition finder (Lanfear et al., 2012) was used to partition genes and to infer the model of nucleotide evolution to use. GTR + Gamma + I trees were constructed via Bayesian inference using mrBayes (in Geneious) (Huelsenbeck and Ronquist, 2001) (Geneious 7 plugin), 10,000,000 iterations and a burn-in of 2,000,000. Maximum likelihood inferences were performed using RAxML, with gene partitions assigned using Partitionfinder (Lanfear et al., 2012). A rapid hill climbing algorithm was implemented with 500 bootstrap replicates.

Results

Sample selection and *Amalda* taxonomy

A 585 bp region of *cox1* was aligned from 61 individuals of the New Zealand *Amalda*. These *cox1* DNA sequences had 79.7% identical sites, and showed no evidence of ambiguities, indels, or stop codons that are indicative of nuclear copies. Haplotypes from representatives of the same species collected at different locations cluster together in the minimum spanning tree (Fig. 3.3)

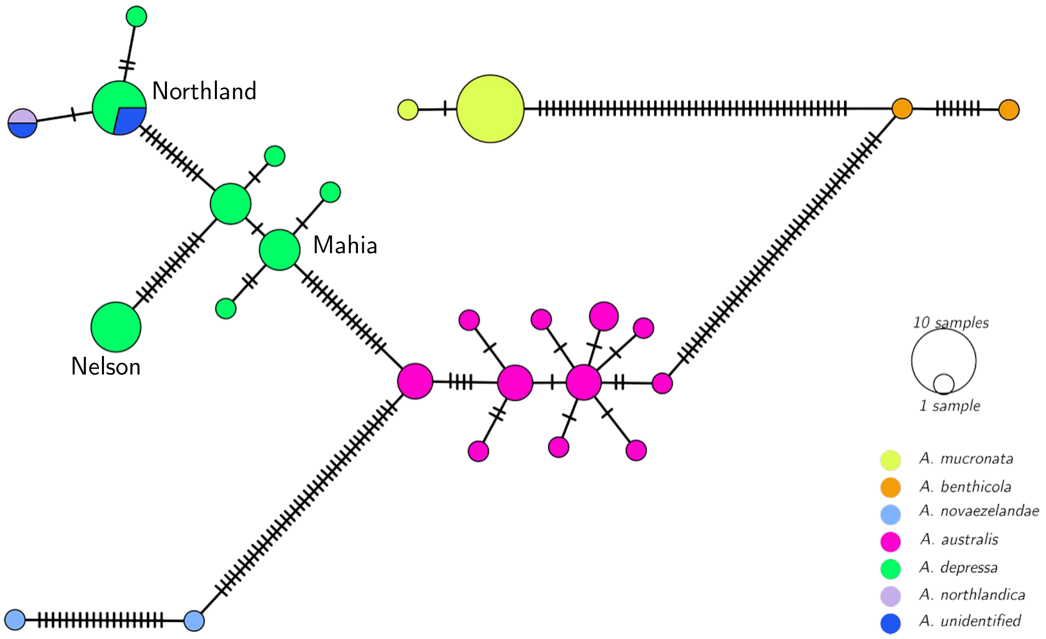


Figure 3.3. Clear taxonomic clustering found with minimum spanning network of mtDNA haplotypes (585 bp of *cox1*) of New Zealand six *Amalda* species. Dash marks on branches indicate single nucleotide substitutions. Collection locations of *A. depressa* are shown to highlight the geographic based genetic structuring in this species.

Analysis of *cox1* haplotypes support current taxonomy. However, haplotypes from the specimen of *A. northlandica* and the unidentified specimens from Spirits Bay are grouped within the *A. depressa* haplotype cluster. Specimens selected for high throughput sequencing therefore represent four distinct New

Zealand species (*A. mucronata*, *A. novaezelandiae*, *A. australis* and *A. depressa*), plus one of the specimens representing a possible unidentified species from the far north of New Zealand.

Taxonomic relationships in *Amalda*

Preliminary phylogenetic relationships were inferred using partial *cox1* sequences from 77 specimens of *Amalda* and related species. All species are part of the family Olividae collected from New Zealand, Australia, New Caledonian, Philippines and Africa. The New Zealand species formed a monophyletic clade sister to a clade with two species from Australia (*A. marginata*, and *A. edithae*) and *A. optima* from Africa. This clade is sister to three New Caledonian taxa (*A. hilgendorfi richeri* and two undescribed species; Fig. 3.4). Four *Amalda* species were selected as suitable outgroups for next generation sequencing: *A. bellonarum A. optima* and *A. fuscolingua* and *A. hilgendorfi richeri.* Suitable material for high throughput sequencing NGS was not available for *A. benthicola* (from New Zealand) and *A. marginata*, *A. edithae.* No DNA was available from *A. bathamae*.. The positioning of *A. benthicola* in the *cox1* tree indicates that it is part of the New Zealand *Amalda* clade. Having the New Zealand species, *A. bathamae* and *A. benthicola* missing from genomic analysis should not unduly effect subsequent interpretation of evolutionary patterns as they are not represented in the fossil record.

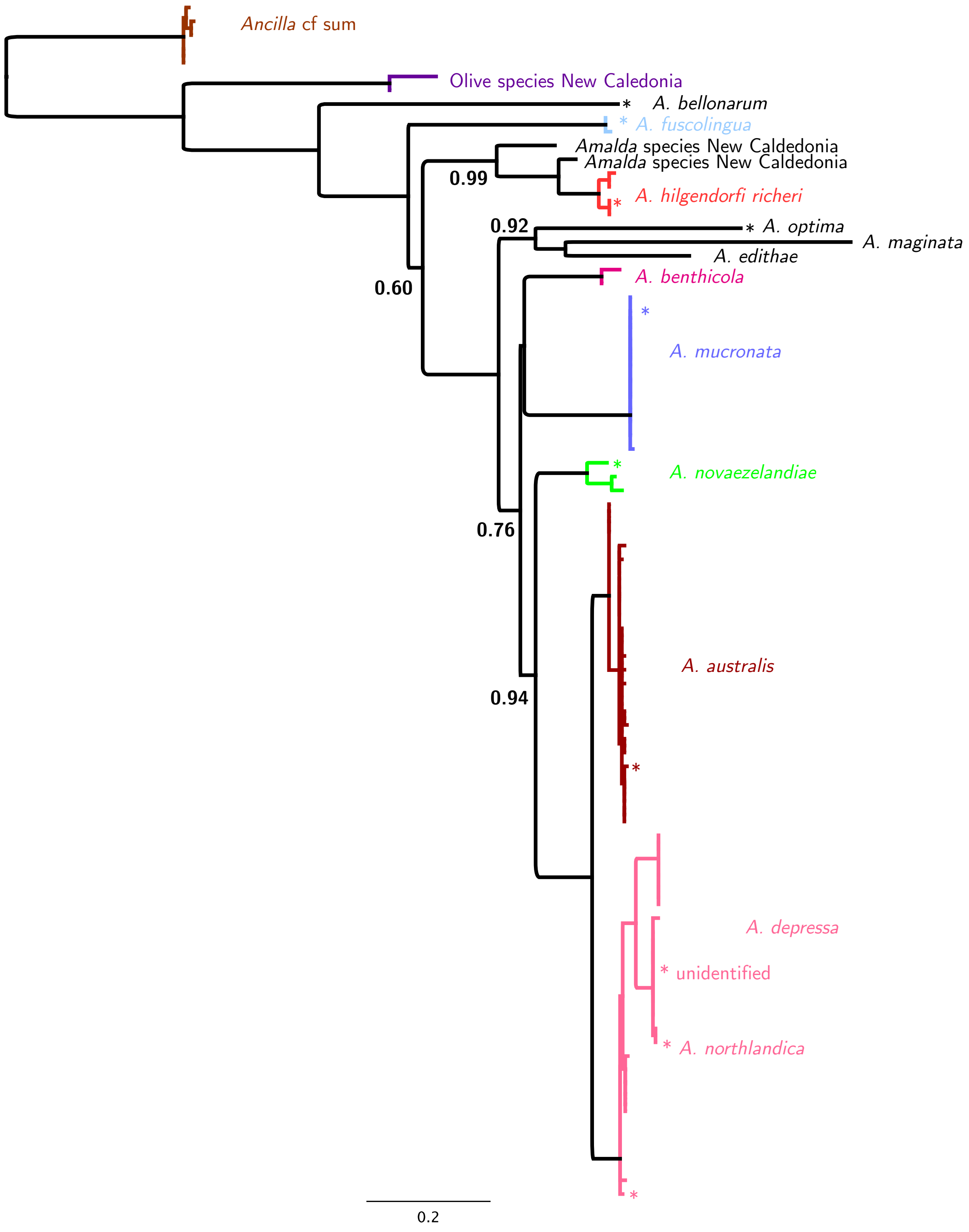


Figure 3.4. Phylogeny of Olive shell species (*Amalda*) inferred from an alignment of 585 bp of the mitochondrial *cox1*. *indicates samples used for whole mitochondrial genome and 45S analysis. Outgroups are a Phillipene *Ancilla* species and an unidentified olive shell species. Bayesian posterior probability indicated where less than 1. (MrBayes Chain length 1,000,000 burn-in 10,000 within species posterior probability omitted)

Mitochondrial genomes and rDNA sequences

Published full mitochondrial genomes of the Neogastropoda are few. Only four of the eight superfamilies have full genomic sequences available for any of their members. Phylogenetic analysis of 11149 bp of concatenated mitochondrial protein coding regions indicate that for specimens included three of the superfamilies form monophyletic clades while Muricoidea is paraphyletic. The Olivoidea are represented solely by *Amalda* specimens (Fig. 3.5).

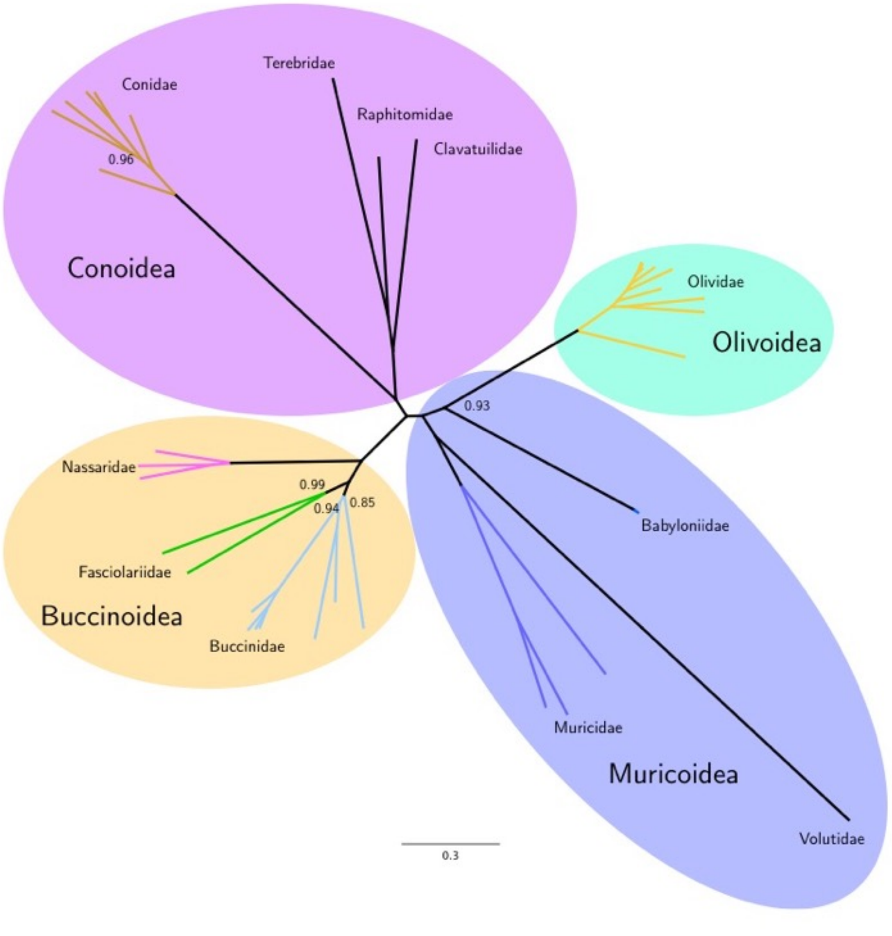


Figure 3.5. Unrooted phylogenetic tree of Neogastropod molluscs based on Bayesian analysis of protein coding genes from Neogastropod molluscs with fully sequenced mitochondrial genomes (11149 bp). Implemented in MrBayes plugin in Geneious 10 1,000,000 iterations 100000 burnin. Posterior probabilities are indicated where less than one. Not all Neogastropod superfamilies or families are represented. Buccinoidea Conoidea and Olivoidea are monophyletic superfamilies. Muricoidea is paraphyletic.

New complete mitochondrial genomes were assembled from nine *Amalda* samples using Illumina sequencing. Mitochondrial DNA sequence length ranged from 15249 to 15403 bp (Table 3.1). There was a wide range in average coverage for the samples; from 67.4 reads per site in *Amalda optima* to 3.9 in *A. hilgendorfi richeri*. In four species there were small areas where coverage dropped to zero and therefore the genomes are not complete (Table 3.1). The mitochondrial genomes of the *Amalda* contain many (36.8%) variable sites, providing information for inferring evolutionary relationships.

Table 3.1. Summary of statistics for mitochondrial genomes and 45S cassettes constructed in this study. *A. northlandica* is from (McComish et al., 2010)

		mt genome				45S			
	Reads	Reads mapped	Mean coverage	% missing	Length	Reads mapped	Mean coverage	% missing	45s length
A. australis	33306422	7657	47.5	0	15403	13489	187.0	0	5984
A. bellonorium	4399343	1491	11.2	0	15249	2954	45.9	0	5820
A. depressa	77366938	5864	18.6	0.4	15347	18079	113.9	0	5809
A. edithae	16977846	2664	17.1	0	15332	16676	31.0	0	5998
A. fuscolingua	3065376	511	4.1	0.7	15357	13361	4.1	0	5813
A. hilgendorfi-richeri	4135126	343	3.7	23.7	14354	3195	41.1	0	5950
A. marginata	15583022	2765	18.5	0	15072	2214	134.8	0	6083
A. mucronata	28217402	4698	30.5	0	15393	34009	279.2	0	5963
A. novaezelandiae	79893938	5099	15.1	0	15331	55096	425.4	0	5839
A. optima	9166880	1158	9.4	0	15339	4562	67.4	0	5974
A. spp (Spirits Bay)	20558288	1538	9.9	11	15358	7479	124.7	0	6010

The full DNA sequences of 45S DNA cassettes were assembled from each specimen (mean length 5971 bp; Table 3.1). ITS 1 ranged in size from 510 to 556 bp, ITS 2 ranged from 124 to 338 bp. The 45S was much more conserved than the mitochondrial genome, varying among species by no more than 4.1%.

Phylogenetic analysis of mtDNA genomes and nuclear 45S produced similar topologies, including monophyly of the six New Zealand taxa (Fig. 3.6). There was some discordance between the mitochondrial genome tree and the nuclear ribosomal DNA tree in the placement of two outgroup species *A. optima* and *A. fuscolingua*; either sister to each other (mtDNA) or *A. optima* was sister to all except *A. bellonarum* (45S).

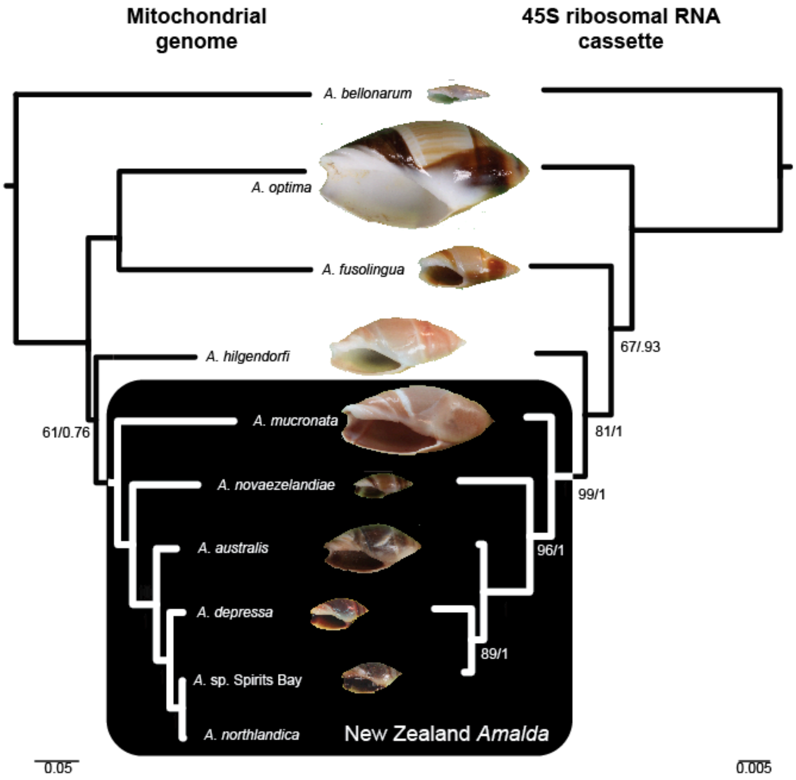


Figure 3.6. *Amalda* Phylogenies based on analysis of mitochondrial genome and 45S sequences. Likelihood scores from RAxML Bayesian posterior probabilities are shown at nodes except where they are certain (100/1).

Discussion

Four species of New Zealand *Amalda* have previously been examined for concordance of taxonomy (based on shell shape and colour) and allozyme loci (Michaux, 1987). Combinations of these marine snail species occur in sympatry, so fixed differences at nuclear loci provide strong evidence of reproductive isolation and support for four taxa (Michaux, 1987). Clustering of mitochondrial haplotypes observed here is consistent with the current species taxonomy and nuclear genetic markers (Michaux, 1987). The inclusion of the New Zealand species *A. benthicola* revealed variation concordant with it being a distinct species. The haplotype from *A. northlandica* as well as the putative new species formed part of a genetic cluster with *A. depressa*. Given that *A. northlandica* is a distinct recognised species (Hart, 1995), it is most likely that the *A. northlandica* specimen used for genetic analysis was misidentified and is actually an *A. depressa* specimen. The unidentified species is likely a colourmorph of *A. depressa.*

Phylogenetic analysis of *Amalda* using new DNA sequence data conflicts with current division of the genus into the subgenera *Gracilispira* and *Baryspira.* These two taxonomic groups were not resolved in any DNA based phylogenetic trees. *A. novaezelandiae* (*Gracilispira*) is nested within the New Zealand group as sister to the clade including *A. australis* and *A. depressa* (both *Baryspira*). In the *cox1* analysis *A. benthicola* (*Gracilispira*) is sister to *A. mucronata* (*Baryspira*), and together sister to other New Zealand *Amalda.*
Amalda novaezelandiae first appears in the fossil record around 5mya (Beu and Maxwell, 1990), before the appearance of *A. depressa.* The DNA based phylogeny for *Amalda* fits approximately with this order of appearance although *A. novaezelandiae* is a representative of the *Gracilispira* group that is recognised

appearing 53mya (Beu and Maxwell, 1990). Genomic mtDNA and 45S indicate that *Gracilispira* is not a distinct clade from the *Baryspira*. Recognising taxonomic groups based on subtle morphological differences in fossil material can be problematic, especially in a group where convergence between taxa based on shell shape is common (Hills et al., 2012).

Monophyly

Few mitochondrial genomes have been sequenced for Neogastropod molluscs. A phylogenetic inference based on protein coding genes of available Neogastropod molluscs from four superfamilies showed that three of the four superfamilies were monophyletic. The exception being Muricoidea which are paraphyletic here, resonating with other analyses which show the resolution of the Muricoidea to be somewhat contentious (Barco et al., 2010; Couto et al., 2016). The Olivoidea are represented only by the genus *Amalda* in the mtgenomic phylogeny. Some indication of the relationships between the clades can be inferred but the lack of representatives of both superfamilies and families means little more can be inferred.

Within *Amalda* there was strong support for the monophyly of the New Zealand species of *Amalda*, this is consistent with a single evolutionary lineage in the region (Cooper and Millener, 1993; Daugherty et al., 1993; Spencer et al., 2009). Monophyly of the New Zealand *Amalda* was supported by phylogenetic inference from both mitochondrial genomes and 45S. The only differences in phylogenetic relationships inferred from mitochondrial genome and 45S was the formation of a clade consisting of *A. optima* and *A. fuscolingua* sister to the clade containing the New Zealand *Amalda*, absent from the 45S phylogeny. Both the *cox1* and genomic data show monophyly of the New Zealand group within a wider context including representatives from Australia and the Pacific covering the closest landmasses to New Zealand and the most likely sources of dispersal to New Zealand.

Low read coverage during assembly of mitochondrial sequences for some samples resulted in some gaps in the *A. depressa*, *A. fuscolingua* and *A. hilgendorfi richeri* and the unidentified *Amalda* species datasets. It is unlikely that this loss of information would be detrimental to the phylogenetic analysis especially when using Bayesian and Likelihood methods (Guillerme and Cooper, 2016; Wiens and Moen, 2008; Wiens and Morrill, 2011). Although the node separating *A. hilgendorfi richeri* and the New Zealand clade had relatively low support, no analysis placed *A. hilgendorfi richeri* within the New Zealand clade. Relatively low node support may be an artefact of the missing data in the *A. hilgendorfi richeri* genome reducing support but not strongly influencing placement.

The inclusion of undescribed species from New Caledonia emphasises the work still required for collecting and describing Pacific olive shells. Lack of sampling (due to extinction or rarity) will always limit phylogenetic inference (Crisp et al., 2011; Grandcolas et al., 2014). Inclusion of the species from the coast of Australia into genomic analyses would be valuable, although the inclusion of two species in the *cox1* phylogeny indicate they are probably outside the New Zealand clade.

Conclusions

Armed with strong evidence that the extant *Amalda* species that occur as fossils are part of a monophyletic New Zealand clade, inferences about the evolutionary patterns seen in the fossil record can be made with more confidence. It is highly likely that morphological changes detected in the New Zealand fossil record are the result of evolutionary events *in situ*. And thus it is less likely that a new morphological form is the result of the arrival of a new species from elsewhere in the Pacific. New phenotypes can be inferred to result from evolution of the local lineages. Support for the monophyly of New Zealand *Amalda* provides an unusually good opportunity to test another prediction of punctuated equilibrium: that phenotypic change will occur simultaneously with speciation (and genetic cladogenesis). However, the unbiased accurate identification of specimens to the correct species lineage based solely on the shells needs to be examined in the light of the difficulty with genetically separating *A. depressa* and *A. northlandica* and the recent collection of specimens with uncertain species designation.

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Supplementary table 3.3. Species used for Neogastropod mollusc phylogeny. Genbank accession numbers indicated where available

Species	Genbank accession number
Cominella adspersa	
Penion sulcatus	
Buccinum undatum	
Volutopsius norwegicus	
Buccinulum pallidum	
Taron dubius	
Glaphyrina caudata	
Amalda northlandica	gi:27048647
hilrich Hilgendorf-richeri	
Amalda fuscolingua	
Amalda bellonarum	
Amalda optima	
Amalda spp	
Amalda mucronata	
Amalda novazelandiae	
Amalda australis	
Amalda depressa	
Nassarius reticulatus	gi:192757886
Cymbium olla	gi:192757858
Conus tribblei	gi:924859541
Conus tulipa	gi:1025726014
Conus textile	gi:110809860
Conus gloriamaris	gi:1025815325
Conus consors	gi:575525360
Conus borgesi	gi:192757844
Terebra dimidiata	gi:213390631
Bolinus brandaris	gi:192757907
Fusiturris similis	gi:192757830
Rapana venosa	gi:157696072
Reishia clavigera	gi:161561766
Lophiotoma cerithiformis	gi:82395857
Babylonia areolata	gi:311788616
Babylonia lani	gi:316992410
Buccinum pemphigus	gi:977902732
Volutharpa perryi	gi:937500896
Ilyanassa obsoleta	gi:78057821
Varicinassa variciferus	gi:702073820

Chapter 4

Clarifying Species Boundaries in the New Zealand Gastropod genus *Amalda* H. and A. Adams, 1853 (Neogastropoda: Olividae) Using Shell Shape Components and Genetic Markers

Introduction

Delimiting evolutionary lineages can be difficult because evolution is continuous and non-directional, and there are many opportunities for boundaries between lineages to be transgressed for example morphological (cryptic species) or genetic (introgression) (Vaux et al., 2016). Thus, it is not surprising that there are so many examples of difficultly in clear delimitation of species (Dowle et al., 2015; Sites and Marshall, 2003; Zuccarello et al., 2015). In studying the evolution of a species, it helps to recognise that species are arbitrarily delineated segments of lineage extending through time i.e. chronospecies (de Queiroz, 2007, 1998; Hunt and Rabosky, 2014). For an extant species this segment of time is the present, and boundaries between lineages can be tested using genetic tools. For fossil species the time range over which a species segment exists can expand to arbitrary boundaries differentiating a species from its ancestor or descendant species. Clarifying these boundaries is difficult but still possible if one assumes that an accepted species in the fossil record has some correspondence to what is regarded as a species in the modern fauna (Allmon, 2016; Jablonski, 2000). This assumption is of great importance when trying to equate patterns seen in the fossil record with theories of evolution. The assumption can be called into question because of the many examples of cryptic species and polymorphic species, especially in marine taxa where strong selection pressures drive both convergence and divergence (Allmon and Smith, 2011; Baker et al., 2004; Hills et al., 2012; Palmer, 1990; Zuccarello et al., 2015). Can we assume then, as in the modern fauna, that most identified fossil species are what they seem, not cryptic nor parts of a polymorphic species? Different species concepts can potentially influence interpretations of the fossil record (Ezard et al., 2012). When examining species closely to interpret evolutionary patterns during lineage splitting, different approaches to defining a species need to be taken into account in order for accurate descriptions of processes to be made.

Genetic tools can provide hypotheses of the evolutionary relationships among individuals, populations, and species. However, aside from a few notable examples of ancient DNA sequences (e.g. Huynen et al., 2003; Orlando et al., 2013; Ritchie et al., 2004) genetic data are constrained to extant species and therefore cannot help with identification of fossil species. Morphological information remains an essential source of information because its application can be extended from modern populations through the fossil record to yield a sequence of data showing evolutionary change. Combining data from genetic and morphometric approaches brings confidence to species delimitation as it provides the opportunity to link past and present forms as lineages, and it allows an inference of reproductive isolation of lineages even when the only data are phenotypic.

New Zealand *Amalda* H. & A. Adams, 1853 comprises species that are morphologically similar to each other with some subtle colouration and shape differences. There is some size variation in adults, which range from around 10 mm to 50 mm (Hart, 1995). These minor colour differences and general similarity of size and shape make it difficult to clearly distinguish some species from one another, especially in the fossil record in which some or all colour is lost. The correct identification of *Amalda* species in the fossil record has broader implications for the understanding of fundamental evolutionary processes. Agreement was found between genetic and morphological species in the New Zealand *Amalda* (Michaux, 1987) and based on simple morphologic measurements, these lineages were estimated to have undergone evolutionary stasis in the fossil record (Michaux, 1989). New Zealand *Amalda* were cited by Gould (2009, 1991) as displaying evidence for punctuated equilibrium. However, it was not demonstrated that individuals could be correctly assigned to separate phenotypic clusters concordant with genetic data without *a priori* identification. Here I examine species delimitation in New Zealand *Amalda* using new tools for documenting both genetic and morphological variation.

Analysis of allozyme data representing sympatric populations demonstrated that some extant species of *Amalda* are reproductively isolated even in sympatry (Michaux 1987). Here I use a combination of nuclear markers (generated via high throughput sequencing) and mitochondrial haplotype data (from PCR and Sanger sequencing) to extend previous work based on allozyme variation (Michaux 1987), and to independently identify specimens of New Zealand *Amalda.* A geometric morphometric approach is used to analyse the shell shape variation of the New Zealand monophyletic *Amalda.* A combination of landmarks and semilandmarks is used to capture the shape of the external morphology of the *Amalda* shells. Landmarks are fixed points at homologous locations on the shell, for example the tip of the teleoconch spire and end of the siphonal canal. Semilandmarks are positioned along curves of the shell such as the external outline and curves of the aperture. Semilandmarks are slid along the curve relative to fixed landmarks in order to remove spurious effects of arbitrary placement on the contour, and to enable comparison of the homologous curves among specimens (Webster and Sheets, 2010; Zelditch et al., 2004). Shell phenotype analysis using this method is effective in discovering variation among ecotypes and species (Collins et al., 2013; Dowle et al., 2015; Hills et al., 2012). Genetic and morphometric variation is compared to test the hypothesis that *Amalda* morphospecies recognised in the current taxonomy are concordant with patterns of genetic and shell shape variation. I aim to identify shell shape characteristics that delimitate species recognised as genetic clusters.

Materials and Methods

Sampling

Extant New Zealand *Amalda* comprise seven recognised, described species from two subgenera (*Baryspira* Fischer 1883: *A. australis* Sowerby I, 1830, *A. bathame*, Dell, 1956 *A. depressa* Sowerby II, 1859 and *A. mucronata* Sowerby I, 1830; *Gracilispira* Olsen 1956*: A. benthicola*, Dell 1956, *A. northlandica* Hart, 1995, and *A. novaezelandiae* Sowerby II, 1859). Another subgenus is recognised in the fossil record the *Spinispira* Olsen 1956. Two other species were formerly recognised but synonomised after consideration of available data (*A. crystallina*, colour form of *A. novaezelandia*, and *A. southlandica*, juvenile *A. mucronata*) (Hart, 1995).

The three species of particular interest, with good sampling of both genetic material and fossil specimens are *A. australis*, *A. depressa*, and *A. mucronata. Amalda australis* lives in sand to a water depth of 20 m below sea level and has a dark shell with a white spiral line along the margin, and a basal groove the same colour as the main body whorl. The shell of *A. depressa* is similar in colour and shape to that of *A. australis* but is generally shorter and the outer lip of the body whorl projects beyond the spire outline. *Amalda mucronata* usually has a larger shell than *A. australis* and *A. depressa*, and it often has a large amount of callusing on the spire. The shell of *A. mucronata* is more brown-orange and lighter than that of *A. australis* or *A. depressa*, with a white basal groove (Hart, 1995)(Table 4.1).

Amalda specimens were collected from coastal marine habitat around New Zealand using dredging or hand sampling (Fig. 4.1 & Table 4.2). Locations were chosen to provide sampling from within the species' distribution providing sampling from distinct geographic regions intended to cover any geographic variation. Sampling numbers were intended to provide enough specimens from each location for population level variation to be investigated. Live *Amalda* specimens were collected from Nelson Harbour during biosecurity monitoring by The National Institute of Water and Atmospheric Research (NIWA). *Amalda* by-catch from Cloudy Bay in the Marlborough Sounds were provided by Cloudy Bay Clams. Specimens collected from Sprits Bay Northland and near the Three Kings Islands were included as they were considered to be putative new or uncertain species. Additional material came from Te Papa Tongawera National Museum of New Zealand. Morphometric analysis included 26 *A. mucronata* samples from Michaux (1987) (collection held by Auckland University), identification of which had been confirmed using nuclear genetic markers. Specimens of *A. northlandica* were included in morphometric analysis without corresponding genetic data. Samples previously recognised as *A. crystallina* were included in some analyses to test the synomisation of the species with *A. novaezealandiae* using geometric morphometric techniques. These specimens were catalogued into museum collections prior to Hart's (1995) work. The New Zealand species *A. bathame*, was not included in the analyses because of the lack of suitable samples for both DNA extraction and morphometric analysis. *A. benthicola* is only included in some genetic analyses.

Table 4.1 List of species with distributions and taxonomically informative characteristics summarised from Hart (1995)

Species	Shell colour	Basal groove colour	Callusing	Outer lip	Relative size	Distribution	Depth
A. australis	dark	dark	sometimes		medium	NI + East coast South Island	<20m
A. mucronata	lighter	white	often		larger	NZ	<450m
A. depressa	dark	dark	sometimes	Projects out	shorter	NI + East coast South Island	<20m
A. northlandica	pale / banded	white	sometimes		small	East coast Northland	<20m
A. novaezelandiae	pale	white	sometimes		small	NI + East coast South Island	<650m

See supplementary Table 4.5 for information about specimens used.

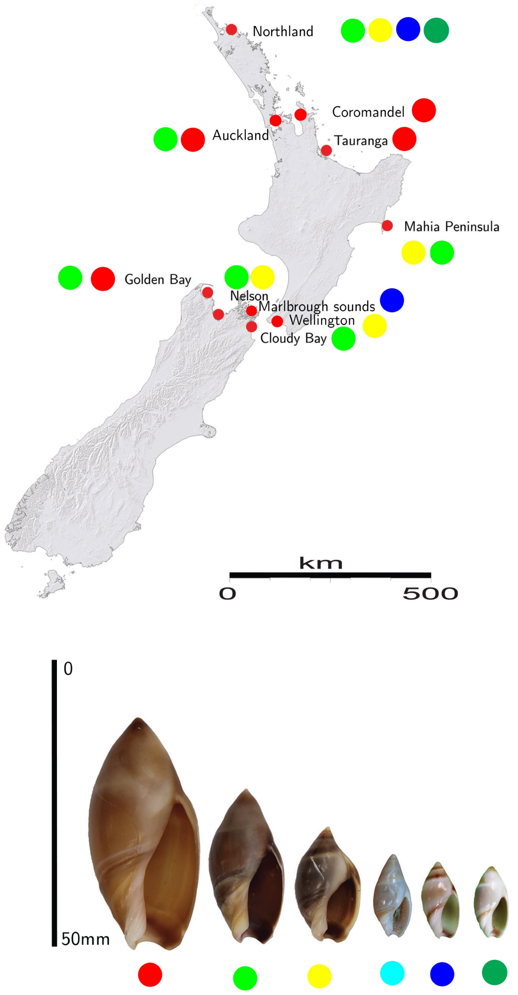

Figure 4.1. Sampling locations of New Zealand *Amalda* used for genetic and morphometric analysis. Shells from left *A. mucronata* (red), *A. australis* (bright green), *A. depressa* (yellow), *A. novaezelandiae* (light blue), *A. northlandica* (dark blue), *A. crystallina* (synonomised with *A. novaezelandiae*)(dark green).

Table 4.2. Collection locations of samples used in morphometric analysis.

Location	*A. australis*	*A. crystallina*	*A. depressa*	*A. mucronata*	*A. northlandica*	*A. novaezelandiae*
Northland	15	27	9	13	12	0
Auckland	36	0	0	12	0	0
Coromandel	0	0	0	13	0	0
Tauranga	0	0	1	0	0	1
Mahia Penninsula	3	0	9	0	0	0
Wellington	0	0	13	0	0	0
Golden Bay	2	0	0	9	0	0
Nelson	9	0	9	0	0	0
Marlbrough sounds	0	0	0	0	0	17
Cloudy Bay	9	0	0	0	0	0
Total	74	27	41	47	12	18

Genetics

Analysis of allozyme data have demonstrated that some extant species of *Amalda* are reproductively isolated even in sympatry (Michaux 1987). Here I use a combination of nuclear markers (generated via high throughput sequencing) and mitochondrial haplotype data (from PCR and Sanger sequencing). Freshly collected live snails were frozen after collection and transferred to the laboratory. Specimens were thawed, and bodies removed from shell before being stored in 95% ethanol until required in the laboratory. DNA was isolated from snail foot tissue using either the Geneaid™ column extraction kit or a modified CTAB extraction protocol (Doyle and Doyle, 1990; Trewick et al., 2009). In most cases the CTAB method produced higher yields of genomic DNA. DNA quantity and quality were assessed using agarose gel electrophoresis and with Qubit™ broad-range DNA assay.

mtDNA sequences

A short fragment of mitochondrial Cytochrome Oxidase subunit I (*COX1*) was amplified using the PCR primers and LCO1490 (Folmer et al., 1994). This region has been used widely in DNA (barcoding) species delimitation (Meusnier et al., 2008). Polymerase chain reactions (PCR) were performed in 20 µl volumes containing: 200 µM dNTPs, 2.5µL NEB thermopol 10x buffer, 1 µM primers, 0.20 U of NEB taq DNA polymerase and 1—10 ng of template DN*A*. Standard thermal cycling conditions were followed, with 50°C annealing temperature and 35 cycles, in a Biometra^{tm} T1 thermocycler. PCR products were sequenced with both primers using BigDye Terminator v3.1 chemistry on an ABI 373. DNA sequences were visualized, checked for errors and ambiguities in Geneious v8 (Kearse et al., 2012). MtDNA haplotypes were compared using minimum spanning networks (Bandelt et al., 1999) generated using PopART (Population Analysis with Reticulate Trees; (Leigh and Bryant, 2015). This allowed unequivocal assignment of specimens to haplotype clusters.

Nuclear markers (DDrad Seq)

Sampling for nuclear markers focused on a pair of species that are morphologically similar and widely sympartric (*A. australis* and *A. depressa*). Anonymous Single Nucleotide Polymorphic nuclear markers (SNP) were generated following the protocol outlined in Peterson *et al.*, (2012) with minor modifications. The enzymes used for digestion of genomic DNA were Nsil HF and MboI, which were selected after a trial of potential enzymes to determine the best cutting efficiency. Whole genomic DNA extracts of 105 *Amalda* samples were digested with both enzymes. Short barcode sequences were ligated to fragments using Invitrogen T4 Ligase to enable identification of individuals after pooling of samples for sequencing. High throughput sequencing was performed using a Illumina Hi-seq platform (New Zealand Genomics Limited) and the resulting data produced were processed using the STACKS pipeline (Catchen et al., 2013).

After initial tests using the STACKS pipeline to assess information content samples with file sizes of less than 2mb were removed from the analysis. These files had low read numbers and their inclusion reduced analytical power downstream. The quality control process resulted in only samples of *Amalda australis* and *A. depressa* being available for further processing. These two species provided data for examining the most closely related species from sympatric distributions. The denovo map function in stacks was used to build a catalogue. A minimum of five reads per stack (-m5) was set and resulted in a reasonable level of coverage for each individual. I allowed a mismatch of 3 bases when combing stacks within an individual (–M3). Up to 5% –n 5 sequence variation per locus was allowed, enabling comparison of two species for which some sequence variation is expected. Otherwise default settings in the STACKS pipeline were used (Catchen et al., 2013).

The final population setting used were -k -p 1 -r 0.4 -f p_value --write_single_snp --fstats --min_maf 0.1. These were chosen based on trials performed (Chapter 2). The data were analysed as a single population to ameliorate the possibility of misidentification of samples. The proportion of individuals that contained a locus (the r value) was set to 0.4 since values above this yield few informative loci, and having missing data is preferential to a large number of lost loci. The first SNP from each stack (=locus) was used so each can be treated independently. A minimum allele frequency of 0.1 was used to reduce the number of rare alleles that could confound results. If the dataset were larger, low frequency alleles could provide important information about diversity but here with small number of individuals they have the potential to be misleading.

Bayesian clustering analysis was applied to resulting genotypes using Structure (Evanno et al., 2005) in order to identify patterns of genetic differentiation in samples of *A. australis* and *A. depressa*, and to identify geographic partitioning. Structure was run with 50000k burnin and 1000000 iterations with K set to 1–6 and the optimal K was chosen using structure harvester (Earl and vonHoldt, 2012).

Morphometrics

Geometric morphometric analysis included 218 *Amalda* specimens from 10 locations around the coast of New Zealand (Table 4.2 & Fig. 4.1).

Digital images of the shells were obtained following the protocol in Dowle (2015) and Chapter two (this thesis). Shells were placed in a bed of contrasting coloured sand with ventral surface upwards and positioned so the aperture was horizontal. Digital images were obtained using a Canon EOS 600d with EF100 mm f2.8 USM macro lens mounted on a high-precision Kaiser stand. Two digital ‘combs’ were positioned over images of each shell using Adobe Photoshop cs6. (Combs were placed as shown in supplementary Fig 4.15). Digitising was undertaken in tpsdig2 2.17 (Rohlf, 2013) on a Wacom Cintiq 22HD tablet. Digitised semi-landmarks were slid using SEMILAND, part of the imp714 package (Sheets, 2012; Zelditch et al., 2004), implementing the Procrustes distance method. Landmark xy-coordinates were then imported into MorphoJ 1.05f (Klingenberg, 2011) for nonparametric statistical analysis. An initial set of three fixed and 42 sliding landmarks were positioned on the curves of the shell. The number of landmarks was reduced to two fixed and 21 sliding landmarks for further analysis (supplementary Fig 4.15). This was done to reduce problems of degrees of freedom where the number of landmarks are higher than the number of samples and the possibility of type I errors, which are more likely with datasets with high numbers of landmarks. Discriminant analysis used leave one out cross-validation and 1000 permutations among groups based on the current taxonomic treatment. I tested the influence of removing some semi-landmarks from the analysis using discriminant analysis with cross-validation and 1000 permutations. Landmarks removed from analysis were chosen after examination of thin plate spline wireframe graphs to maintain informative loci.

To quantify the amount of error introduced into analysis from the digitising process, a disparity test was performed in Geomorph v 3.0.3 (Adams and Otárola-Castillo, 2013) implemented in R programing environment (R Development Core Team, 2014). To test placement, the shell was placed and photographed and removed, this was repeated five times and each photograph digitised for analysis. To test digitisation error alone a single photo was duplicated five times and the digitisation process performed on each duplicate. The effect of other photographic variables, camera height and position of shell within frame, have been tested and found to be small (Collins and Gazley, 2017) and were not tested in this study.

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Preliminary species classification was based on collection identifications (from museum material) or subjectively by the investigator based on characteristics described by Hart (1995) (see Table 4.1). Species identification for shells from Michaux's collection had been corroborated by analysis of nuclear genetic markers (Michaux 1987), and genetic analysis in my study provided a subset of specimens with genetic identification. Downstream analyses were performed without *a priori* identification to explore the statistically significant clusters in the morphometric data. Shell shape variation was examined with MorphoJ. Shape was assessed using principal component analysis (PCA) across all individuals and all retained landmarks. A broken stick test was implemented using the Vegan (Oksanen et al., 2015) package in R (R Development Core Team, 2014) to determine how many statistically significant, informative principal components were present, using Eigenvalues produced in MorphoJ. The dataset was subdivided into separate parts for subsequent analyses. With each subset of the data, shell shape variation was reanalysed using PCA of uncorrelated principal shell shape components before a model-based clustering approach was employed.

For each subset of the morphological data, a model-based clustering approach was used to explore the distribution of variation and to test for natural clusters within the *Amalda* dataset. Model based clustering was conducted using the Mclust v5.0.2 package (Fraley and Raftery, 2002) in the R programing environment. The Mclust algorithm (Fraley et al., 2012) uses a general model in which the total dataset is considered as a mixture of multivariate normal datasets, with a selection of covariance structures and vectors of expectation (Nanova, 2014). Unlike discriminant analysis, Mclust analysis does not require prior information about specimen identity to classify sample data (Fraley and Raftery, 2003, 2002, 1999) and it is, therefore, objective and repeatable. The best model and optimal number of clusters in the data are selected based on Bayesian Information Criteria (BIC), using the value of the maximized log likelihood, with a penalty for the number of parameters in the model (Cordeiro-Estrela et al., 2008; Fraley and Raftery, 2003, 2002, 1999; Nanova, 2014). In

Mclust BIC is x-1 so the higher the BIC score, the lower the global average and median classification uncertainty, and the better the model fits the dataset (Cordeiro-Estrela et al., 2008; Fraley and Raftery, 1999). *Amalda* morphometrics were analysed first as a whole group and then in smaller groups for pairwise comparisons focused on the analysis of putative taxa.

Discriminant analysis was used to attempt assignment of specimens without genetic confirmation to a species group based on a training dataset of specimens for which I had both genetic and morphometric data. Discriminant analysis was implemented using MclustDA in the programme Mclust v5.0.2 (Fraley et al., 2012; Fraley and Raftery, 2002, 1999). This approach allowed us to estimate the potential mis-assignment of specimens to species.

Results

Genetic analysis and species delimitation

Mitochondrial DNA sequence variation was examined in a 585 bp region of *cox1* from 67 *Amalda* specimens. Haplotype variation resolved five clusters in a medium joining network analysis (Fig. 4.2). These five clusters correspond to the five described New Zealand species that were sampled, but neither of the putative new taxa are distinguished.

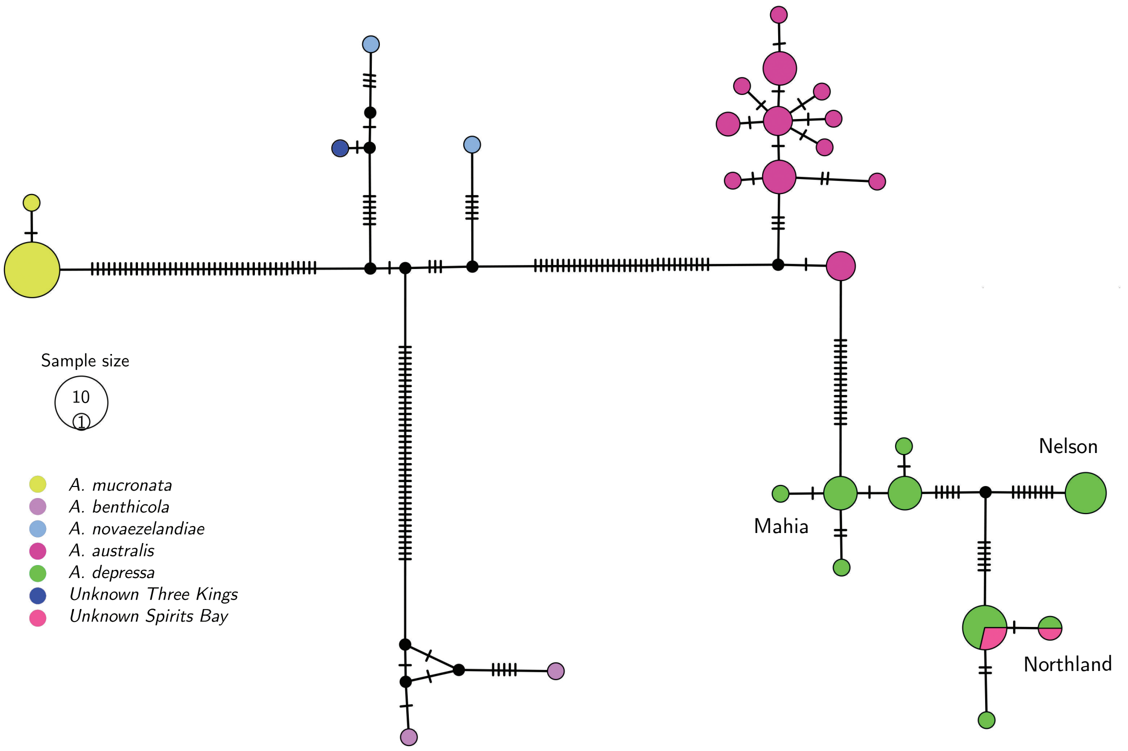
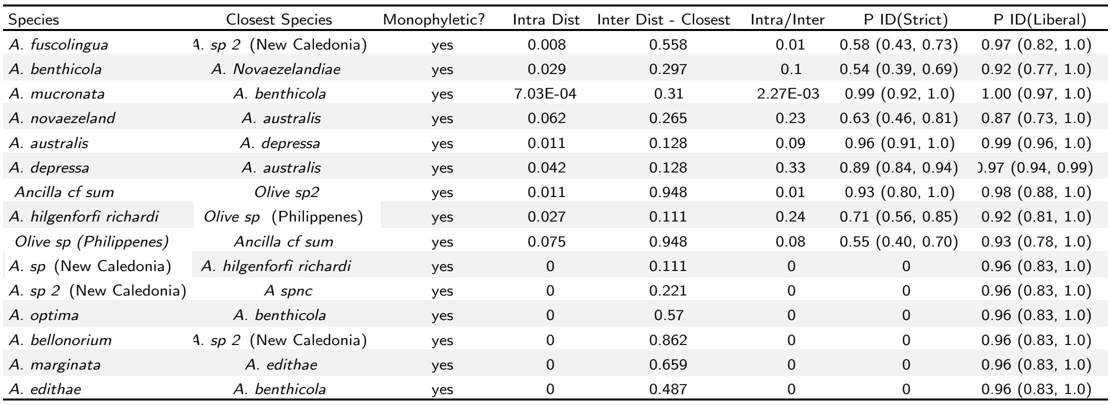


Figure 4.2. Median joining network of mitochondrial *cox1* haplotypes from New Zealand *Amalda* snails. Coloured based on putative species identification. Circle size indicates number of samples with that haplotype. Hash marks indicate single nucleotide sequence differences. Sub-clusters of *A. depressa* haplotypes are associated with different locations.

The *A. mucronata* sample revealed little mtDNA diversity with only two haplotypes detected in a sample of 12 snails from two locations. The limited sampling locations of *A. mucronata* may have contributed to the lack of diversity, but results are concordant with the low nuclear diversity resolved from allozyme data (Michaux, 1987). In contrast, *A. depressa* has haplotype variation concordant with geography; distinct haplotypes come from the three collecting locations (see Fig.4.1). The specimens from Spirits Bay that were identified as a possible new species based on shell colour have the same haplotype as *A. depressa* from Northland. The putative taxon from near the Three Kings Island has a haplotype that clusters within the range of *A. novaezlandiae* mtDNA diversity. Samples of *A. novaezelandiae* and *A. depressa* have haplotypes that differ by up to 3.2% and 4.3%, respectively. *A. novaezelandiae* and *A. depressa* thus have correspondingly high intra-inter ratios from the Geneious™ (Kearse et al., 2012) species delimitation tool (Table 4.3). High intra-inter ratios between haplotype clusters can indicate species groupings (Churchill et al., 2014; Masters et al., 2011), but at least for *A. novaezelandiae* the low sample size probably explains the lack of haplotypes with fewer differences. The *cox1* haplotypes from *A. depressa* formed three distinct genetic clusters corresponding to the geographic origin.

Table 4.3. Summary of within and between genetic distances for mtDNA *cox1* partial sequence as an output from the Geneious species delimitation tool.



Species	Closest Species	Monophyletic?	Intra Dist	Inter Dist - Closest	Intra/Inter	P ID(Strict)	P ID(Liberal)
A. fuscolingua	*. sp 2* (New Caledonia)	yes	0.008	0.558	0.01	0.58 (0.43, 0.73)	0.97 (0.82, 1.0)
A. benthicola	*A. Novaezelandiae*	yes	0.029	0.297	0.1	0.54 (0.39, 0.69)	0.92 (0.77, 1.0)
A. mucronata	*A. benthicola*	yes	7.03E-04	0.31	2.27E-03	0.99 (0.92, 1.0)	1.00 (0.97, 1.0)
A. novaezeland	*A. australis*	yes	0.062	0.265	0.23	0.63 (0.46, 0.81)	0.87 (0.73, 1.0)
A. australis	*A. depressa*	yes	0.011	0.128	0.09	0.96 (0.91, 1.0)	0.99 (0.96, 1.0)
A. depressa	*A. australis*	yes	0.042	0.128	0.33	0.89 (0.84, 0.94)	.97 (0.94, 0.99)
Ancilla cf sum	*Olive sp2*	yes	0.011	0.948	0.01	0.93 (0.80, 1.0)	0.98 (0.88, 1.0)
A. hilgenforfi richardi	*Olive sp* (Philippenes)	yes	0.027	0.111	0.24	0.71 (0.56, 0.85)	0.92 (0.81, 1.0)
Olive sp (Philippenes)	*Ancilla cf sum*	yes	0.075	0.948	0.08	0.55 (0.40, 0.70)	0.93 (0.78, 1.0)
A. sp (New Caledonia)	*A. hilgenforfi richardi*	yes	0	0.111	0	0	0.96 (0.83, 1.0)
A. sp 2 (New Caledonia)	*A spnc*	yes	0	0.221	0	0	0.96 (0.83, 1.0)
A. optima	*A. benthicola*	yes	0	0.57	0	0	0.96 (0.83, 1.0)
A. bellonorium	*. sp 2* (New Caledonia)	yes	0	0.862	0	0	0.96 (0.83, 1.0)
A. marginata	*A. edithae*	yes	0	0.659	0	0	0.96 (0.83, 1.0)
A. edithae	*A. benthicola*	yes	0	0.487	0	0	0.96 (0.83, 1.0)

Nuclear markers: SNPs

SNP data were produced for two species that are sympatric and morphologically very similar; *A. australis* and *A. depressa*. RAD-Seq data were processed using the stacks pipeline process-radtags in STACKS. Due to fragmented DNA, poor digestion, or poor tag ligation, not all individuals yielded adequate coverage. After initial Denovo SNP building individuals with a large proportion of missing data (>80%) were removed, leaving 23 individuals comprising a dataset with 700 loci. Samples from Mahia Peninsula (three putative *A. australis* and eight *A. depressa*) and Doubtless Bay (four *A. australis* and six *A. depressa*) account for 21 of the 23 specimens the remaining two samples were from Nelson (*A. depressa*) and Cloudy Bay (*A. australis*). Evidence for four genetically distinct groups (Fig. 4.3) was inferred from the Bayesian assignments of individual genotypes from STRUCTURE (optimal K of 4). Groupings inferred from the nuclear SNP data were identical to those inferred from their mtDNA haplotype data and from morphometric analysis.

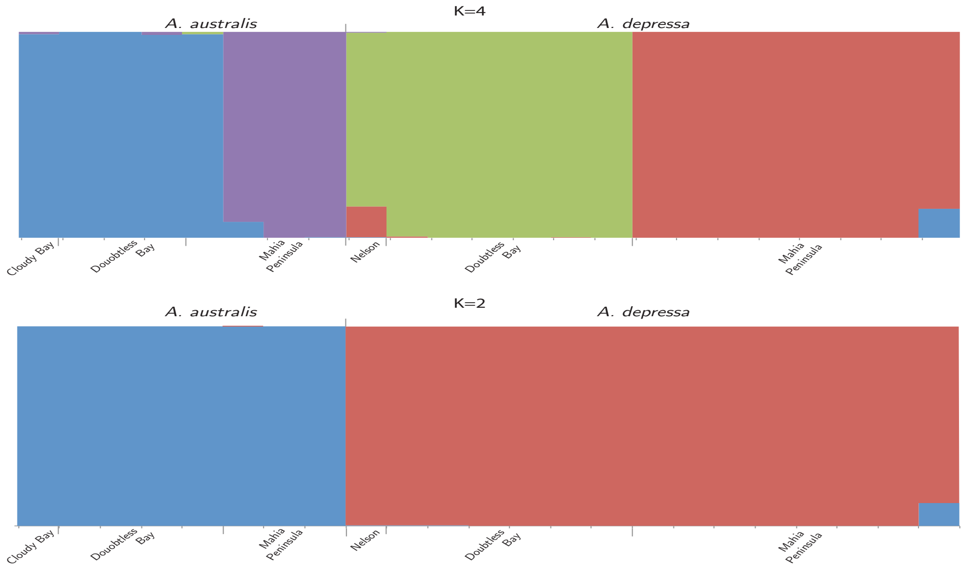


Figure 4.3. Structure output of nuclear data from 23 *Amalda* individuals from four locations. Top, four genetic groups are recognised (K=4) as best fit for the data by Structure Harvester. Two *A. australis* groups (blue and purple) and two *A. depressa.* (red and green). Bottom, two clusters are recognised (at K=2) and correspond to the two species tested.

Full morphological *Amalda* dataset

Initial analysis of the geometric morphometric data included all New Zealand *Amalda* specimens that had been identified to any of the seven currently recognised species. Classification was based on traditional morphological determinations and/or available genetic data (Hart, 1995). Shell shape variation was reduced to uncorrelated principal components (PCs) for further analyses. The first principal component of shape (PC1) explains 50% of the sample variance, and from the warp grid diagram (Fig. 4.4) one can see that this axis involves variation in relative spire height and relative width of the top of the aperture (taller shells have narrower apertures). The second principal component of shape (PC2; Table 4.4) explains almost 30% of the sample variation and involves variation in relative length of the aperture and width of the shell. The first three principal components account for a total of 84.6% and all contain statistically significant information (broken stick analysis of Eigenvalues).

When the first three principal components are used to cluster specimens without *a priori* identification, the best fitting model has four clusters which show correspondence to the preliminary species identifications for four of the seven recognised species of New Zealand *Amalda* (Fig. 4.5 bottom). One cluster corresponds to specimens of all the *A. novaezelandiae* (including specimens catalogued as *A. crystallina)*, along with a single *A. australis* specimen. Another cluster consists only of *A. depressa* specimens, although not all (61%). The third cluster is a group of *A. mucronata* (33%) and two *A. australis* specimens. The fourth cluster contains the remaining *A. australis* (96%) and the remaining *A. depressa* and *A. mucronata* specimens (orange) (Fig. 4.5.). Assignment probabilities to each cluster for every specimen are shown in Fig. 4.6. Interestingly, when the first two principal components are used, the clustering more closely corresponds to the species groups of *A. australis* (92%), *A. depressa* (78%) and *A. mucronata* (83%). The *A. crystallina* and *A. northlandica* specimens group either with the *A. australis* cluster or with the *A. novaezelandiae* cluster (Fig. 4.6).

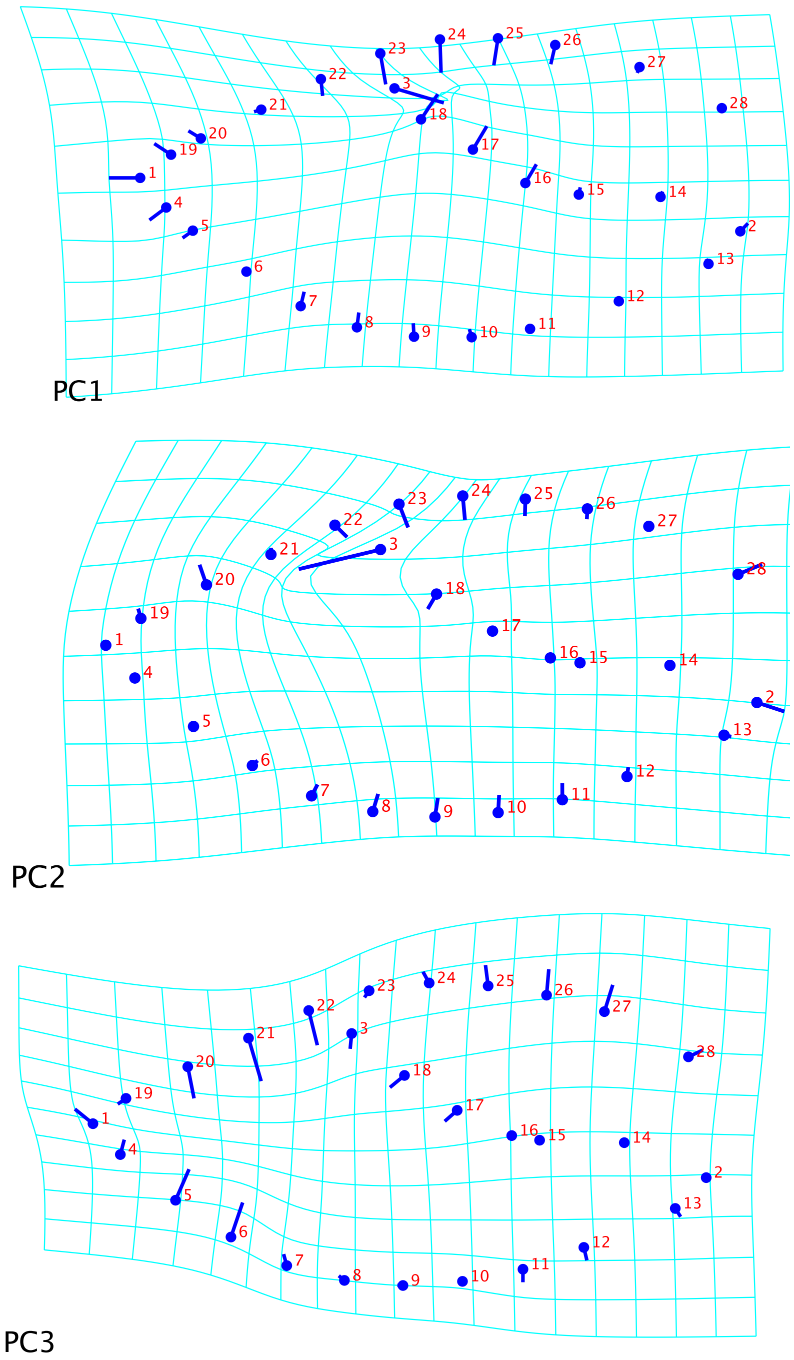


Figure 4.4 Thin plate spline warp grid for the first three principal components of *Amalda* morphometric analysis in MorphoJ. Lollipops indicate the amount of variation for each landmark incorporated into each principal component. PC1 shape changes are mostly associated with the length and width of the shell and aperture. PC2 is the shape of the aperture width of the shell and PC3 the shape of the spire and outer lip of the aperture.

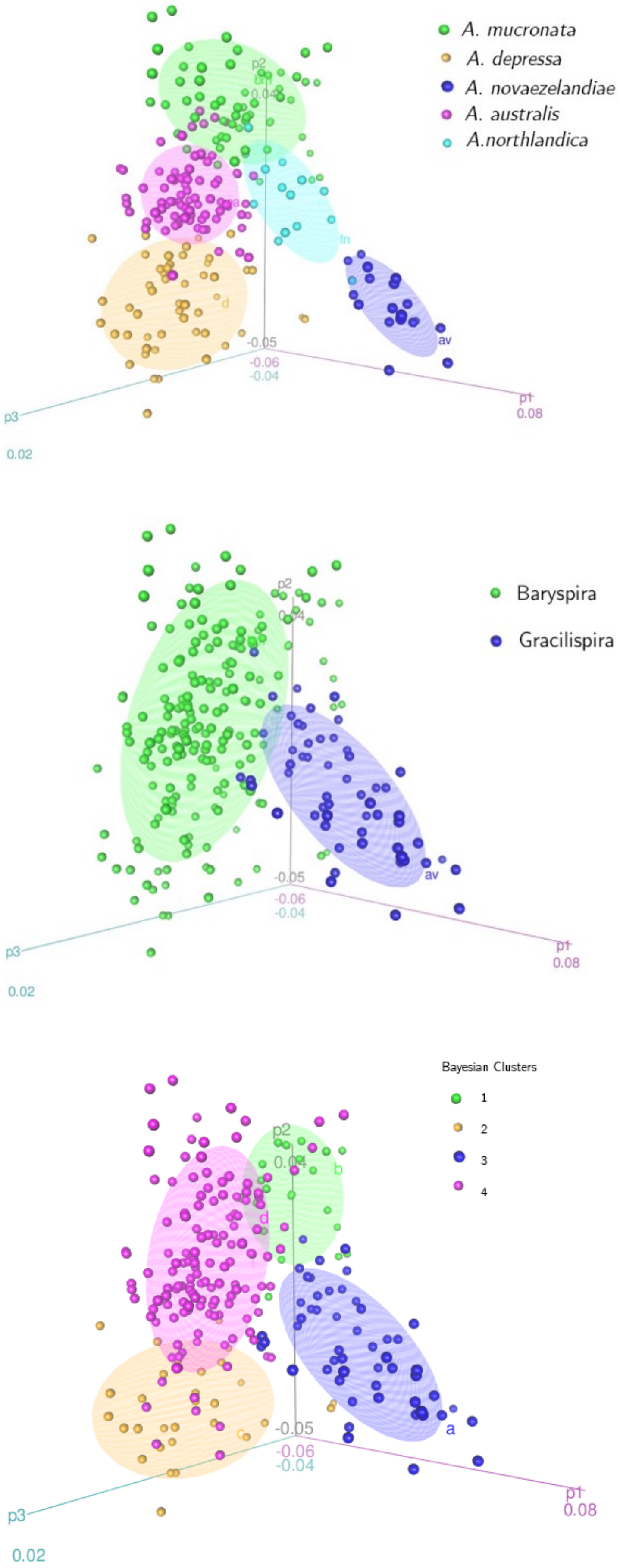


Figure 4.5. MClust 3D representation of first three principal components as recognised as significant in broken stick analysis of *Amalda* specimens from New Zealand enabling comparison of morphospace distributions. Specimens coloured by species excluding *A. crystallina* (top). By sub genera, Baryspira and Gracilispira including crystalline (middle) and by Bayesian cluster analysis including *A.* crystalline (bottom). The best fit model chosen was EVE (ellipsoidal, equal volume and orientation) model with 4 components, BIC 3955.347.

Table 4.4 The amount of variation explained by the first three principal components and the cumulative values

PC	Eigenvalues	% Variance	Cumulative %
1	0.00075384	50.366	50.366
2	0.00041847	27.959	78.325
3	0.00009427	6.299	84.623

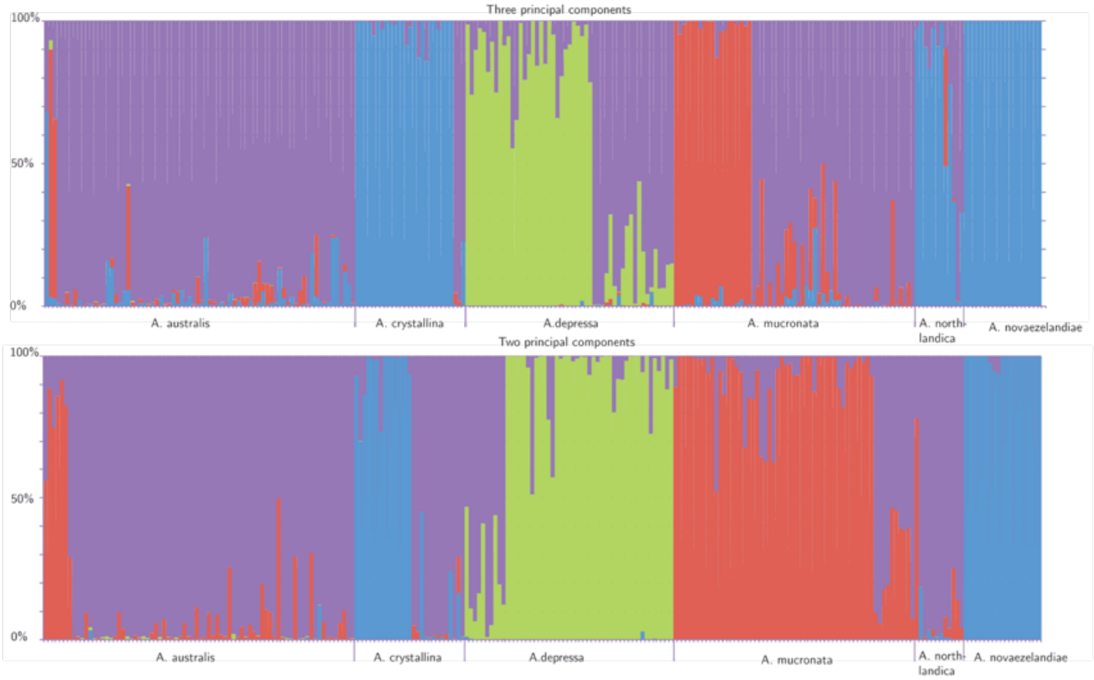


Figure 4.6. Bayesian assignment probabilities of individual *Amalda* snails to one of four clusters (K=4) ordered by species and cluster assignment based on the first three principal components (top panel), and the first two principal components (lower panel) *A. novaezelandiae* *A. northlandica* and *A. crystallina* specimen are clustered together with high probability. *A. australis* individuals are mostly clustered together with some *A. depressa* and *A. mucronata* specimens. Analysis of the first two PCs more closely matches *A. australis*, *A. depressa*, *A. mucronata*, and *A. novaezelandiae* species groupings. *A. crystallina* is split between two clusters and *A. northlandica* is grouped with *A. australis*.

Three sets of specimens have some ambiguity regarding their taxonomy. Recently collected specimens from the far north coast of New Zealand were initially considered putative new or uncertain species. These specimens clustered morphologically with *A. depressa*, and based on genetic results it seems that they are colour morphs of *A. depressa*. *Amalda northlandica*, which as yet has not been conclusively identified genetically had a distribution in morphospace overlapping with *A. australis* (Fig. 4.7.). *A. australis* and other specimens of the *Gracilispira* group, *A. northlandica* and *A. novaezelandiae* (including specimens that had been classified as *A. crystallina*), were analysed to test for distinct morphological variation that might indicate *A. northlandica* is a discrete taxon, as they had an apparent continuous distribution in morphospace (Fig. 4.7.).

With this subset of data, the PC variation was recalculated, which resulted in only a single principal component (73.8% of variation) being statistically informative (broken stick test). When this single PC was analysed in Mclust, two morphological clusters were found to be the best fit to the data (model E (univariate, equal variance) BIC 591.9245). Putative *A. novaezelandiae* and putative *A. australis* specimens formed separate clusters. Putative *A. northlandica* specimens were all part of the *A. australis* cluster but putative *A. crystallina* specimens were split between the two clusters (Fig. 4.7).

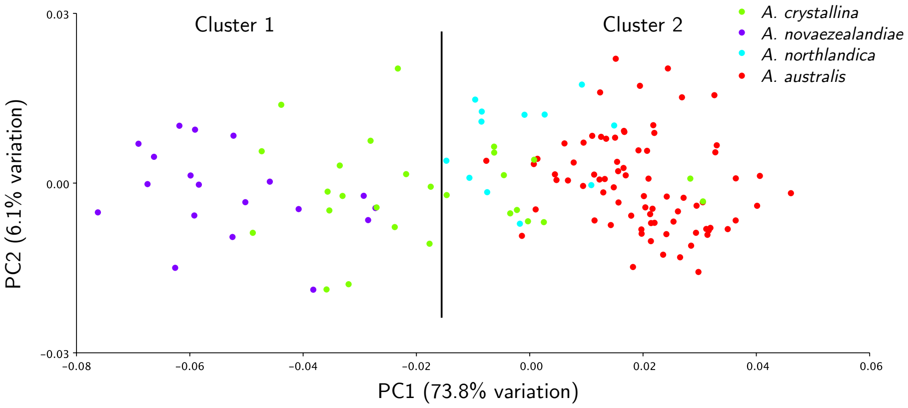


Figure 4.7. First two principal components from morphometric analysis of *A. australis*, *A. crystalline*, *A. northlandica* and *A. novaezelandiae*. Two clusters are were found as best fit to the data by Mclust, one containing *A. novaezelandiae* and some *A. crystallina* and the second cluster containing *A. australis*, *A. northlandica* and some *A. crystallina*.

Combining datasets

Although *a priori* 'expert' clustering of specimens distinguishing morphologically distinct taxa, only the preliminary identification of *A. novaezelandiae* specimens seemed to be 100% in agreement with Bayesian clustering (Fig.4.5). To determine whether the mismatch between preliminary identification and morphological clusters was the result of species overlap, or incorrect identification, genetic and morphologic datasets were combined. To test for concordance between genetic and morphological clusters, the morphological dataset was reduced to only specimens for which genetic data that identified them to either *A. australis*, *A. depressa* or *A. mucronata* were available. This sample of 73 specimens has corresponding mtDNA haplotype data, nuclear data from the ddRAD analysis, or allozyme confirmation (Michaux 1987), and two dimensional landmark morphometrics of shell shape. Morphometric analysis of these specimens identified three principal shape components that explained 83.6% of variation, and that were statistically informative (broken stick test). The largest principal shape component shows concordance with currently recognised putative species cluster, although PC2 is needed to separate some *A. australis* from the other species (Fig. 4.8.). The clustering of the species groups in morphospace indicates, that as with the genetics, there are morphologic clusters corresponding to species delimitation. Importantly, some specimens identified by genetics cannot be separated based on PC1 and PC2 of shell shape variation.

Bayesian assignment of specimens with genetic confirmation of species ID using the first two principal components found the data was best fit to a model with only two morphological clusters. One cluster is closely aligned to the *A. depressa* genetic cluster and the second cluster is *A. mucronata* specimens with *A. australis* being split between the two clusters (Fig. 4.9. & 4.10).

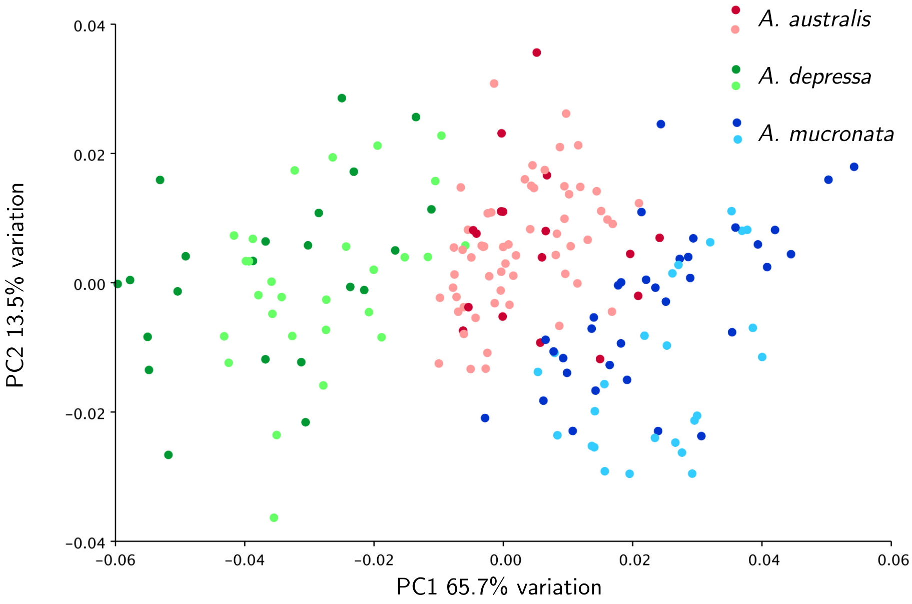


Figure 4.8. Principal component analysis of two dimensional landmark morphometrics of *A. australis* *A. depressa*, and *A. mucronata*. Dark colours represent specimens for which there is both genetic data and morphometric data and the light colours represent specimens for which there is only morphometric data. The three species form clouds in morphospace with some overlap.

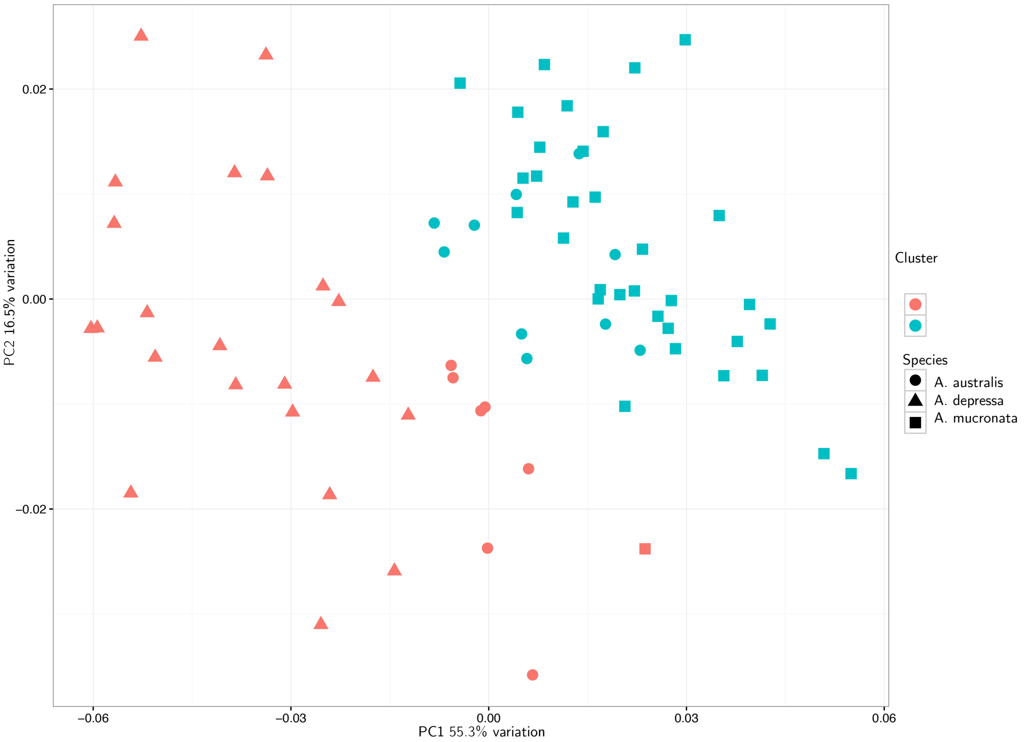


Figure 4.9. Shell shape variation of three New Zealand *Amalda* species identified with genetic data but clustered without *a priori* assignment. Using first two principal components two morphometric clusters are recognised using VEE model in Mclust (Bic score 722).

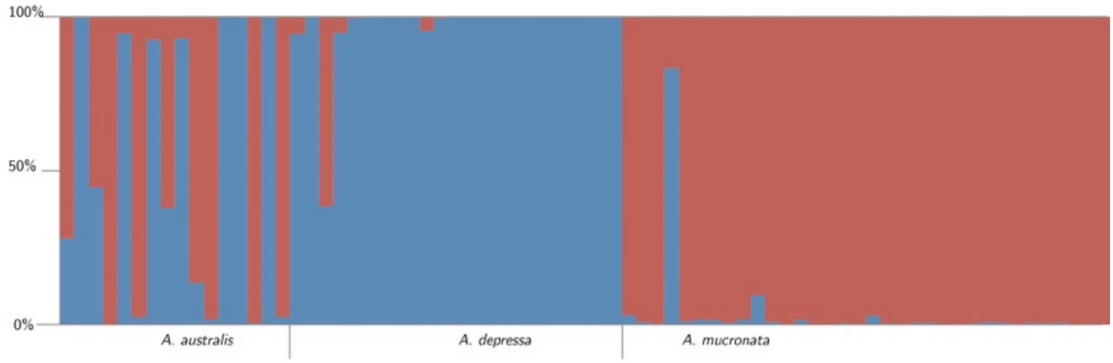


Figure 4.10. Bayesian assignment probabilities of individual specimens (that have genetic data) into two morphological clusters based on the first three principal components of shell shape from specimens identified using genetics; K = 2.

A discriminant analysis test was performed on the first two principal components for the *A. australis*, *A. depressa* and *A. mucronata* dataset. Samples with confirmed genetic assignment to one of the three recognised species was used as the training dataset. The remaining specimens were tested against this. Training error was = 9.5% and Test error = 14.2 % (Fig. 4.11). If the third PC was included in the test the error in the test dataset rose to 35.4%. This indicates that although PC3 is recognised as statistically informative it confounded species identification.

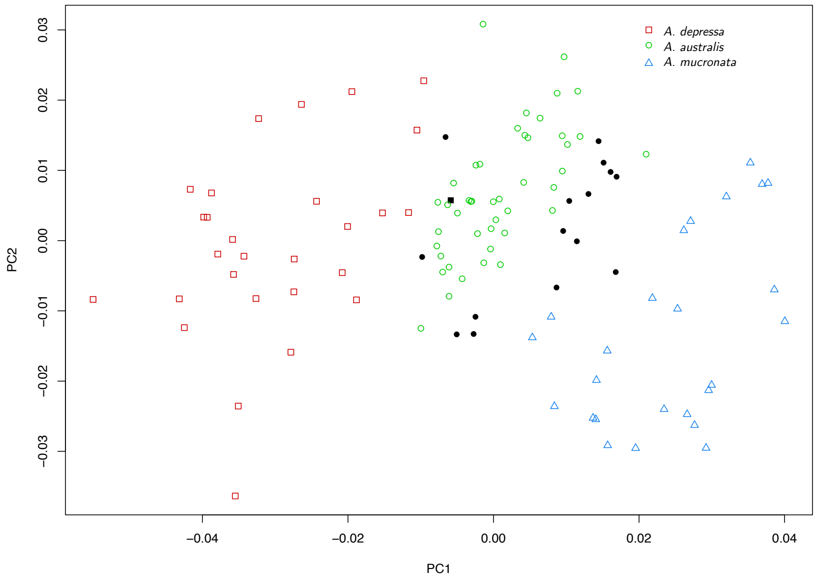


Figure 4.11. Discriminate analysis of *A. australis* *A. depressa* and *A. mucronata* using the first two principal components. The training dataset consists of samples with genetic information and a test dataset of the remaining recent samples of those species. Black shapes indicate specimens assigned to a species different from their *a priori* ID.

The shell shape variation did not easily divide specimens of *A. australis*, *A. depressa* and *A. mucronata* into their corresponding species clusters. Because *A. australis* is morphologically intermediate between *A. depressa* and *A. mucronata*, a comparison of pairs of taxa might help resolve the expected clusters. Thus the morphological dataset was reduced to include species pairs either with a preliminary identification of *A. mucronata* or *A. australis* (n = 135) or *A. australis* and *A. depressa* (n = 128).

For the *A. australis* and *A. mucronata* pairing the shape variation was re-assessed and four principal components were found to be significant (broken stick test) and to capture 82.5% of variation. Cluster analysis without *a priori* classification of specimens and using the first four principal components of the morphological data identified two clusters closely matching species delimitation (Model VEI, BIC 3394) (Fig. 4.12). One cluster contains all the putative *A. mucronata*, specimens with assignment probabilities of >0.70. However, this cluster also contains 14% of the putative *A. australis* specimens. The second cluster contains only putative *A. australis* specimens.

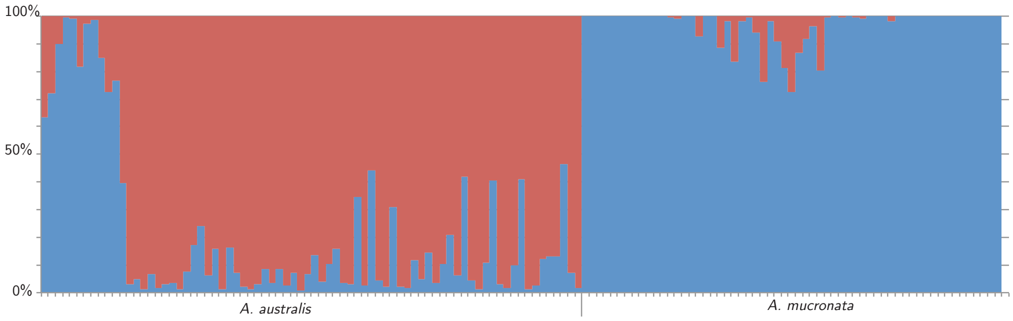


Figure 4.12. Bayesian cluster assignment probabilities of specimens identified as *A. australis* and *A. mucronata* from Mclust based on the first 4 principal components (78.5% variation). All *A. mucronata* samples are clustered together with 14% of *A. australis* specimens

In the same manner the shape variation of *A. depressa* and *A. australis* (n = 128) was re-assessed and for this dataset three principal components were found to be significant (broken stick test) and captured 77.1% of the variation. Cluster analysis without *a priori* classification of specimens and using the first three principal components (77.1% variation) of the morphological data identified two clusters (Model VEI, BIC 2255.634)(Fig. 4.13). These two clusters are strongly aligned to preliminary species designation. Only eight specimens were assigned to clusters not matching their identification. One *A. australis* specimen clustered with the *A. depressa* samples (assignment probability of 0.85). Seven *A. depressa* specimens clustered with the *A. australis* (assignment probability of 0.54–0.91).

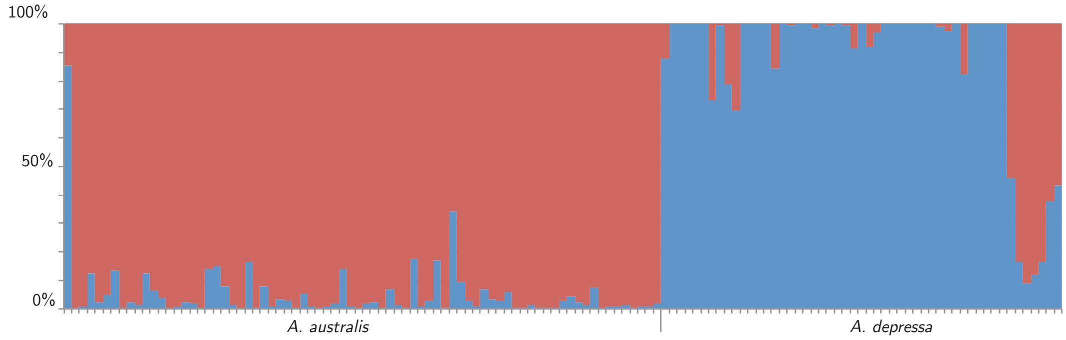


Figure 4.13. Assignment probabilities of *A. australis* and *A. depressa* based on Mclust analysis of first three principal components capturing 77.1% of the variation. Strong correlation between cluster assignment and species designation is seen with a single *A. australis* and seven *A. depressa* being grouped differently from their recognised taxa.

Discussion

Sometimes determining where the boundary lies between species is made difficult not by blurred evolutionary distinctions, or debate about species concept, but simply because of a paucity of clear morphological differences. There is clear genetic separation between the New Zealand *Amalda* species examined here, even though shell traits do not always clearly distinguish species. These clear genetic differences are seen in both mitochondrial and new nuclear data, confirming results found with allozyme variation by Michaux (1987). In fact, the diversity found in *Amalda* mitochondrial *cox1* sequences mirrors the pattern in the nuclear genome described by Michaux (1987), with lowest diversity in *A. mucronata*, and highest diversity (including distinct clades) in *A. depressa* and *A. novaezelandiae*. Importantly, the ability to delimit taxa using genetic tools encompasses the species with established fossil records: *A. mucronata*, *A. australis*, *A. depressa* and *A. novaezelandiae*. In the closely related (and morphologically similar) species *A. australis* and *A. depressa*, I found genetic structuring that is concordant with geographic structuring. Both species have distinct genetic clusters originating from Mahia Peninsula. *A. depressa* also had distinct *cox1* haplotypes from other populations sampled in Northland and Nelson. Samples of *A. australis* from other areas did not have distinct clades. An east coast North Island cluster is found in other marine organisms (Buchanan and Zuccarello, 2012; Hannan et al., 2016; Will et al., 2015, Chapter 2). This geographic based structuring is not reflected in the morphometric analysis where there are no apparent geographically based morphology patterns.

Traditional taxonomy of snails relied on experts comparing shell shape, size, colour and, in some cases, soft-part anatomy, and determining what variation was biologically significant. In the case of New Zealand *Amalda*, a combination of shell colour, shape and size has been used to recognise five distinct species. There is concordant evidence from genetic markers to support all but one of these distinct species. Two–dimensional landmark geometric morphometric analysis is able to capture the same shell shape variation used by the taxonomists and can be applied in an unbiased manner. When the datasets are limited to specimens with both genetic and morphometric information (*A. australis*, *A. mucronata* and *A. depressa)*, I see the clustering of the species but cannot assign specimens with 100% accuracy. Perhaps I should consider this an unrealistic goal. There is some overlap at the edge of the *A. mucronata* and *A. australis* regions in morphospace, highlighting the high intra and low inter species variation.

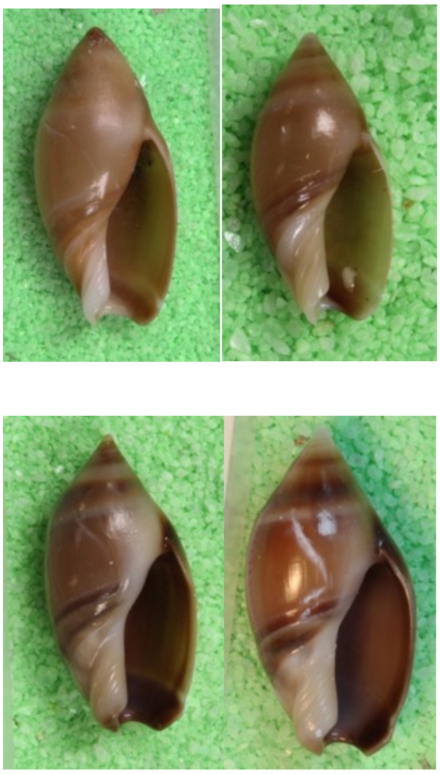


Figure 4.14. Photographs of *Amalda* specimens used in morphometric analysis showing similarities between specimens genetically confirmed as belonging to separate species but which have similar morphology. Top left *A. mucronata*, Top right *A. australis*, bottom left *A. australis* bottom right *A. depressa.* Photos are at different scales.

The New Zealand *Amalda* species form morphometric clusters that correspond to their recognised taxonomy and genetic clusters. Bayesian cluster analysis of the landmark morphometric data indicates that there are four morphological clusters. These clusters approximately correspond to the recognised species *A. depressa*, *A. novaezelandiae*, predominantly *A. mucronata* (plus some *A. depressa)*, and one with all *A. australis*, and *A. northlandica* (plus a few *A. mucronata* and *A. depressa*). The capacity of Bayesian cluster analyses to resolve clusters corresponding to species groupings is strongly influenced by the selection of data being analysed. There are marked differences when the number of PC's being analysed is changed. In some cases, further PCs increased resolution of groups, for instance *Gracilispira* (*A. novaezelandiae* and *A. northlandica*, Fig. 4.6), but for other species the increased PC's obscured species groupings somewhat. The lack of consistent morphologic clustering of species limits the use of Bayesian cluster analysis as a diagnostic tool in this genus, and implies morphologic homogeneity. However, this result does not exclude use of Bayesian assignment as a helpful tool in recognising species groupings. The strong clustering of shell shape within the *Amalda* two dimensional landmark morphologic cloud shows that shell shape characteristics that are taxonomically informative are not distinct enough to be recognised within a Bayesian framework as separate clusters. Discriminant analysis using genetically confirmed taxa corroborates the general support for morphologic clusters for each taxon, but again shows that there is not complete separation of the groups and some specimens are difficult to identify based on just shell shape.

Of the *Amalda* species investigated, the *A. novaezelandiae* cluster is most strongly differentiated. This differentiation is based largely on the shape of the aperture relative to the shell, along with a slight narrowing of the shell. The next most distinct phenotype is *A. depressa*, the most distinguishing feature of which is the projection of the outer lip beyond the outline (Hart, 1995).

A. australis, *A. mucronata* and *A. northlandica* each have taxonomically informative characteristics (such as shell colour) that are not captured by two dimensional landmark based morphometric analysis. There are distinct coloration differences among the three species, enabling easy identification of extant specimens, although within species colour variation is evident when *A. crystallina* is included. However, colouration is lost in the fossilization process and so is not helpful in identifying specimens in the fossil record. Another characteristic helping to distinguish *A. australis* and *A. mucronata* is the callusing on the spire of the shell. The callusing is easy to identify by eye but may not have much impact on the shape difference captured by my morphometric methods. *A. mucronata* can grow to be the largest of the species but there is a considerable overlap in adult size between *A. mucronata* and *A. australis.*

Geometric morphometric analysis implies no evidence to support a number of putative species established recently based on colour variation. *A. crystallina* was recognised by Hart (1995) as being a pale colour form of *A. novaezelandiae.* My analysis of shell morphology of museum collections suggests that specimens previously identified as *A. crystallina* are probably colour forms as Hart (1995) suggested, but specimens could in fact be colour forms of more than one species, *A. novaezelandiae* and *A. australis* (Fig. 4.7.).

Hart (1995) formalised the species designation of *A. northlandica* mostly based on the distinct banded pattern. Hart (1995) aligned it to *A. novaezelandiae* based on spire shape. Interestingly, *A. northlandica* is not recognised in the fossil record. This is probably because most fossil collections (and Michaux's work) were undertaken before *A. northlandica* was described by Hart (1995), and it is likely that fossil *A. northlandica* is not recognised because without colour it is difficult to distinguish it from *A. australis.* The specimen used for genetic work that was putatively identified as *A. northlandica* (McComish et al., 2010) is genetically indistinguishable from *A. depressa* (see Chapter 3). The shell from the sequenced individual was not available for morphometric analysis, but other shells classified as the same species cluster with *A. novaezelandiae* as part of the *Gracilispira* group (within the full *Amalda* dataset) (Fig. 4.5). This shell shape similarity is why they have been considered closely related (Hart, 1995). However, when shells classified as *A. northlandica* were analysed with the two most morphologically similar species (*A. australis* and *A. novaezelandiae*), *A. northlandica* clusters more strongly with *A. australis*. Together *A. australis* and *A. northlandica* form a single cluster with *A. northlandica* specimens clustering at one end of the distribution (Fig. 4.7.). *A. northlandica* is currently recognised as a distinct species despite the recognised colour variation found in some *Amalda* species in Northland (Hart, 1995; Michaux, 1987). My morphometric analysis of shell morphology (without genetic confirmation) suggests the possibility that *A. northlandica* specimens represent a colour morph of *A. australis* or *A. novaezelandiae* or both. No definitive genetic data are currently available for *A. northlandica* because of the difficulty collecting fresh specimens, and so confirmation of its evolutionary relationships to other New Zealand *Amalda* remains elusive.

Conclusions

Given the concordance of genetic distinction and morphological clustering I can have some confidence in my species delimitations. The majority of New Zealand *Amalda* shells can be allocated to their correct species using geometric morphometric analysis. Given the amount of morphological variation in each of the species, and apparent overlap in morphospace at the edges of species shape range, there is always the possibility of some misidentification using morphology alone (as required for fossil material). However, I can be confident that the *Amalda* species are reproductively isolated even in sympatry, and I consider that for the majority of specimens, morphological identification should not be problematic. Confidence in interpreting the fossil record requires further exploration (see Chapter Five).

As the New Zealand *Amalda* species are all separately evolving metapopulations it is reasonable to accept that there may be some overlap in morphospace. Each species will have its own evolutionary constraints acting on phenotype. As they are closely related and living in similar environments, it is likely there are both convergent and divergent selective pressures leading to superficial similarities between the species, creating overlapping phenotypic distributions.

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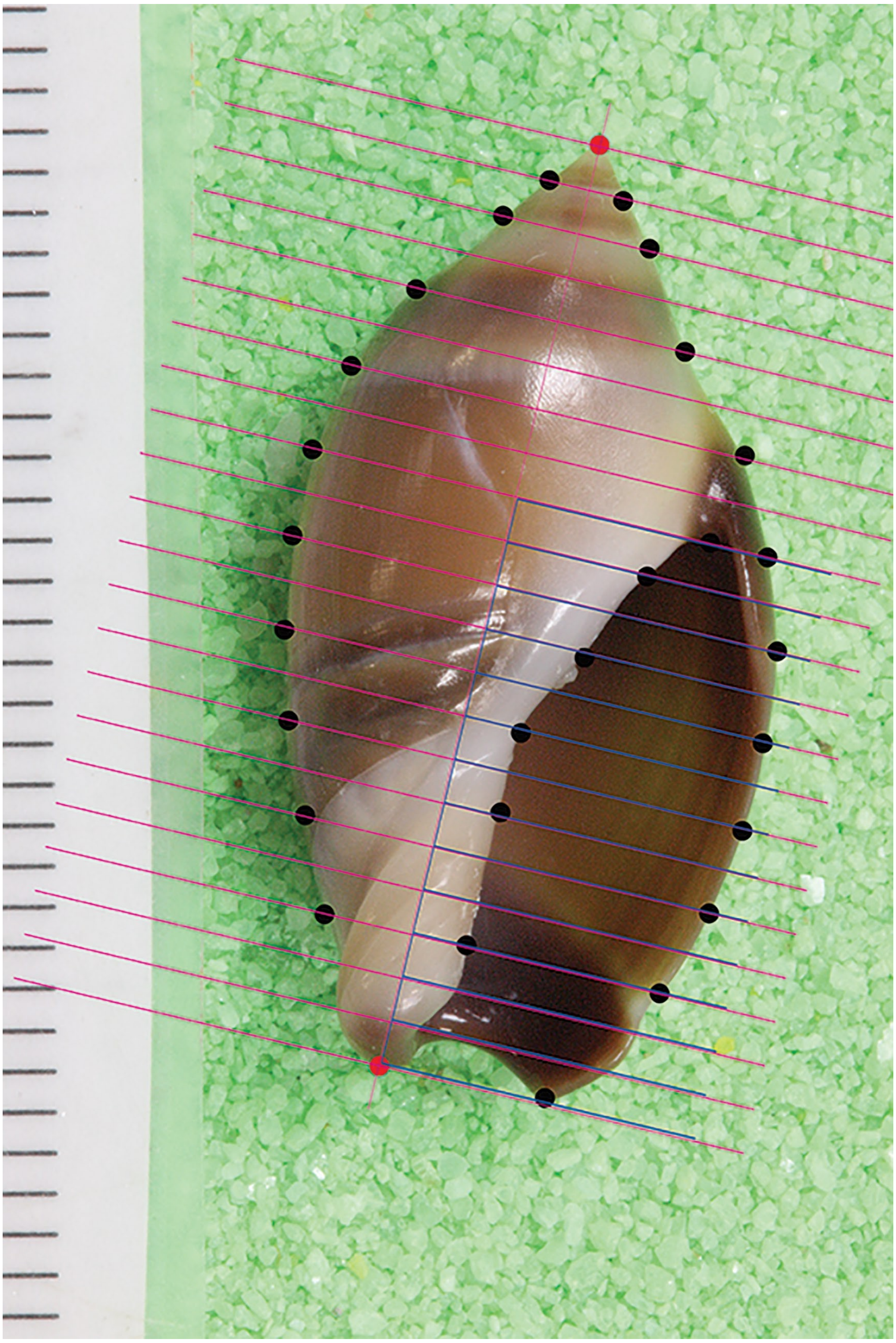
Supplementary Table 4.5 Specimens and collections used in genetic and morphometric analysis

Sample	Species	Photo	Allozyn	COI	ddrad
individuals					
mg13001	A. depressa	y		y	
mg14047	A. mucronata	y		y	
mg14048	A. mucronata	y		y	
mg14049	A. mucronata	y		y	
mg14050	A. mucronata	y		y	
mg14051	A. mucronata	y		y	
mg14061	A. mucronata	y		y	
mg14062	A. mucronata	y		y	
mg14063GB	A. mucronata			y	
mg14064GB	A. mucronata			y	
mg14066	A. australis	y		y	
mg14067	A. australis	y		y	
mg14068	A. australis	y			
mg14069	A. australis	y			
mg14070	A. australis	y		y	
mg14071	A. australis	y			
mg14072	A. australis	y			
mg14073	A. australis	y			
mg14074	A. australis	y			
mg14075	A. depressa	y		y	
mg14076	A. depressa	y		y	
mg14077	A. depressa	y		y	
mg14078	A. depressa	y		y	y
mg14079NE	A. depressa			y	
mg14080	A. depressa	y		y	
mg14081	A. depressa	y			
mg14082	A. depressa	y		y	
mg14139	A. australis	y			
mg14141	A. australis	y			
mg14142	A. australis	y		y	
mg14144	A. australis	y			
mg14146	A. australis			y	
mg14147	A. australis	y			
mg14150	A. australis	y			
mg14152	A. australis			y	y
mg14153	A. australis			y	
mg14155	A. australis	y			
mg14156	A. australis	y		y	
mg14159	A. australis	y		y	
mg14160	A. australis	y			
mg14163	A. australis	y			
mg14176	A. mucronata	y		y	
mg14177	A. mucronata	y			
mg14190	A. depressa	y			
mg14192	A. australis	y			
mg14193	A. australis	y		y	y
mg14194	A. depressa	y		y	y
mg14195	A. depressa	y			y
mg14197	A. australis	y			
mg14198	A. australis	y			y
mg14200	A. australis	y		y	
mg14202	A. australis	y			
mg14203	A. australis	y			
mg14204	A. australis	y			
mg14205	A. depressa	y			
mg14208	A. depressa	y		y	y
mg14220	A. australis	y		y	
mg14222DB	A. australis			y	
mg14223DB	A. australis			y	
mg14224	A. australis	y		y	
mg14230	A. australis				y
mg14235	A. australis	y		y	
mg14236	A. depressa	y			
mg14237	A. australis	y		y	
mg14245	A. depressa	y		y	y
mg14246	A. depressa	y		y	y
mg14249	A. australis	y		y	
mg14265	A. australis				y
mg14266DB	A. depressa			y	
mg14270	A. mucronata	y			
mg14273	A. depressa	y		y	y
mg14275	A. australis	y		y	
mg15001	A. depressa	y		y	y
mg15002	A. australis	y			
mg15003	A. australis	y			y
mg15004	A. australis	y		y	y
mg15005	A. australis	y		y	y
mg15006	A. depressa	y		y	y
mg15007MP	A. depressa			y	y
mg15008	A. depressa	y		y	y
mg15009	A. depressa	y		y	y
mg15010MP	A. depressa			y	y
mg15011MP	A. depressa			y	y
mg15012	A. depressa	y		y	y
mg15013	A. depressa	y		y	
MG15014MP	A. depressa			y	
mg15015	A. depressa	y			
mg15016	A. depressa	y			
mg15017	A. depressa	y			
MG15014MP	A. depressa			y	
mg15030	A. australis	y		y	
mg15031	A. australis	y			
mg16001	A. depressa	y		y	
mg16002	A. depressa	y		y	
mg16003	A. depressa	y			
mg16004	A. depressa	y			
mg16005	A. depressa	y			
mg16007CR	A. benthicola			y	
benthicolamg16009CR	A. Benthicola			y	
mg16012	A. northlandica			y	
mg15032GBnv	A. northlandica			y	
shell collections					
m_090698	A. australis	y			
GS15443	A. australis	y			
arwgx0028	A. australis	y			
M_075037	A. Crystallina	y			
M_035596	A. Crystallina	y			
drtpx0047	A. depressa	y			
drwgx0022	A. depressa	y			
mrakx0069	A. mucronata	y			
AKL111358	A. mucronata	y	y		
AKL11365	A. mucronata	y	y		
m_090012	A. northlandica	y			
M_054721	A. novaezelandiae	y			



Supplementary Figure 4.15 Amalda depressa shell showing placement for photographing with aperture up. Two combs are overlaid, Pink for external shell curves and blue for internal aperture curve. Initial landmarks were placed at all curve and comb intersections. The final landmarks used are shown in red (fixed landmarks) and black circles (semi-landmarks)

Chapter 5

Modelling Phenotypic Evolution Through Time in Three New Zealand *Amalda* Lineages

Introduction

Our understanding of biological evolution is informed by observation of both living biodiversity and the fossil record, although the different temporal scales involved can lead to different interpretations of both rate and mode of change. The modern biota provides information about current diversity and patterns existing now and genetic tools are able to look at evolution over generations and infer molecular phylogenies. Palaeontology provides an insight into morphological evolution over a much longer time frame but the fossilisation process and specimen discovery leave us with a much reduced set of informative characters to work with. Critical then is confirmation that evolutionary units observed in the extant biota are equivalent to those documented by palaeontology. A key problem is that analysis of modern biodiversity frequently uses genetic data in addition to behavioural, geographic, morphological, and ecological information to establish hypotheses of taxon boundaries (Hey, 2006; Sites and Marshall, 2003). On the whole only morphological information is available for fossils and this means clarification of lineage homology needs careful consideration. This is especially important if the implications of theory founded on observations of the fossil record are being assessed in the neobiota.

Documenting the fossil record as a means to infer evolutionary process has a long history reaching back to the birth of evolutionary theory (Darwin, 1859; Hutton, 1795). In the ensuing years better descriptions of the fossils have been made and used to help understand evolutionary trajectories of fossil lineages (Hunt et al., 2015; Jablonski, 2000; Roopnarine et al., 1999; Simpson, 1944).

Describing how the phenotypes of lineages have changed through time has provided one of the most well-known and perhaps controversial ideas in evolutionary biology, that of Punctuated Equilibria (PE) (Eldredge and Gould, 1972; Gould and Eldredge, 1977). This idea brought to attention the concept that evolution might not be (simply) a continuous gradual process, happening over long periods of time as lineages diverge. Rather, morphological change might occur in rapid bursts, associated with speciation, interspersed with longer periods of morphological stasis. This theory was in part able to account for the apparent lack of transitional fossils found in the fossil record because they were present for geologically very short periods of time and therefore very rare. Recently, increasingly complex models have made possible more detailed examination of evolutionary trajectories through morphospace and time (Monteiro, 2013). Mathematical models of evolution such as random walk (Brownian motion), stasis or directional change, go some way to describing models of morphological evolution and identifying what must be observed statistically to fit these models (see Fig. 5.1).

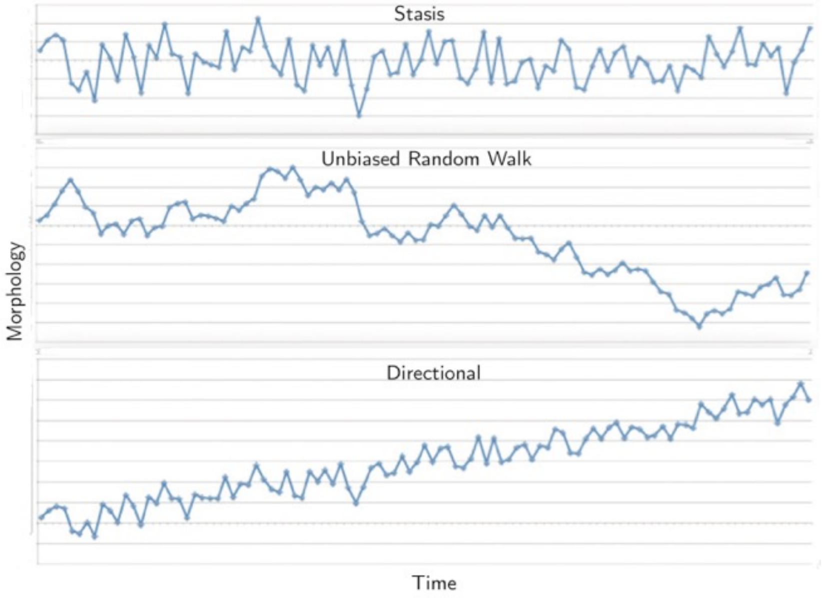


Figure 5.1. Three models of phenotypic change through time accommodate minor short term variation while tracking larger scale trend. Stasis with variation occurring around a constant mean, Unbiased Random Walk variation occurring in any direction and Directional where variation occurs around a shifting mean

The PE model only has two predictions, that for relatively long periods there is morphologic stasis (resulting from constraining selection for an optimal phenotype), and occasional brief bursts of rapid morphologic change associated with speciation. In contrast directional evolution, General Random Walk (GRW) (gradualism), implies ongoing, largely constant evolution or change with directionality driven by selection. A third model of morphological change through time is the Unbiased Random Walk (URW) (removal of selective pressure (Benton, 2015)). Random walk has been considered to be a null evolutionary hypothesis (Bookstein, 2012) but with improved statistical tools (e.g. Hunt et al. 2015), URW can be treated as an equal alternative hypothesis to directional evolution or stasis. The PE model is a long term evolutionary model with stasis as one factor. Stasis should be detectable in the study of evolutionary lineages if PE were the model under which evolution occurred. The detection of stasis is not conclusive support of PE but rather another piece in the body of evidence supporting PE. In this chapter I have the tools available to explicitly test for the presence of stasis as a prediction of PE. The fossil shells available do not extend to cover the putative speciation events. Inferences about the timing of speciation events using the fossil dating and geneic dating can help discount these occurring during periods of stasis, but not confirmation of concurrence of speciation and morphologic change.

With increasing complexity in evolutionary models, simplistic descriptions of the morphological evolution of species are being superseded by explanations that are more flexible and incorporate more details (Hunt, 2013, 2006). Evolutionary processes operating on a trait can shift within the evolution of a lineage over time and it is now accepted that different traits can follow different models of evolution in the same lineage over the same time period (e.g. Hunt, 2006; Hopkins & Lidgard, 2012; Hunt *et al.*, 2015; Voje, 2016). This mixed model explanation reinforces the fact that evolution is the result of many forces acting on different traits simultaneously. Completeness of the fossil record can also influence what patterns are inferred, for example, as collections (time series) cover longer periods, data are more likely to fit a model of morphological stasis as mean direction of long term random walk trajectories become closer to zero by chance and complex models rather than favour simple ones as shifts in modes are morle likely to be covered. (Hunt et al., 2015). As more examples of morphological stasis in the fossil record are reported, there is more support for one of the underlying components of PE. There is a long running debate over the association between rapid morphological change and speciation (Lieberman and Eldredge, 2014; Pagel et al., 2006; Pennell et al., 2014a, 2014b; Venditti and Pagel, 2014) which is linked to debate about species concepts (and speciation). Nevertheless, there is at least agreement that in some situations describing the pattern as that of PE is apt (Jackson and Cheetham, 1999; Mattila and Bokma, 2008; Strotz and Allen, 2013). Evidence for directional morphological change has been demonstrated only rarely (5–13% of studies) compared to either stasis or URW (Hunt et al., 2015; Voje, 2016). While directional trait evolution is rarely detected in studies many biologists probably still envisage morphological evolution happening this way (Hunt, 2007; Hunt et al., 2015; Sherratt et al., 2016).

By using landmark morphometrics I here investigate evolutionary models in mathematically independent shell shape components within lineages over time. Although shell shape traits are not necessarily biologically independent within a lineage, if the traits are uncorrelated they can be modelled separately. I revisit a group of snail species that in the past were demonstrated to show morphological stasis in the fossil record (Michaux, 1989). New Zealand representatives of the neogastropod genus *Amalda* were held up by Gould (2009, 1991) as evidence for punctuated equilibrium. *Amalda* is a genus of marine gastropods in the Olividae that are mostly confined to temperate regions (Olson, 1956). The benthic habitat of *Amalda* species lends itself to high levels of fossilisation, and in New Zealand *Amalda* have a continuous fossil record (from around 45 million years ago (i.e. *A. morgani*) in Eocene fossil beds (Beu and Maxwell, 1990)). There are seven extant species recognised from New Zealand, four of which are recorded in the fossil record: *A. mucronata*, *A. australis*, *A. depressa* and *A. novaezelandiae* (Beu and Maxwell, 1990; Olson, 1956). Three of these species: *A. australis*, *A. depressa and A. mucronata*, are examined in this study because of their fossil record and the availability of modern samples from which genetic lineages can be determined. New Zealand *Amalda* species have been examined using shell shape variation and genetic data confirming that they represent distinct lineages that are morphologically very similar (Michaux, 1987). Using traditional linear measurements of various homologous features on the shell, Michaux (1987) demonstrated stasis over 2-3 million years in three species of New Zealand *Amalda A. australis*, *A. mucronata* and *A. novaezelandae*. Michaux combined shell measurements to produce a single canonical variate value summarising their position in a ten dimensional space (Michaux, 1989). In contrast, I will use a two dimensional landmark geometric morphometric dataset to perform multivariate analysis in order to derive mathematically independent elements of shell shape.

Access to a comprehensive collection of fossil *Amalda* provided the opportunity to compare shape variation among temporal populations. Analysis was able to incorporate more intricate morphometric analysis and more fine scale geologic age dating than in previous studies (Michaux, 1989). Improved geological dating is due in part to the recent integrated chronologic and sequence stratigraphic framework established for the Wanganui Basin using a combination of cyclostratigraphic, biostratigraphic, magnetostratigraphic and tephrochronologic data (Carter and Naish, 1998). First, I look for concordance between extant samples and fossils identified as the same species, using the landmark morphometrics of three lineages to verify that what have been accepted as lineages in the fossil record are recognisably distinct. Second, using temporal populations partitioned into time bins (reflecting the geologic horizons the fossils were collected from) I will analyse shell size and principal components of shell shape to test for fit to alternative evolutionary models and see how well each conform to three possible evolutionary models; directional, URW and stasis.

Materials and Methods

Sampling

Specimens of *A. australis A. depressa* and *A. mucronata* were collected from coastal marine habitat around New Zealand using dredging or hand sampling, directly by my team or via contributors. Locations were chosen to provide sampling from within the species' distribution providing sampling from distinct geographic regions intended to cover any geographic variation. Sampling numbers were intended to provide enough specimens from each location for population level variation to be investigated. Live *Amalda* specimens were collected from Nelson Harbour during biosecurity monitoring by The National Institute of Water and Atmospheric Research (NIWA). *Amalda* by-catch from Cloudy Bay in the Marlborough Sounds were provided by Cloudy Bay Clams. Fossil specimens were accessed from collections held by Te Papa Tongawera Museum of New Zealand, GNS Science, Te Pū Ao and Tāmaki Paenga Hira, Auckland War Memorial Museum (see supplementary table 5.2). Museum material had been identified to species by collectors and museum curators, usually Fleming Beu Marshall and Powell. These collections included the specimens used by Michaux (1989).

Morphometrics

A geometric morphometric approach was used to analyse the shape variation of the New Zealand *Amalda* species *A. australis*, *A. depressa and A. mucronata.* Shell phenotype analysis using this method is effective in discovering variation between ecotypes and species (Collins et al., 2013; Dowle et al., 2015; Hills et al., 2012). Digital images of the shells were obtained following the protocol in Dowle (2015) and chapters 1 & 3.

A combination of landmarks and semilandmarks was used to capture the shape of the external morphology of the *Amalda* shells. Landmarks were fixed points at homologous locations on the shell, the tip of the teleoconch spire and end of the siphonal canal. Semilandmarks were positioned along curves of the shell such as the external outline and curves of the aperture. For analysis of fossil specimens the outer aperture curve was not included due to the high rate of damage to this fragile part of the shell (Fig. 5.2). Semilandmarks are slid along the curve relative to fixed landmarks in order to remove spurious effects of arbitrary placement on the contour and to enable comparison of the homologous curves between specimens (Webster and Sheets, 2010; Zelditch et al., 2004).

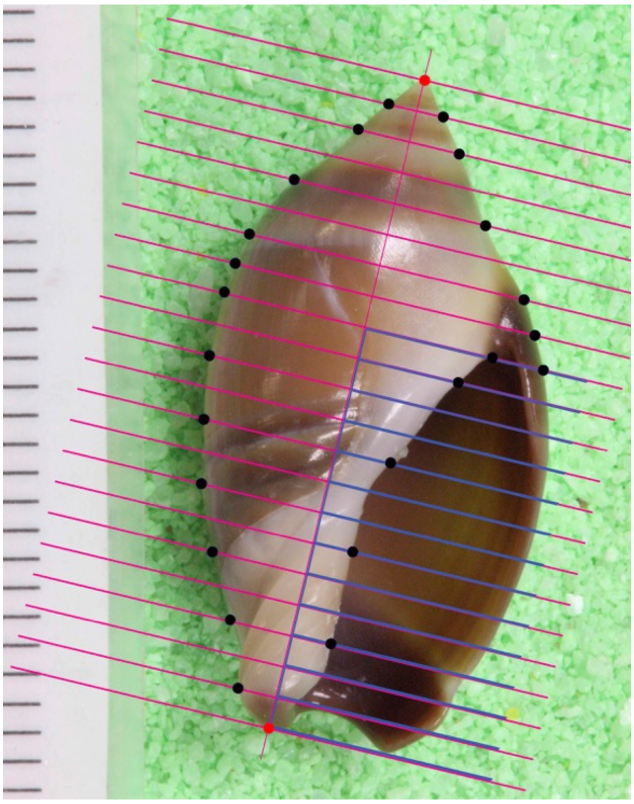


Figure 5.2. *A. depressa* shell showing placement for photographing with aperture up. Two combs are overlaid, Pink for external shell curves and blue for internal aperture curve. Initial landmarks were placed at all curve and comb intersections. The final landmarks used are shown in red circles (fixed landmarks) and black circles (semi-landmarks)

Initial species identification of museum material was based on identifications by experts. For recently collected specimens shell shape, colour and location were used for preliminary species assignment with further confirmation provided for some samples by genetic markers (mtDNA sequence and/or ddRAD; chapter 2 & 3). Identification of recent specimens used from Michaux's shell collection had been confirmed by unique allozyme alleles (Michaux 1987). In order to fit evolutionary models to the morphological data one must assume samples represent a single biological lineage through time, and therefore correct species identification is important. Initial shell shape comparisons were performed with all three species to get an overall idea of classification. Further analyses were then performed with subsets of the data using the morphologically closest pairs of taxa to improve species identification.

By combining evidence from shell shape and genetic markers, the majority of extant specimens could be unequivocally identified to species level, but morphological separation of *A. australis* from *A. depressa* or *A. mucronata* was not possible for about 14% of specimens (see chapter 4). Principal component analysis was performed with fossil and recent specimens together. Species morphology formed reasonably clear clusters with some overlap, however some specimens fell well outside the main body of species clusters. All specimens that were at the limits of the morphospace region for their species were re-examined to determine if their preliminary species assignment resulted from an accidental misclassification or misplacement into collections. After consultation with molluscan taxonomists Alan Beu (GNS) and Bruce Marshall (Te Papa) several specimens were determined to have been incorrectly catalogued in museum collections and were duly reassigned. However, there were also several specimens that were inconsistently placed by the principal component analysis for which re-examination of the digital image did not clearly suggest misidentification.

These specimens were left with their original species assignment. This process highlights the potential for misidentification of *Amalda* shells, especially for fossil material as there are several species with recognised phenotypic variation in the extant fauna (Hart, 1995; Michaux, 1987). Preliminary tests removing ambiguous specimens indicated that their presence or absence had little effect on results an so they were included in analysis after reassignment if necessary.

The Procrusties superimposition, done using Semiland within Coordgen, of the snail shell shape of the samples effectively standardises the variables in preparation for PCA. In MorphoJ the Eigenvalues are then scaled by the total variance before variance is calculated resulting in a dimension free index (Young, 2006). Shell shape variation was examined with MorphoJ (Klingenberg, 2011) using a standard principal component analysis (PCA) across all individuals and all landmarks. A broken stick test was implemented using the Vegan (Oksanen et al., 2015) package in R (R Development Core Team, 2014) to determine how many statistically significant, informative principal components were present, using Eigenvalues produced in Morphoj. The dataset was subdivided into separate parts for subsequent analyses. With each subset of the data, shell shape variation was reanalysed using PCA of uncorrelated principal shell shape components before a model-based clustering approach was employed.

For each subset of the morphological data a model-based clustering approach was used to explore the distribution of variation and to test for natural clusters within the *Amalda* dataset, using the Mclust v5.0.2 package (Fraley et al., 2016; Fraley and Raftery, 2002) in the R programing environment. The Mclust algorithm uses a general model in which the total dataset is considered as a mixture of multivariate normal datasets, with a selection of covariance structures and vectors of expectation. Unlike discriminant analysis, Mclust analysis does not require prior information about specimen identity to classify sample data (Fraley and Raftery, 2003, 2002, 1999) and it is, therefore, objective and repeatable. The best model and optimal number of clusters in the data are selected based on Bayesian Information Criteria (BIC), using the value of the maximized log likelihood, with a penalty for the number of parameters in the model (Cordeiro-Estrela et al., 2008; Fraley and Raftery, 2003, 2002, 1999; Nanova, 2014) . In Mclust BIC scores are multiplied by -1, meaning that the higher the BIC score, the lower the global average and median classification uncertainty, and the better the model fits the dataset (Cordeiro-Estrela et al., 2008; Fraley and Raftery, 1999).

Discriminant analysis is used to attempt assignment of fossil specimens to species groups based on a training dataset of modern examples of the three *Amalda* species, implemented using MclustDA in the programme Mclust v5.0.2 (Fraley et al., 2012; Fraley and Raftery, 2002, 1999). This approach allows one to obtain an indication of the potential for mis-assignment of specimens to species.

Evolutionary Model

Analysis of evolutionary model for each lineage employed PaleoTS which analyses variance of fossil series with a likelihood approach. The best fit model is chosen based on the Akaiki information criterion and Akaiki weights (Hunt, 2006). First, simple models were tested: directional, random walk, or stasis. These models are all special cases of random walk in which two parameters are of importance the mean at each step in the sequence (μ_{step}) giving a value to directionality and the variance (σ^2_{step}) representing volatility. When μ_{step} is 0 within bounds of limited variation stasis is appropriate. However, when μ_{step} is 0 but with large σ^2_{step} random walk is a better descriptor of the pattern. If μ_{step} is positive or negative then the pattern can be considered as directional(Hunt, 2006). Complex models were also tested which incorporate more than one model of evolution to explain morphological change over time (within a single lineage), were also tested. Six additional models were tested: strict stasis (no change in mean and no variation through time), punctuation, stasis followed by random walk, stasis followed by directional, random followed by stasis and directional followed by stasis (Hunt, 2006; Hunt et al., 2015). Shape variation was examined using significant principal components generated in MorphoJ for each inferred lineage through time separately. Most fossil horizons provided more than five specimens per lineage (=species) resulting in narrow variances around sample means, but some fossil horizons had few representatives. These specimens were placed into an age bin with samples from the age bin that was the closest in time to ensure each time series examined had at least five samples. If possible, specific horizons were dated otherwise the midpoint of their recognised geological age was used (details provided in supplementary Table 5.2).

Results

Two dimensional landmark morphometric analysis was performed on 715 individual shells including both recent and fossil specimens: *Amalda. mucronata* - 58 recent, 254 fossil specimens; *A. australis* - 75 recent, 154 fossil and *A. depressa* - 61 recent, 113 fossil.

Amalda morphospace

Sampling error introduced by the digitisation process was tested for (as described in Chapter 1). Error was found to be 0.3% for digitisation alone and 0.6% for shell placement and photography together. Therefore, the digitisation process is considered negligible in influencing the results from the current dataset. The variation of shell shape in the overall sample of more than 700 was reduced to three significant uncorrelated shape traits (principle principal components) that explained 82.9% of the shape variation (Fig. 5.3). PC1 is the correlated variation in the curve of the shell from relative spire height and width, and this shell trait contains more than half the shape variation in this dataset (Fig. 5.3). Principal component analysis (PC1 & PC2 plot) of the complete fossil and recent material shows clustering of the separate species with modest overlap at the cluster edges. However, the 90% confidence mean ellipses of all species are clearly distinct for PC1 indicating that each species is forming a cluster in morphospace. The distribution of fossil specimens within the PC1 and PC2 morphospace was larger than that occupied by the recent specimens (Fig. 5.4)

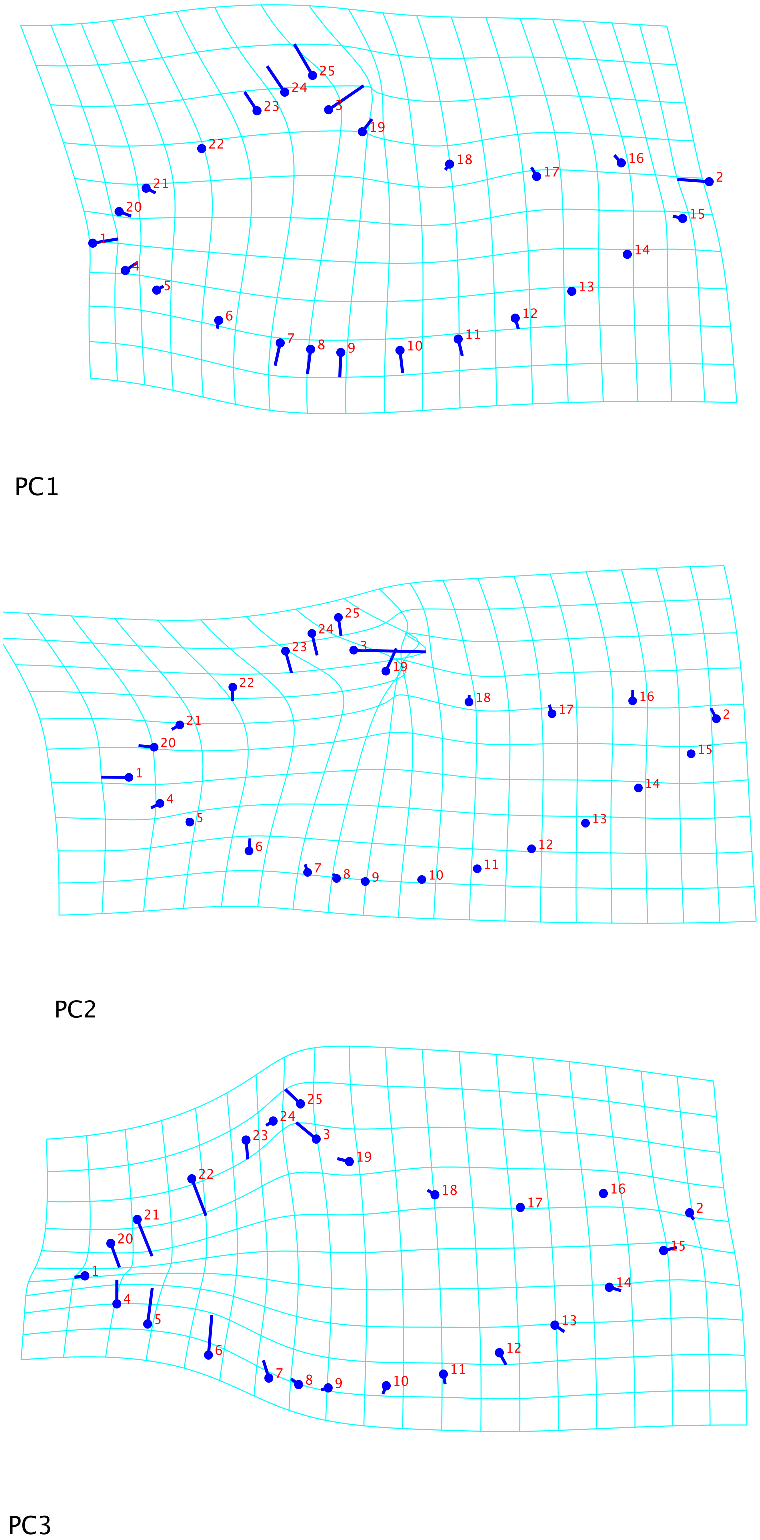


Figure 5.3. Thin plate spline warp grid diagram for the first three principal components of *Amalda* morphometric analysis in MorphoJ. Lollipops represent a scaled indication of the amount of variation for each landmark incorporated into each principal component. PC1 (top) accounting for 53.2% of the variation. Shape changes are mostly associated with the length and width of the shell and aperture. PC2 (middle), 22.0% variation, relative length of the aperture and height of the spire and PC3 (bottom), 7.7% of variation, the shape of the spire.

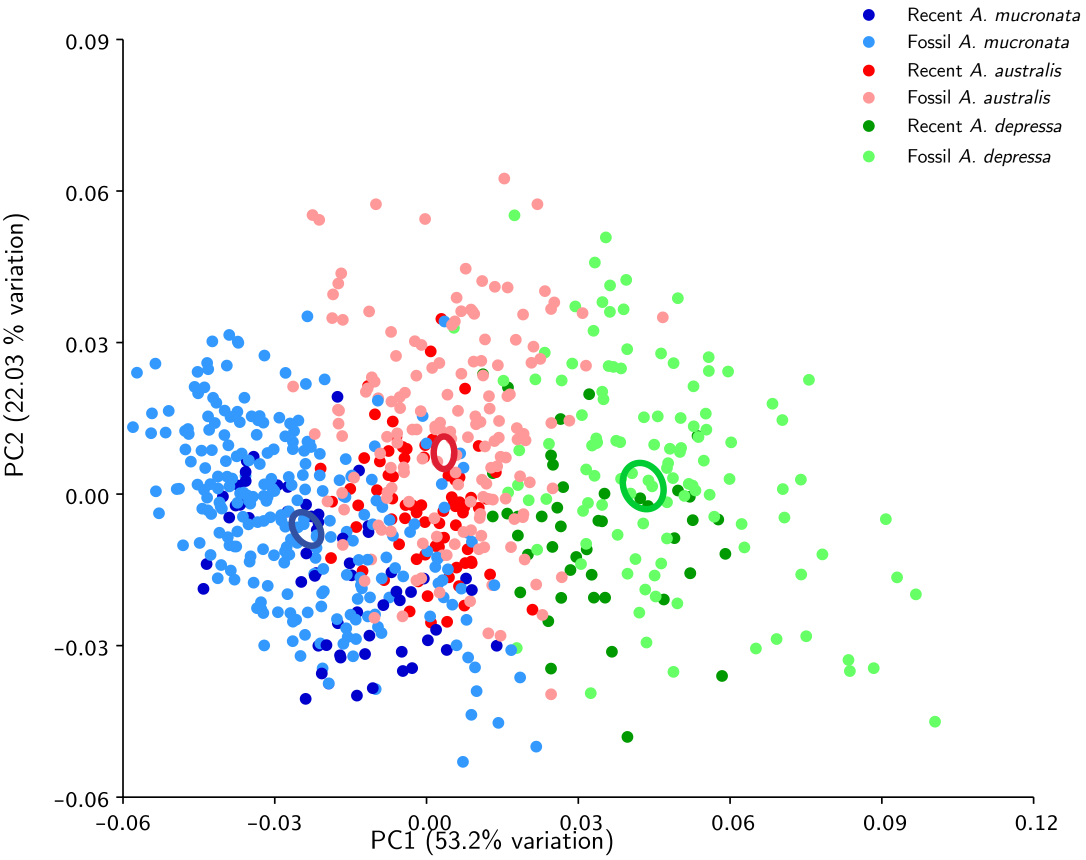


Figure 5.4. Distribution of recent and fossil *Amalda* specimens in morphospace based on the first two principal components. Morphospace distributions of the three species, identified and colour coded prior to analyses overlap. Dark colours indicate recent specimens and pale colours indicate fossil specimens showing the greater variation seen in the fossil samples compared to the recent specimens. 90% confidence ellipses of means are shown for the three species.

Morphometric clustering without priors

Bayesian cluster analysis without *a prior* assignment of specimens, using the first three PCs, found three morphological clusters (Fig. 5.5). One cluster was closely associated with samples recognised as *A. mucronata.* The two other clusters contained both *A. australis* and *A. depressa* samples. The *A. australis* and *A. depressa* were split by the clustering algorithm perpendicular to an axis on which *A. australis* and *A. depressa* are best separated based on currently recognised identification (Fig. 5.5). The disagreement between clusters determined by Bayesian assignment and preliminary species identification is apparent in the assignment probabilities of each specimen, grouped by preliminary species identification (Fig. 5.6). When only the first two PC's were used, two clusters are recovered, a cluster of *A. mucronata* and a cluster of both *A. depressa* and *A. australis* together (Fig. 5.7).

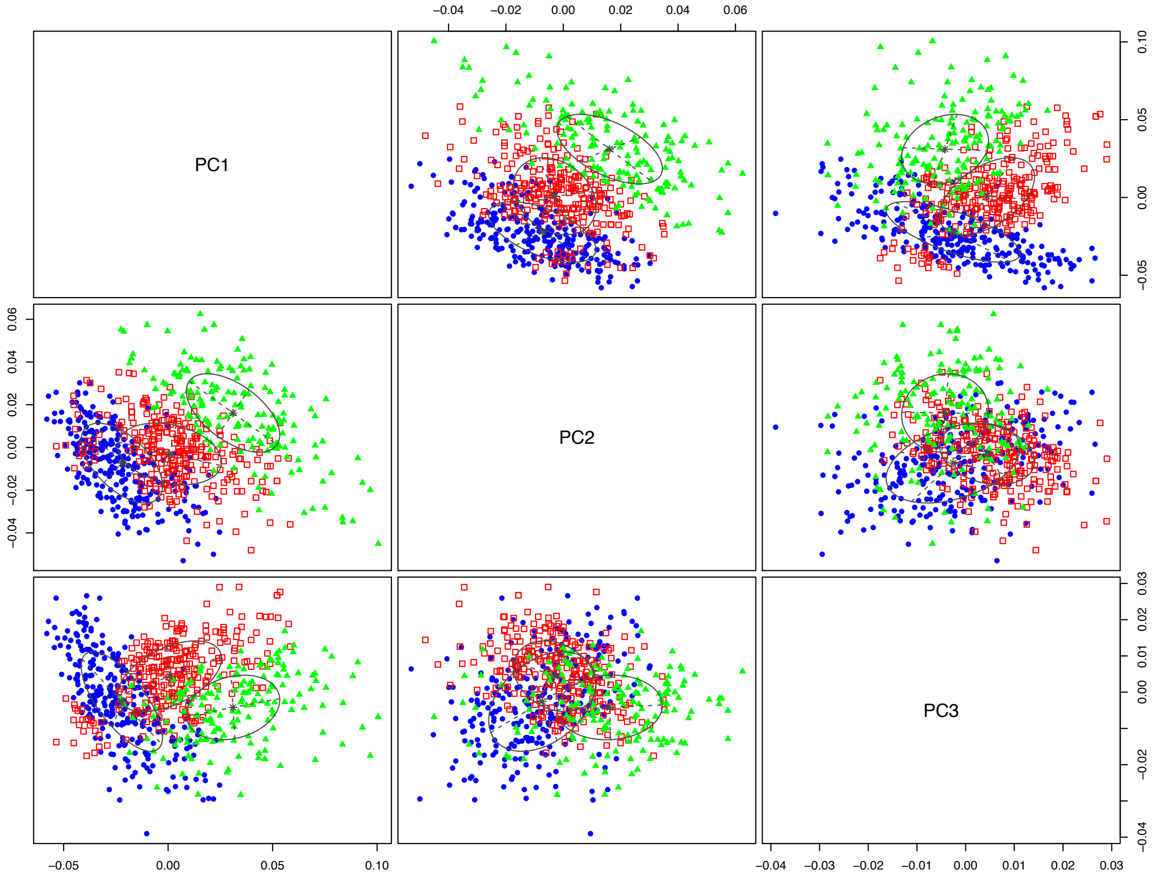


Figure 5.5. Bayesian cluster analysis of the first three principal components of shell shape from three New Zealand *Amalda* species. Three clusters shown by the different colours are inferred using a EEV (ellipsoidal, equal volume and shape) model with 3 components: BIC 10980.

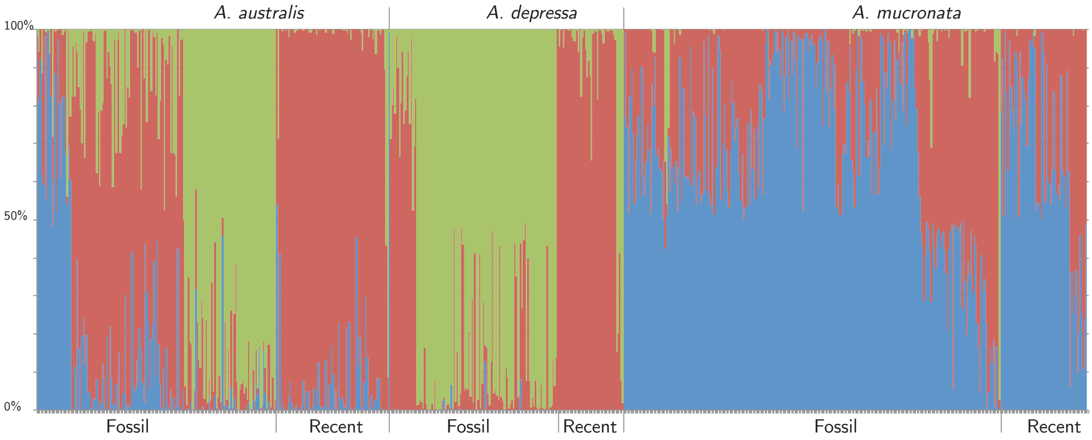


Figure 5.6. Assignment probabilities of *Amalda* specimens into the three clusters determined by Bayesian analysis of three PCs in Mclust. 80% of *A. mucronata* specimens are clustered together with 15% of the *A. australis* samples. 60% of *A. australis* specimes are clustered together with 20% of *A. mucronata* and 40% of *A. depressa*. 60% of *A. depressa* and 40% of *A. australis* specimenscluster together. Each sample is assigned to one of three clusters based on the highest P score.

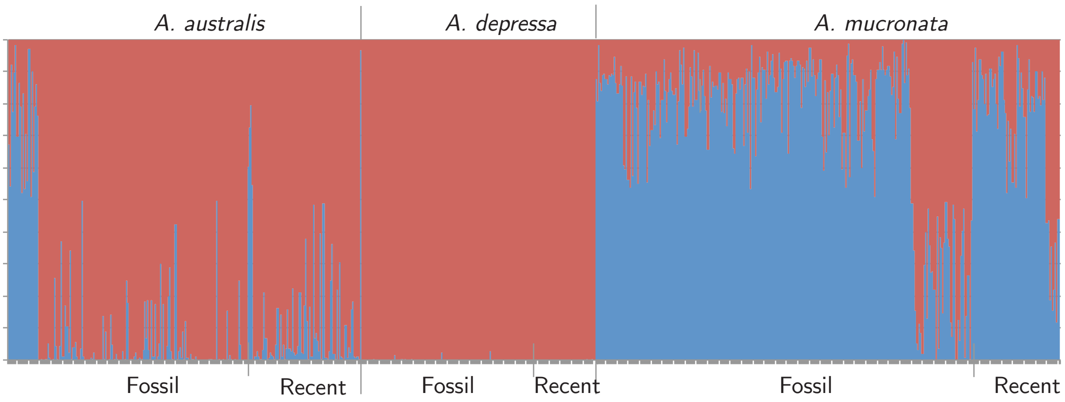


Figure 5.7. Assignment probabilities of *Amalda* specimens into the two clusters chosen by Bayesian analysis of PC1 and PC2 in Mclust VVV (ellipsoidal, varying volume, shape, and orientation) model with 2 components: BIC 10971. 84% of *A. mucronata* are assigned to a cluster correctly while 89% of *A. australis* and 99% of *A. depressa* specimens are assigned to a single cluster.

Because most *A. mucronata* specimens formed a single cluster closely matching *a priori* assignment, a separate Bayesian clustering analysis with just the specimens that had been preliminarily identified as either *A. australis* or *A. depressa* was undertaken. As principal component analysis summarises all the variation in the group, by removing *A. mucronata* from the analysis only the variation within *A. australis* and *A. depressa* is tested and may provide better separation of the two species than when the three species are combined. The first three significant PCs were used and the model that best fitted the shape variation had three morphological clusters; one closely matching *A. depressa* and the *A. australis* specimens divided into predominantly fossil and recent groupings (Fig. 5.8).

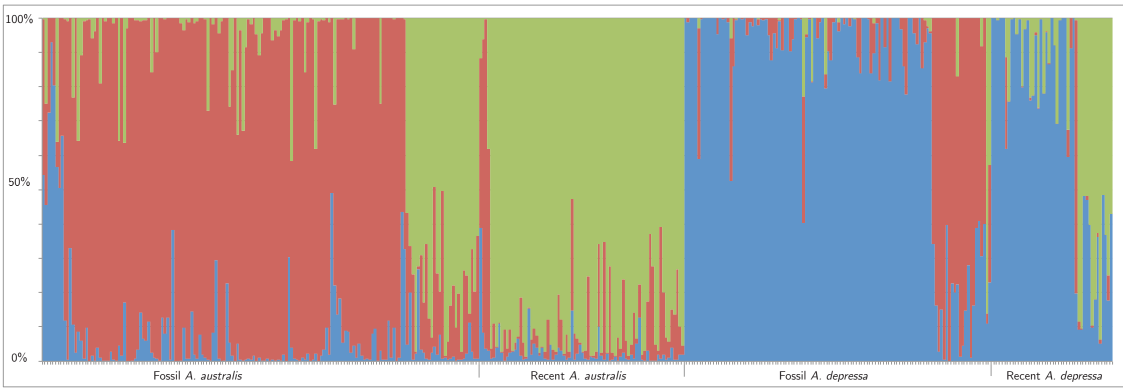


Figure 5.8. Assignment probabilities of *A. australis* and *A. depressa* into the three clusters chosen by Bayesian analysis in Mclust VEV (ellipsoidal, equal shape) model with three components: BIC 6268.265

Discriminant testing

Genetic identification of New Zealand *Amalda* specimens revealed that genetically distinct lineages can be difficult to distinguish using shell shape characteristics (Chapter 4). Morphological variation in the three species make results in a minority of individuals being morphologically cryptic. To assign fossil specimens to one of the three possible species their shell shape variation was examined using a training dataset. The modern *Amalda* specimens were confirmed as being correctly identified based on genetic genetic evidence and used as the training dataset. Discriminant testing of the fossil dataset based on the training dataset found up to 20% of samples misallocated according to their preliminary species identification. Even when genetically identified specimens were used, shape similarity among these *Amalda* species resulted in difficulty identifying species boundaries. When all three species were tested together there was a 9% error in the training set. When the fossils were examined, 19% were assigned to a species other than their prior identification (Fig. 5.9). As Bayesian assignment models revealed that limiting datasets to include just two species helped identify informative shape variation, discriminant analysis testing was performed with subsets of the data using two species groups at a time. When phenotypically closest species pairs were tested the training error was 4% for *A. australis* and *A. depressa*, with a testing error of 20% (Fig. 5.10) *A. australis* and *A. mucronata* had a training error of 2% and 11% testing error (Fig. 5.11; testing and training results can be seen in Supplementary Table 5.3).

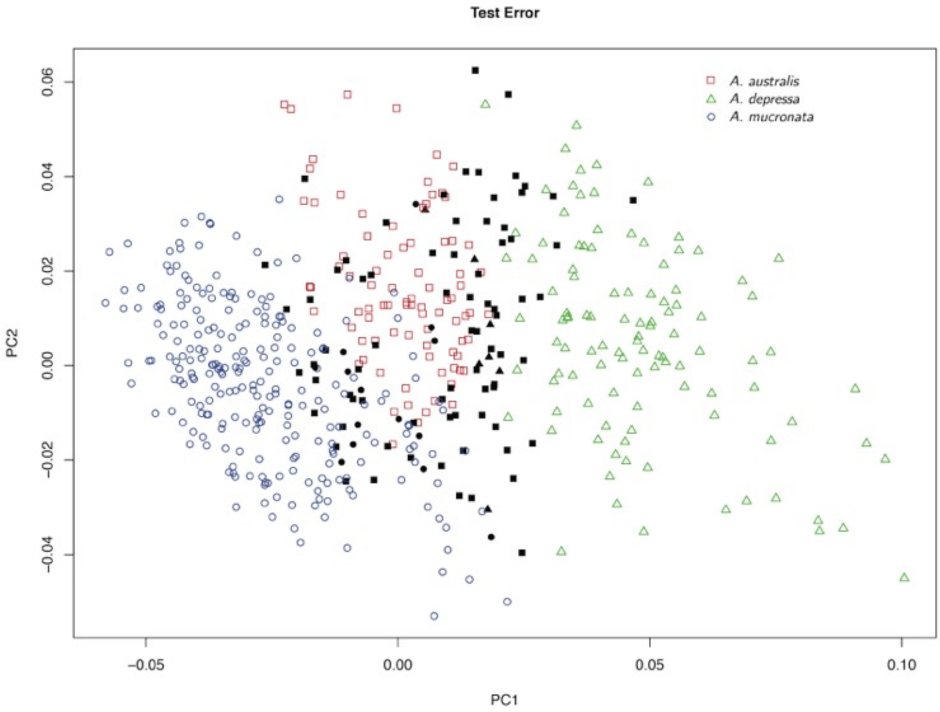


Figure 5.9. Discriminant testing of fossil *A. mucronata*, *A. australis* and *A. depressa* after training with data from genetically identified extant snail shells. Empty shapes indicate specimens correctly assigned to preliminary species identification. Black shapes indicate specimens with incorrect or equivocal assignment.

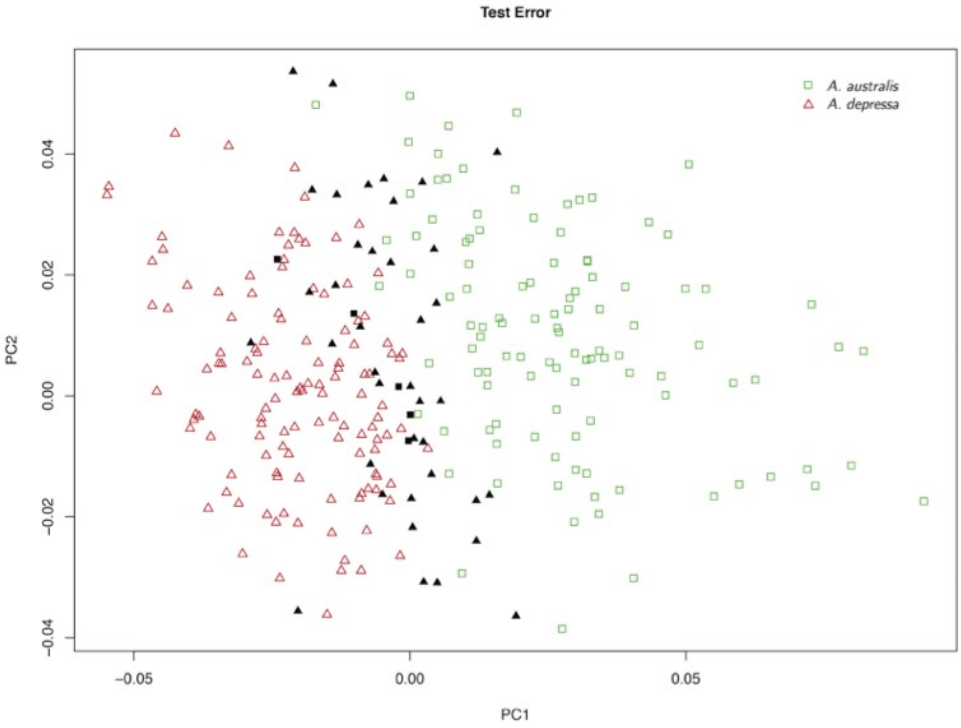


Figure 5.10 Discriminant testing of fossil *A. australis* and *A. depressa* specimens after training with genetically identified extant snail shells. Empty shapes indicate specimens correctly assigned to preliminary species identification. Black shapes indicate specimens with incorrect or equivocal assignment

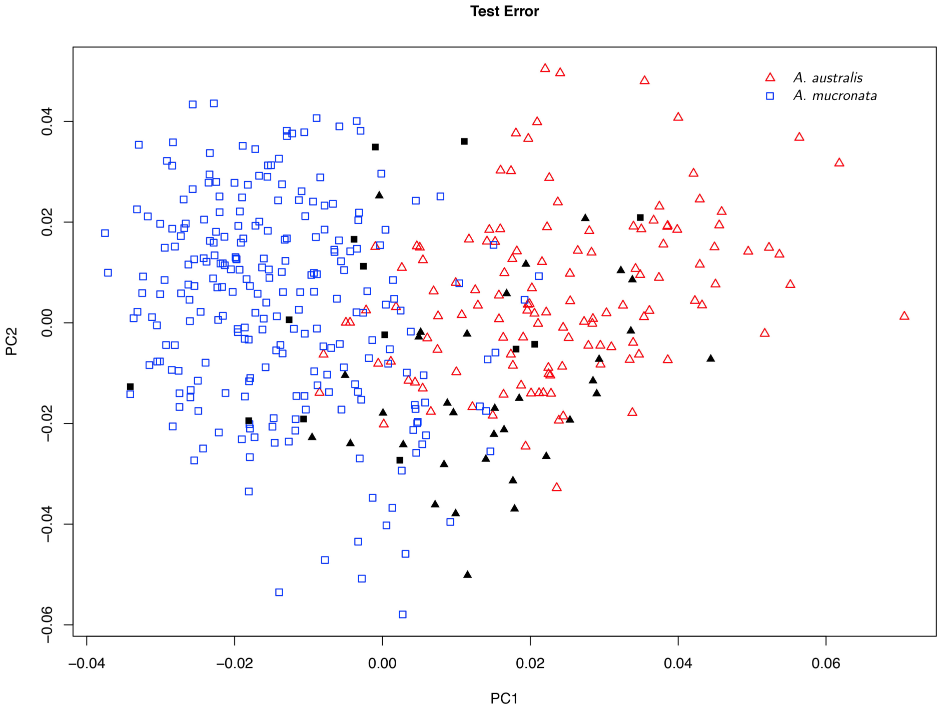


Figure 5.11. Discriminant testing of fossil *A. mucronata* and *A. australis* specimens after training with genetically identified extant snail shells. Empty shapes indicate specimens correctly assigned to preliminary species identification. Black shapes indicate specimens with incorrect or equivocal assignment

Evolutionary model

Both cluster analysis and discriminant analysis support the assumption that the majority of fossil *Amalda* specimens are correctly identified (84–89%) and so enabled examination of shell shape evolution.

For each fossil time series, the mean and variance of each shell trait was calculated and used to model morphological evolution. First, the most taxonomically informative shell shape trait (first PC1 from analysis of the group as a whole) was used to determine the best fit evolutionary model for each independent lineage (species) (Fig. 5.12). For further analysis shell shape variation for each lineage was analysed separately using principal components determined as being significantly informative (broken stick test), and shell size. For each *Amalda* lineage, the time series consisted of between nine and 15 time points spanning 2–3 million years, with each time point represented by four or more specimens. For each independent lineage (species) the best fit evolutionary model was determined for each significant PC. All three lineages tested showed evidence of stasis and Unbiased Random Walk (URW) as the most likely model explaining the evolution of the shape characters tested. Stasis was the model that fitted the most time series, accounting for eight of the thirteen traits. For *A. australis*, during two million years, PC1 from whole group analysis indicated URW (Fig. 5.12) and for independent lineage analysis four of five traits fitted a model of morphological stasis (Fig. 5.13). PC3 in *A. australis* was found to have a complex model favoured over simple models, stasis followed by random walk was slightly favoured over random walk (Akaike weights 0.464 and 0.434 respectively). The *A. depressa* lineage showed morphological stasis for PC1 from whole group PCA (Fig. 5.12) and only one trait from independent analysis (PC2; Fig. 5.14). *A. depressa* did not have enough time series to include analysis of complex models. The *A. mucronata* lineage had URW as best fit for whole group PC1 (Fig. 5.12) and had two morphological traits from independent analysis (size and PC3) that showed stasis over three million years (Fig. 5.15). *A. mucronata* had no traits that favoured complex models over simple ones. No traits in any of the three *Amalda* lineages best fitted a model with a directional trend (GRW).

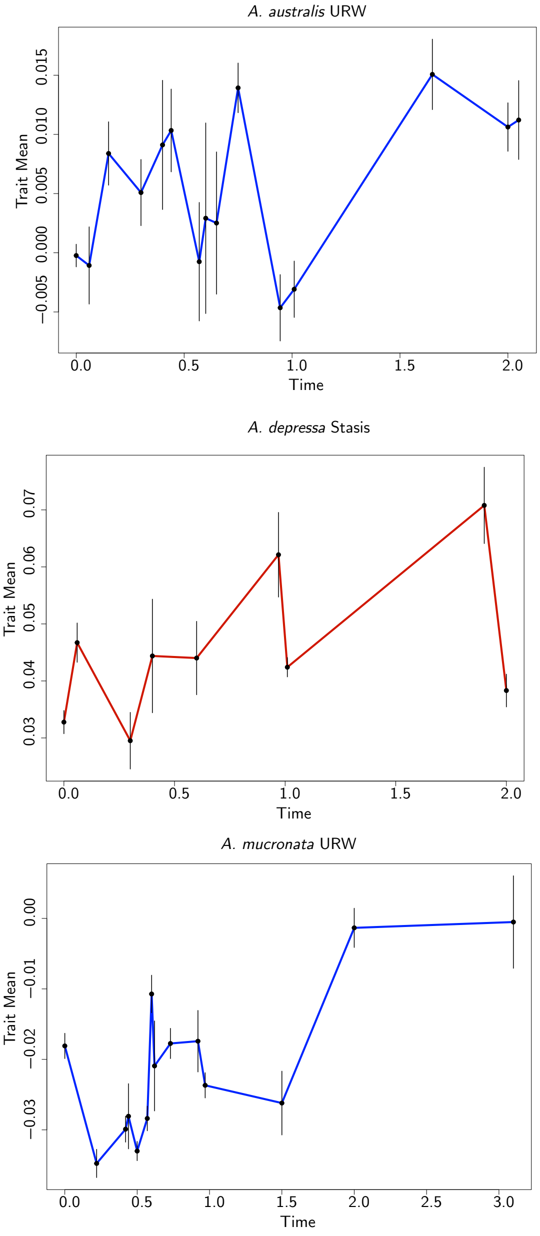


Figure 5.12 Variation through time of PC1 of shell shape variation in fossil *Amalda* analysed as three species together showing both stasis and URW evolutionary models. Time in millions of years from present(mya) (left). *A. australis* weakly fits the URW model with Akaiki weight 0.602, *A. depressa* fits the stasis model (Akaiki weight 0.921) and *A. mucronata* weakly fits the URW model (Akaiki weight 0.514)

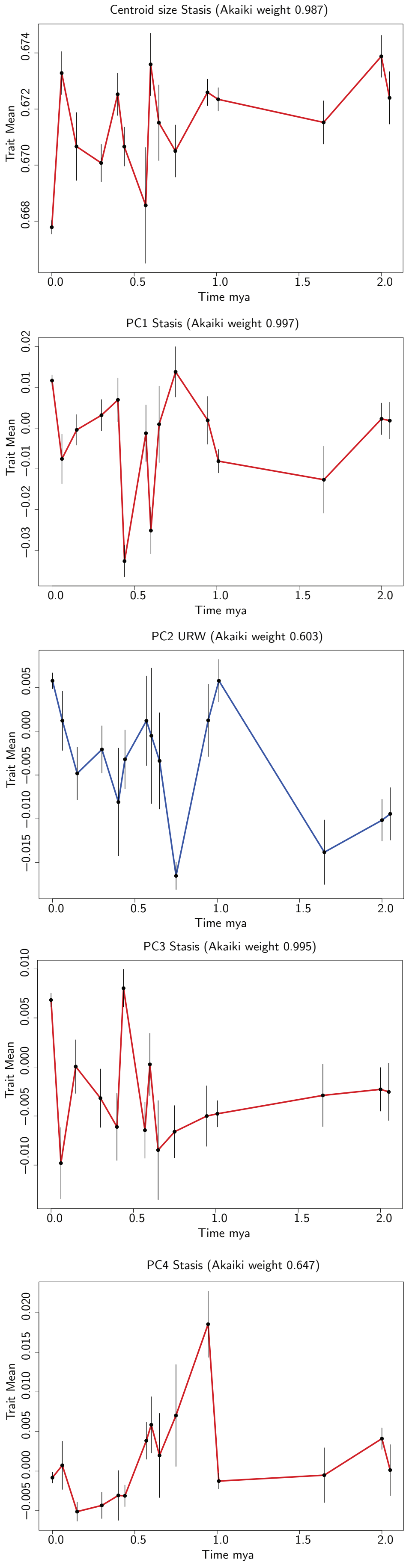


Figure 5.13. Variation through time of centroid size and principal components of shell shape variation in the *A. australis* lineage, showing both stasis and URW evolutionary models. Time in mya from present (left) Centroid size has strong support for stasis (Akaike weight 0.987), PC1 strong stasis (Akaike weight 0.997), PC2 weak support for URW model (Akaike 0.603) and PC3 strong support for stasis (Akaike weight 0.995), PC4 moderate stasis (Akaike 0.647). When analysed with complex models included, the best fit model for PC3 slightly favoured a complex model of stasis-random walk (Akaike weight 0.463) over stasis (Akike weight 0.434)

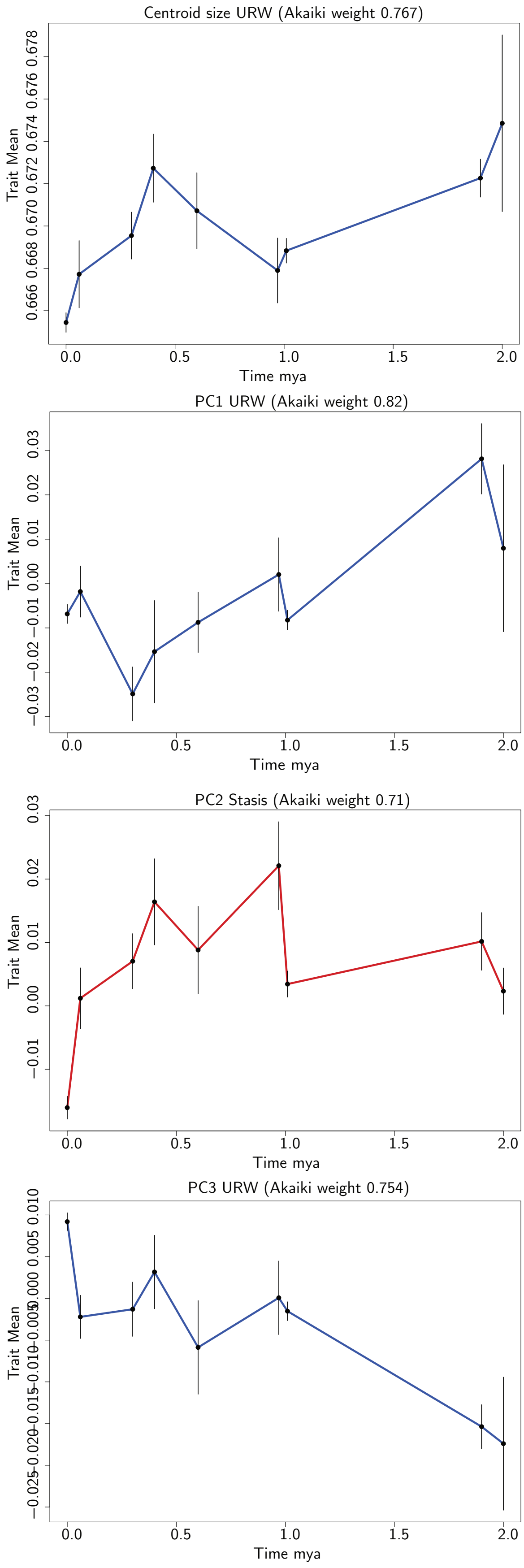


Figure 5.14. Variation through time of principal components in the *A. depressa* lineage. Showing the presence of both stasis and URW. Time in mya from present (left) Centroid size moderate URW Akaike weight 0.767, PC1 weak URW Akaike weight 0.820, PC2 moderate stasis Akaike 0.710 and PC3 moderate URW Akaike weight 0.754

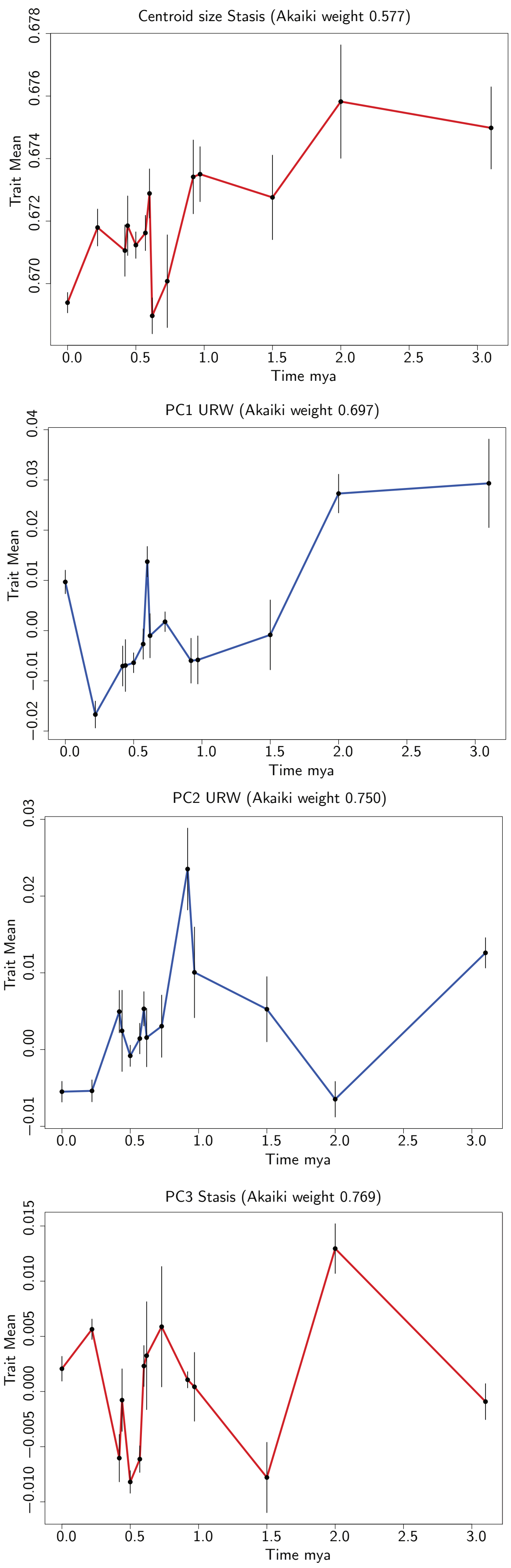


Figure 5.15. Variation through time of principal components in the *A. mucronata* lineage. Showing the presence of both stasis and URW. Time in mya from present (left) Centroid size weak stasis Akaike weight 0.577, PC1 Weak URW Akaike weight 0.697, PC2 moderate URW Akaike 0.750 and PC3 moderate stasis Akaike weight 0.769

Because of the overlapping morphospace distributions of the lineages the possibility of misidentification of *Amalda* specimens in this dataset is recognised. To test the possible impact of ambiguous specimens in the analysis I removed eight *A. australis* and 34 *A. depressa* and 54 *A. mucronata* fossil specimens that the model based clustering had assigned to lineages other than their prior identification (Fig. 5.6 & 5.8) from the dataset and repeated the analysis. This had little effect on the fit of the evolutionary models and all traits examined were inferred to have evolved according to the same model as seen in the full dataset. As an alternative all specimens clustered together were tested as groups (Fig.5.6) Cluster 1, which was composed of 261 putative *A. mucronata* and 22 putative *A. australis* were analysed as a lineage. All three principal components tested showed stasis as best fit. Cluster 2, 149 *A. australis* 57 *A. depressa* and 63 *A. mucronata* had stasis as best fit for PC1 and PC3 and URW for centroid size and PC2. Cluster 3 57 *A. australis* 102 *A. depressa* and a single *A. mucronata* best fit stasis for PC1 and PC2 and URW for centroid size (Table 5.1).

Sampling biases resulting from collecting recent specimens as opposed to fossil specimens may preferentially favour shells of different developmental stage and influence results (Jablonski et al., 2003; Kidwell, 2001). I tested for any impact of this by repeating the analysis with recent specimens removed. Again this had little effect with only one trait for one species being assigned a better fit to an alternative model from stasis to URW (Table 5.1)

Table 5.1 Best fit model for morphologic change through time for different groups tested. Lineages as identified by species, specimens whose ID is consistent with cluster analysis, and Bayesian clusters from PC analysis of all specimens. The numbers in brackets are the Akaike weights for each model tested

test group	Centroid size	PC1	PC2	PC3	PC4
A. australis	stasis (0.987)	stasis (0.997)	URW (0.603)	stasis (0.995)	stasis (0.647)
A. depressa	URW (0.767)	URW (0.82)	stasis (0.71)	URW (0.754)	
A. mucronata	stasis (0.577)	URW (0.697)	URW (0.75)	stasis (0.769)	
A. australis = cluster	stasis (0.952)	stasis (0.987)	URW (0.473)	stasis (0.882)	stasis (0.764)
A. depressa = cluster	URW (0.638)	URW (0.830)	stasis (0.520)	URW (0.745)	
A. mucronata = cluster	stasis (0.770)	URW (0.814)	URW (0.680)	stasis (0.709)	
Cluster 1	stasis (0.779)	stasis (0.802)	stasis (0.918)	stasis (0.785)	
Cluster 2	URW (0.661)	URW (0.665)	stasis (0.981)	stasis (0.994)	
cluster 3	URW (0.563)	stasis (0.987)	stasis (0.759)		
A. mucronata fossil	stasis (0.79)	URW (0.706)	URW (0.706)	stasis (0.632)	
A. australis fossil	stasis (0.858)	stasis (0.999)	stasis (0.749)	stasis (0.996)	staisis (0.883)
A. depressa fossil	URW (0.500)	URW (0.724)	stasis (0.502)	URW (0.812)	

Discussion

It has been suggested that the New Zealand *Amalda* provide strong support for the theory of punctuated equilibrium (Gould, 1991, 1991). Re-examination of the fossil *Amalda* material studied by Michaux (1989) has shown that assignment of fossil material to the three morphologically similar species *A. depressa*, *A. australis* and *A. mucronata* is by no means simple and straightforward. Modern material of these three species can be confidently distinguished using genetic data, data that confirms they represent distinct evolutionary lineages. However even the modern material is difficult to identify using just shell shape traits (Chapter 4).

I start with the hypothesis that the same three *Amalda* species in the extant fauna are represented in the fossil material. These three separate lineages are *A. australis A. depressa* and *A. mucronata* extending into the fossil record. I have tested the separation of the three lineages using various means both in the modern fauna with genetic and morphometric analysis and the fossil record. I have found no strong evidence to suggest rejection of these species hypothesises so I accept the simplest explanation being that the taxonomic analysis and classification species and specimens into species groupings are largely correct. I have accepted that there will be some misidentifications of specimens and have explored the downstream effect of these identifications.

Morphological clusters of fossil and recent specimens representing the three lineages are apparent in morphospace using the first two principal components of morphometric variation (Fig. 5.2). Having recognisable clusters containing both fossil and modern representatives gives us confidence that the majority of fossil specimens are correctly classified. More overlap among species in morphospace was apparent in the fossil record compared to the modern samples, which is probably a reflection of the increased amount of variation recorded in the fossil dataset. This contrasts somewhat with the previous work using a different summary of shell shape variation, which concluded that morphologic variation in fossil *Amalda* species fell within the range of variation observed in recent specimens (Michaux, 1989). The current landmark morphometric dataset did not include landmarks pertaining to the outer lip of the aperture because of the frequency with which fossil samples were damaged in this region. Landmarks on the outer lip of the aperture had been used with the modern specimens and were important components of both the first and third components of that analysis. This may have reduced available variation required to separate species, but the most taxonomically informative landmarks pertaining to relative length and width of the shell and the shape of the spire were included.

Having phenotypically similar species is going to create problems for morphological species delimitation leading to potential misidentification of samples and means that separate species clusters are not always captured by the Bayesian analysis. Despite this, the vast majority of specimens fit morphologically to species clusters. Of the three species *A. mucronata* is the most easily recognised species assuming correct expert identification with 84% of recent and fossil samples resolved as part of a distinct morphological cluster based on just shell shape. When a training dataset of genetically identified specimens was used, discrimination had only an 11% error rate. The two species *A. australis* and *A. depressa* could more accurately be separated into clusters when analysed without *A. mucronata* specimens. I was able to test the morphologic assignment of specimens into species because I have a dataset that includes a set of genetically confirmed samples rather than undefined fossil specimens. The discriminant tests showed that when testing all three species together or in pairwise comparisons >= 80% of fossil samples were assigned to their preliminary species classification. I am unable to determine whether miss-assigned samples are misidentified or morphologic ambiguous specimens. It seems that in most cases the first principal component is the most taxonomically informative, by the third PC variation has little correlation with a priori species identification.

Variation in independent shell characteristics through time: models of evolution.

Surprisingly few studies have modelled independent morphological traits in the same lineage over the same time frame (Hopkins and Lidgard, 2012; Hunt et al., 2015; Voje, 2016). The use of principal components generated from 2d-landmark morphometric methods enables us to do this with mathematically uncorrelated elements of shell shape, which were independent of shell size. One goal of this study was to attempt confirmation or otherwise of morphologic stasis in *Amalda* lineages which would allow for the possibility of punctuated equlibrium as a mode of evolution. How well each of three models of shape variation through time (directional, random walk, or stasis) fitted the morphologic data was compared. I found that all three lineages studied showed at least one of their shell traits fitted a model of stasis better than any other model. *A. australis* had the most traits that fitted the stasis model, i.e. shell size and three of the four principal shape components spanning over two million years. *A. mucronata* also had stasis indicated for its shell size and one of the three principal components tested. *A. depressa* had one principal component conform to the stasis model but centroid size and two of the three principal components best conformed to the URW model. For traits not showing stasis for *A. australis* and

A. mucronata, URW was the most appropriate model. The recognition of stasis as an evolutionary mode in all three species goes some way to corroborating the results found by Michaux (1989) that *A. mucronata* and *A. australis* species have essentially fluctuated around an optimal (unchanging) mean over 2-3 million years. These three species inhabit near shore environments which could be considered dynamic environments, being susceptible to short term (storm action) or longer term factors (sea level fluctuations associated with the Pleistocene glaciations). Fluctuating environmental conditions have been argued as both favouring and against stasis occurring in the fossil record (Haller and Hendry, 2014; Sheldon, 1996).

I accept that there may be some specimens in my analysis that are misidentified but I am confident at least 80% of specimens have the correct assignment. Due to the potential error rate in classification of specimens I removed all specimens from the analysis with low assignment probabilities to test the influence on evolutionary model assignment. Inferred models of morphological evolution were not strongly influenced by these ambiguous specimens. I am confident fossil species as recognised reflect a continuum with the modern representatives at the tip, reconfirming the relationship between extant species and fossil lineages and that any errors in classification have not significantly influenced the identification of evolutionary models within each lineage. Alternative analyses involving removal of specimens that had conflicting species identification and cluster assignment did little to alter the inferred model of evolution. This gives us some confidence in the robustness of the inferences made regarding evolutionary mode against misidentification of a proportion of specimens. Again when specimens identified as clusters (Fig. 5.6) were analysed as single lineages mixed models were found to fit best. Stasis was favoured as the best fit model of evolution in cluster one. The effect of the clustering algorithm choosing clusters that have specimens with similar phenotypes, could have an effect of reducing the amount of variation apparent in a putative lineage and could favour stasis; however, finding both stasis and URW in the other two clusters does negate this, indicating mixed models are pervasive in this dataset.

The strength of this study is that I have mathematically independent shape and size traits of a lineage that clearly reveal that the same lineage can exhibit more than one mode of morphological evolution. The recognition of different evolutionary models found between traits within a species is consistent with the idea of mosaic evolution, in which evolutionary units are a combination of traits and these can evolve in differing ways (Gerber and Hopkins, 2011; Hopkins and Lidgard, 2012; Klingenberg, 2008; Stebbins, 1983). That different models were recognised within the different lineages seems to fit well with the idea that evolution is taking place in dynamic environments with countless number of pressures having some level of influence on species.

Constraining (stabilising) selection is thought to be responsible for morphological stasis (although palaeontologists suggest gene flow could also be the explanation (Hansen and Houle, 2004)). The unbiased random walk model suggests that drift rather than selection is the main process influencing the trait. In contrast, directional selection is likely to result in directional evolution of trait variation, a model of morphological evolution not seen in New Zealand *Amalda* lineages.

Divergence times

The timing of lineage splitting in relation to morphologic change is another fundamental premise of PE. The appearance of novel forms in the fossil record is one of the primary ways of inferring the dates of lineage splitting (and of fundamental importance in developing molecular clocks). Accurate estimates of lineage splitting events are highly dependent on sampling probabilities, how certain it is that the sampling effort has sampled fossils when they first appeared, capturing the divergence time. The sampling probabilities of the New Zealand Cenozoic marine fossil record are well understood (Cooper et al., 2006; Crampton et al., 2006, 2003). Earliest reliable records for all three species *A. mucronata*, *A. australis* and *A. depressa*, as recorded on the FRED fossil data base, predate the periods of stasis inferred in this study. Divergence time estimates can be made using DNA mutation rate estimates. Getting accurate mutation rates is a limiting factor in determining accurate dates and even related lineages can have differing molecular clock rates (Hills et al. in press). Invertebrate mitochondrial DNA nucleotide substitution rates are generally somewhere in the range of 2–0.7% per million years, although rates vary greatly between groups so using rates from other taxa to infer speciation dates must be done with caution (Knowlton and Weigt, 1998; Lynch and Jarrell, 1993). Using the rates reported for marine snails (0.5–0.07%) provides a conservative range (Ozawa and Okamoto, 1993; Reid et al., 1996). Nucleotide substitutions per site based on phylogenetic trees inferred from coding mitochondrial genes were used to infer first appearance of the lineages now recognised as *A. mucronata*, *A. australis* and *A. depressa*, and these all pre-date the periods of stasis observed in the fossil record. Periods of morphological stasis observed were not concurrent with estimated lineage splitting dates, and thus fossil *Amalda* meet another prerequisite for punctuated equilibrium.

Conclusions

Genetic and morphological lines of evidence indicate that the taxa *A. mucronata*, *A. australis* and *A. depressa* are recognisably separate lineages extending from the present back millions of years into the fossil record. The phenotypic similarity of the species is recognised in the modern fauna and the difficulty distinguishing the taxa is more pronounced with fossil specimens, given the greater morphological diversity exhibited and the loss of some distinguishing features due to the fossilisation process. I recognise that this may mean that some specimens in both this analysis and the previous study (Michaux, 1989) are misclassified but I have explored the effect of this and can conclude that the estimated level of misclassification is likely to have very little influence on inferences drawn about models of evolution in these lineages. Misidentification is occurring at the edges of the species clusters (where the lineages have similar phenotypes) and therefore might increase the recorded variation within each species but removal of ambiguous specimens had little effect on evolutionary inferences. My results indicate the presence of stasis over long timeframes during the evolution of *Amalda* species and that stasis is not concurrent with lineage splitting events. The presence of stasis is a crucial result and is compatible with punctuated equilibrium as a description of how *Amalda* evolved in New Zealand. By continuing to combine molecular phylogenetic data with fossil evidence I can look for correlations between shifts in morphology and inferred lineage splits within this genus.

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Supplementary Table 5.2. Fossil collection dates and localities used in analysis

AU Number	Fossil Record Number	AGE	Locality
912	N138/f393 R22/f393	MIS 9, 300 ka	Landguard Bluff
923	N137/f352 R22/f6352	150ka	Castlecliff Beach
925	N137/f351 R22/f6351	MIS 11, 400 ka	Castlecliff Beach
926	N137/f350 R22/f6350	MIS 11, 420 ka	Castlecliff Coast
930	N137/f429 R22/f6429	440 ka	Castlecliff Beach
934	N137/f357 R22/f6357	440 ka	Wanganui Coast
939	N137/f368 R22/f6368	MIS 14?, 570 ka	Castlecliff C. 1-1/2 ml W. of end of Springdale Rd at "The
940	N137/f690 R22/f6690	570 ka	The Pinnacles Castlecliff Coast
975	N137/f469 R22/f6469	440 ka	Castlecliff Coast
976	N137/f516 R22/f6516	MIS 13, 500 ka	Castlecliff Coast - "The Buttress" - 90 ch NW of Castlecliff
981	N137/f516 R22/f6515	500 ka	Castlecliff Coast - "The Buttress" - 90 ch NW of Castlecliff
987	N69/f520 W15/f7520	600 ka	Northern Coastal Section Whale Island
990	N69/f501 W15/f7501	600 ka	Ohope Beach, Fleming's Loc GS 3893
1001	N137/f360 R22/f6360	570 ka	Wanganui Coast
1002	N137/f571 R22/f6571	570 ka	Castlecliff Coast between "Pinnacles" & "Buttress"
1005	N138/f461 S22/f6461	MIS 15, 650 ka	Wangaehu Valley SW of Mechan Trig, beside farm road
1006	N138/f525 S22/f6525	650 ka	Wangaehu River, W bank
1008	N137/f373 R22/f6373	700 ka	Coast c 2 ml SE of Kai Iwi Beach
1026	N138/f417 R22/f7417	MIS 19 base, 800 ka	Mt Jowett, spur 30 ch SE of
1027	N137/f393 R22/f6393	MIS 23, 920 ka	Beach E of Kai Iwi R. mouth
1028	N137/f393 R22/f6393	920 ka	Beach E of Kai Iwi R. mouth
1029	N138/f404 R22/f7404	MIS 25, 950 ka	Kaimatira Bluff E side Wanganui River
1034	N137/f392 R22/f6392	920 ka	Wanganui Coast 1/2 ml SE Kai Iwi River mouth
1035	N137/f395 R22/f6395	950 ka	Kai Iwi Beach Road
1036	N137/f398 R22/f6398	MIS 27, 970 ka	Wanganui Coast, Okehu Stm mouth
1037	N137/f449 R22/f6449	950 ka	Wanganui Coast, Okehu Stm mouth
1043	N137/f404 R22/f6404	MIS 27, 970 ka	G & Northwestern Rd., Kai Iwi
1044	N137/f402 R22/f6402	970 ka	Roadside, Okehu Valley
1045	N137/f397 R22/f6397	970 ka	Wanganui Coast, Okehu Stm mouth
1048	N143/f498 S23/f6498	Tainui Shbed, 500 ka	Rangitikei Valley, W side, upstm from Onepuhi Br.
1052	N62/f505 Y14/f7505	MIS 7, 220 ka	As above NO, East Cape
1054	N62/f505 Y14/f7505	MIS 7, 220 ka	As above
1062	N137/f299 R22/f6299	400 ka	Wanganui Coast
1063	N137/f352 R22/f6352	150ka	Castlecliff Beach
1069	N78/f582 W15/f9582	600ka	Cutting on NE side of Wainui Rd., just E of Wainui Stm mouth
1070	N78/f582 W15/f9582	600ka	As above
1072	N137/f414 R22/f6414	MIS 59, c. 1.67 Ma	Coast W of Ototoka Stm mouth
1073	N137/f417 R22/f6417	MIS 57, c.1.65 Ma	Wanganui Coast, SE of Ototoka Stm mouth
1104	N137/f373 R22/f6373	MIS 17, 700 ka	Coast c 2 ml SE of Kai Iwi Beach
1107	N137/f415 R22/f6415	MIS 59, c. 1.67 Ma	Maxwell Coast, W of Ototoka Stm mouth
1142	N139/f472 T22/f8472	2.0 Ma	Hunterville - Taihape main road, C. 6 ml from Hunterville
1172	N165/f1203	2.0 Ma	East bank Ruakokopatuna River, 20 m upstream from Hautotara
1284	N62/f505 Y14/f7505	MIS 7, 220 ka	Left of main road 3-3/8 ml E of Cape Runaway P.O.
1289	N62/f589 Y14/f7589	MIS 7, 220 ka	Te Piki
1387	N137/f350 R22/f6350	420 ka	Upper Castlecliff s/b 20' above top of S.C. Siltstone
1611	N137/f567 R22/f6567	MIS 5a, 60 ka	Waverly Beach
1613	N137/f692 Q22/f7692	MIS 5a, 60 ka	Immediately E of Low Waipipi Shellbed
1935	N129/f617 Q21/f6617	3.3 Ma	0-2.5m above base of cliff 325-375m east of Inaha stream mouth
2386	N135/f543	3.5 Ma	Kereru Rd, 50-80m W of bridge
2423	W21/f8497a	1010 ka	Kereru Rd, E for 100 yds from junction with Wharikira Stn Rd
2637	N62/f505 Y14/f7505	MIS 7, 220 ka	Black Reef, Cape Kidnappers
4407	N137/f353 R22/f6353	500 ka	The Pinnacles
5519	Q21/f3	60ka	In cliff 200m W of stream 0.95km NW Waihi Beach
5521	Q21/f5	60ka	200m up Denby Road stream from sea below carpark
5526	Q21/f10	60ka	0.6km SE of Rifle Range Stream
5534	Q21/f18	60ka	0.6km SE of Waihi Beach track
5547	Q21/f30	60ka	On cliffs 0.15km E of Rifle Range Stream
5572	Q21/f56	60ka	On right hand side of Waihi Beach track when facing the sea
5628	W15/f9583	600ka	Road cut Wainui Road, c 700m N of Te Kooti Road
5709	N134/f712 U21/f8712	Wn, c. 2 Ma	Shellbed exposed in fallen boulder upper Okauawa Stream
5733	N146/f498b	1080 ka	Road cut 180m along Maharakeke Rd from Main Rd
6474	N135/f518	1010 ka	Black Reef section
6475	N135/f W21/f8518	1010 ka	West of Black Reef shell bed, Kidnappers section
6476	W21/f8551	1010 ka	Maraetotara Valley, east branch, 100 - 150' above river, 100 yds upstream from earlier collection
6477	N135/f497	1010 ka	Maraetotara Valley, 100 - 150 ' above river level
6478	N135/f561	1010 ka	20' above base, 30 ch W of Black Reef 60' above sea level
6479	N137/f518 R22/f6518	MIS 15, 620 ka	Fall of Lower Castle Cliff Shellbed at Toms conglomerate
7125	N137/f350 R22/f6350	320 ka	Wanganui coast
7852	R22/f6690	570 ka	Pinnacles, Wanganui
8420	R22/f108	920 ka	Mowhanau Beach, south of Kai Iwi Stream
8592	N129/f551 Q21/f6551	Wp, c. 3.3 Ma	Waihi Beach, Hawera
8992	N63/f532 Z14/f9532	20 Ma	400 yds north of N63/f530
9639	N137/f516 R22/f6516	500 ka	Tainui Shell Bed, Castlecliff Coast - "The Buttress" 90 ch NW of Castlecliff
9642	N137/f397 R22/f6397	MIS 27, 970 ka	Okehu Shelly Grit, Wanganui Coast
11037	N02/f7598	18 Ma	Parengarenga Harbour

Supplementary Table 5.3 Results from Discriminant tests showing the error found during training using the modern samples and when using the fossil dataset is tested against the training set.

A.australis, A. depressa and *A. mucronata*				*A. australis* and *A. depressa*				*A. australis* and *A. mucronata*			
MclustDA model summary:				MclustDA model summary:				MclustDA model summary:			
log.likelihood	n	df	BIC	log.likelihood	n	df	BIC	log.likelihood	n	df	BIC
1582.523	180	20	3061.188	1239.912	134	22	2372.1	1809.171	136	36	3441.5
Classes	n	Model	G	Classes	n	Model	G	Classes	n	Model	G
A. australis	77	XXI	1	A. australis	77	XXX	1	A. australis	77	XXX	1
A. depressa	45	XXI	1	A. depressa	57	VVI	2	A. mucronata	59	EVE	2
A. mucronata	59	EII	2								
Training classification summary:				Training classification summary:				Training classification summary:			
Predicted				Predicted				Predicted			
Class	*A. australis*	*A. depressa*	*A. mucronata*	Class	*A. australis*	*A. depressa*		Class	*A. australis*	*A. mucronata*	
A. australis	72	1	3	A. australis	76	1		A. australis	77	0	
A. depressa	5	40	0	A. depressa	4	53		A. mucronata	3	56	
A. mucronata	7	0	52								
Training error =	0.08889			Training error =	0.03731			Training error =	0.02206		
Test classification summary:				Test classification summary :				Test classification summary:			
Predicted				Predicted				Predicted			
Class	*A. australis*	*A. depressa*	*A. mucronata*	Class	*A. australis*	*A. depressa*		Class	*A. australis*	*A. mucronata*	
A. australis	86	35	41	A. australis	106	56		A. australis	128	34	
A. depressa	5	108	2	A. depressa	1	120		A. mucronata	13	243	
A. mucronata	13	1	241								
Test error =	0.18233			Test error =	0.20141			Test error =	0.11244		

Supplementary Table 5.4. PaleoTS analysis of species variation through time for shell characteristics. The model with the highest Akaiki weight score is considered the best fit and Akaiki scores above 0.7 are strong (Hunt 2006). Omega and Vstep scores are an indication of the variance through time a proxy for the amount of evolution occurring

A. australis					*A. depressa*					*A. mucronata*				
Centroid					Centroid					Centroid				
	logL	K	AICc	kaike.wt		logL	K	AICc	kaike.wt		logL	K	AICc	kaike.wt
GRW	69.66215	3	-131.1425	0.002	GRW	43.41678	3	-76.03355	0.133	GRW	68.46434	3	-128.5287	0.101
URW	69.53377	2	-134.0675	0.01	**URW**	**42.77186**	**2**	**-79.54373**	**0.767**	URW	67.96372	2	-130.8365	0.322
Stasis	**74.08437**	**2**	**-143.1687**	**0.987**	Stasis	40.73837	2	-75.47674	0.1	**Stasis**	**68.54709**	**2**	**-132.0033**	**0.577**
omega	1.65E-06				vstep	0.999428				omega	3.38E-06			
PC1					PC1					PC1				
	logL	K	AICc	kaike.wt		logL	K	AICc	kaike.wt		logL	K	AICc	kaike.wt
GRW	38.49821	3	-68.81461	0.001	GRW	28.57378	3	-46.34756	0.119	GRW	43.02935	3	-77.6587	0.144
URW	38.49216	2	-71.98433	0.003	**URW**	**28.10625**	**2**	**-50.21249**	**0.82**	**URW**	**42.9539**	**2**	**-80.81689**	**0.697**
Stasis	**44.46313**	**2**	**-83.92626**	**0.997**	Stasis	25.51097	2	-45.02194	0.061	Stasis	41.47708	2	-77.86326	0.159
omega	0.0002474				vstep	76.23623				vstep	109.7875			
PC2					PC2					PC2				
	logL	K	AICc	kaike.wt		logL	K	AICc	kaike.wt		logL	K	AICc	kaike.wt
GRW	54.21321	3	-100.2446	0.141	GRW	27.49426	3	-44.18852	0.027	GRW	51.5823	3	-94.76461	0.189
URW	**54.07311**	**2**	**-103.1462**	**0.603**	URW	27.38736	2	-48.77472	0.264	**URW**	**51.30481**	**2**	**-97.51871**	**0.75**
Stasis	53.21485	2	-101.4297	0.256	**Stasis**	**28.37736**	**2**	**-50.75472**	**0.71**	Stasis	48.78128	2	-92.47166	0.06
vstep	32.59403				omega	9.34E-06				vstep	24.11353			
PC3					PC3					PC3				
	logL	K	AICc	kaike.wt		logL	K	AICc	kaike.wt		logL	K	AICc	kaike.wt
GRW	52.32009	3	-96.45835	0.001	GRW	33.64015	3	-56.4803	0.142	GRW	50.85682	3	-93.31364	0.037
URW	52.26914	2	-99.53827	0.004	**URW**	**32.9092**	**2**	**-59.8184**	**0.754**	URW	50.85258	2	-96.61426	0.194
Stasis	**57.8529**	**2**	**-110.70579**	**0.995**	Stasis	30.9325	2	-55.86501	0.104	**Stasis**	**52.22816**	**2**	**-99.36541**	**0.769**
omega	2.66E-05				vstep	30.79204				omega	6.13E-05			
PC4														
	logL	K	AICc	kaike.wt										
GRW	56.34521	3	-104.5086	0.061										
URW	56.33056	2	-107.6611	0.293										
Stasis	**57.12368**	**2**	**-109.2474**	**0.647**										
omega	3.89E-05													

Chapter 6

General Discussion

Species delimitation

In the context of this work I sought to identify species boundaries from concordance of both genetic and morphologic markers. Morphologic characteristics have provided vital information for taxonomic work in gastropods (e.g. Ponder and Lindberg, 1997) and provide a link to ancestral lineages in the fossil record. More recently genetic information has been incorporated into phylogenetic work providing a more robust framework in which to examine evolutionary relationships (Hills et al., 2012; Lawler and Duda Jr., 2017; Reid, 1989; Reid et al., 1996). Diagnostic features in snails are not widely shared across different groups, a problem confounded by both phenotypic plasticity and homogeneity. In investigating marine gastropods in New Zealand I found that delimitating species boundaries is not always straightforward. Both *Amalda* and *Buccinulum* have a history of recognised miss-identification, miss-classification, and taxonomic revision (Hart, 1995; Ponder, 1971). In the molecular analysis of *Buccinulum* I have uncovered some anomalies between the mitochondrial data and the morphology, which may necessitate a revision of the taxonomy. The morphologically distinct *B. colenosi* occupies the coast between the ranges of two *B. vittatum* subspecies. Over the same coast, the sympatric whelk, *Cominella maculosa*, showed no shell shape differentiation. The mtDNA showed strong geographic structure within *Buccinulum* but not within *C. maculosa.* A group of haplotypes belonging to *B. v. vittatum* are differentiated from the two other taxa, *B. v. littorinoides* and *B. colenosi.* Initial investigation of nuclear genetics using SNP data suggests a similar pattern of *B. v. vittatum* being distinct from *B. colensoi* and *B. v. littorinoides,* which together form a single genetic cluster. This dataset illustrates that when more than one trait or marker is used to investigate population differentiation and explore species delimitation it is possible that lack of concordance will be revealed. Morphology is under selection, while much of the genome is neutral providing the opportunity for divergent phenotypic and genetic patterns. One cannot assume that morphology, manifesting as morphospecies, is a true picture of where gene flow ends when for example, instances of hybridisation and gene flow patterns allow different loci to have alternate histories, leading to different phylogenetic patterns (Harrison and Larson, 2014; Mallet, 2005; Vaux et al., 2016)..

Identifying evolutionary lineages within the extant New Zealand *Amalda* had a different set of problems. There is approximate concordance between molecular phylogenetics and morphologically recognised species using a range of genetic markers (allozymes – (Michaux, 1987), mitochondrial DNA and ribosomal DNA sequences – this study) and morphometric data (classical – (Michaux, 1989) and landmark morphometrics – this study). These methods show little conflict with the accepted species taxonomy, but the long held understanding that there are two subgenera, *Baryspira* and *Gracilispira*, is in conflict with the genetic markers and not fully resolved morphometrically. *A. novaezelandiae* traditionally in *Gracilispira,* is the most morphologically distinct of *Amalda* analysed using landmark morphometrics showing some concordance to the idea of distinct clades. The morphometric data from *A. northlandica* show it is not clearly separated from *A. australis* in the *Baryspira* group but was included in a morphologic cluster with *A. novaezealandiae* in one of the cluster analyses. The genetics show that *Gracilispira* is not monophyletic, suggesting that the subgeneric classifications are not applicable to the modern fauna. These conflicting taxonomic inferences highlight problems with interpreting evolutionary lineages in a largely morphologically homogeneous genus. *Amalda* species from around the world can have very similar gross morphology which can lead to problems of cryptic species. In the past *A. northlandica* may have been grouped with *A. australis* (or *A. depressa*) based on their similarity (pending genetic confirmation of their species classification) until their taxonomic status as a species was determined by Hart (Hart, 1995). Alternatively, small phenotypic differences have the possibility of being overly emphasised in identification, for example colouration which led to the identification of *A. crystallina* as a species rather than colour forms of other species.

While mostly confirming species classifications, with a few instances of uncertainty, my analysis has also highlighted the problem of sample identification in the fossil record. While some overlap in morphospace between *Amalda* species in the modern fauna was recorded there was an increased overlap within the fossil specimens. The process of fossilisation is at some level a destructive one. It is highly unlikely that a specimen can go through the fossilisation process without losing some character definition. The most commonly lost is the colour, which has some taxonomic informative value in *Amalda.* Also lost through wear and tear can be features like the thin outer edge of the aperture or the definition of characters like spire or end of the siphonal canal as they are worn away. These seemingly minor modifications to the shell may increase the variance seen within a group, leading to increased overlap of similar species in morphospace. The amount of overlap was reflective of what might be expected given the biological and fossilisation processes influencing final shell shape, but still reflect real morphologic clusters (Crampton pers comm). In palaeontology, identification of species will not always identify evolutionary lineages (Hills et al., 2012) but in my case my data support that the identified fossil species correspond with varying degrees to genetic clusters indicating distinct lineages.

Here in chapter five I have an example of three species all distinct enough to have been recognised by taxonomists and later confirmed as genetically distinct, but over millions of years to have maintained similar morphologic evolutionary trajectories in that they have retained very similar phenotypes. At times they come close to inhabiting a space between morphospecies and cryptic species. These species have some level of sympatry with overlapping bathymetric and geographic ranges and so experience similar environmental conditions. Therefore selective pressures could be working to constrain morphologic separation on these three species.

The *cox1* data show higher genetic diversity in *A. depressa*, then *A. australis* and *A. mucronata*, counter to what might be expected if diversity increased along with species duration, although possible with phenomena such as bottlenecking reducing diversity. The higher diversity in *A. depressa* and *A. australis* the youngest species as inferred by fossil presence and phylogenetically may be concordant with the idea that early in a species history diversity increases as it moves to fill evolutionary potential space. This is recognised in morphometric studies (Uyeda et al., 2015) but runs counter to what is expected in genetic patterns, with new species arising from clusters with restricted genetic diversity relative to the entire metapopulation (Bolnick and Fitzpatrick, 2007; Mayr, 1963).

Evolutionary models

One of the primary objectives of the project was to investigate the possibility that *Amalda* provide support for the punctuated equilibrium hypothesis. To do this the mode of evolution needed to be chosen from a set of possibilities using morphologic variance through time. Of the possible models, stasis within a lineage is predicted by PE (Eldredge and Gould, 1972). I found stasis to be the most likely model of evolution for at least one trait in each of the three species tested corroborating the results of Michaux (1989). With the increased resolution my methods afforded, it was not simply stasis that I found. Differing models were allowed for with different shape components, highlighting the complicated process that is evolution. The presence of stasis allows PE to be considered as a viable explanation for the pattern of evolution in *Amalda*.

Alongside the presence of stasis in the fossil record I also noted the presence of Unbiased Random Walk as the probable mode for some traits in all three species. The two modes, stasis and URW, are by far the most common seen in the fossil record accounting for 87-95% of datasets examined (Hunt, 2007; Hunt et al., 2015). Both modes having been found in all three species tested, showed that independent traits of shell shape components can have different evolutionary modes and are possibly under the influence of different evolutionary pressures (Hopkins and Lidgard, 2012). The presence of differing modes for traits within a species has been noted and is recognised as mosaic evolution.

Future work

The fossil collection examined was not comprehensive enough to encompass lineages that included speciation events that would have been necessary to conclusively indicate PE. To do so I would need to access the fossil collections of species putatively suggested as ancestral to the species present today. While there are theories on decent in *Amalda* it is uncertain in some cases which fossil lineages are ancestral to the modern species in such a morphologically homogenous genus (Beu and Maxwell, 1990). It is possible that the problem of species identification in the fossil record will remain an issue, which may impact identification of continuous lineages necessary to show speciation. However, monophyly within the extant New Zealand species (as shown in this work) does reduce the likelihood that species invasions will mislead inferences of lineage splitting in the fossil record.

The time frame covered by my analysis extends into the millions of years providing an interesting contrast with studies that are of more concise time periods (e.g. Bocxlaer and Hunt, 2013; Hunt, 2008) and possibly representing the early stages during which patterns of bounded evolution begin to change to a more divergent pattern (Uyeda et al., 2011). The predominance of URW in *A. depressa* the youngest species, based on the FRED fossil database, may be a reflection of this idea inasmuch as we may be seeing the effects of unbounded evolution and increased diversity. *A. depressa* also has the highest level of genetic variation of the species tested. Could unbounded evolution cause rapid genetic diversification concordant with morphology or is this an artefact of *A. mucronata* and *A. australis* passing through a genetic bottleneck or a lack of sampling of the true diversity of the species tested? The timeframe during which these three species have existed encompasses a wide range of environmental changes the most notable being the vast temperature fluctuations and associated sea level changes associated with the Pleistocene glaciations. These are likely to have been important factors influencing the histories of the species investigated.

The importance of integrated data sets

Perhaps the most important outcome of this study is the building of a large data set that incorporates large-scale genetic data with a morphologic data set, which covers multiple species and extends millions of years into the fossil record. These integrated data sets are seen as being important tools in clarifying taxonomies and evolutionary models (Hunt and Slater, 2016; Schlick-Steiner et al., 2010). Data sets that include extensive fossil records linked to large amounts of genetic data are uncommon. There are a few examples mostly from unicellular organisms, foraminifera, and a few others including plants ostracods molluscs and some vertebrates (Bocxlaer and Hunt, 2013; Grey et al., 2012; Hunt, 2007, 2006; Manos et al., 2007; Morlon et al., 2011). Combined datasets are invaluable in the study of evolutionary trends allowing the formulation and testing of hypotheses not possible with only recent or fossil specimens. Pennell and Harmon (2013) suggest that we are coming to an impasse in the integration of phylogenetics and palaeontology. This may be true but I have shown that they can be used concurrently to help create a better understanding of evolutionary processes. Beyond inferring evolutionary models integrated datasets are becoming increasingly important for resolving aspects of the circumstances surrounding the evolution of species from evolutionary models to understanding diversity and the conditions driving speciation (Fritz et al., 2013; Quental and Marshall, 2010).

Increasingly complex methods to process increasing amounts of data are continuously being developed for the testing of evolutionary hypotheses (Hunt et al., 2015). I have revisited a collection of samples and applied more advanced analyses to them, and it is likely that in the future this will happen again as full genomic sequencing becomes more accessible and more powerful morphometric tools are developed. This thesis used some of the most up to date methods available and goes some way to forming a better understanding of the relationships between genetics and morphology and using these to explain the evolution of species. The thesis highlights how fundamentally important defining evolutionary lineages for trying to infer evolutionary patterns is and some of the challenges in doing so. The major finding of this thesis is that the models of evolution tested are not mutually exclusive. I have seen both models differing between traits within lineages, and between lineages from similar timeframes and locations. In doing this I have also confirmed stasis in the fossil record in *Amalda* species, a prerequisite for punctuated equilibrium.

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