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Evolutionary lineages and the diversity of New Zealand true whelks

A thesis presented in partial fulfilment of
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Felix Vaux
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Abstract

Biological evolution fundamentally operates according to the basic principles of variation, heritability and selection, but it generates the astounding complexity of nature. One of the greatest challenges for evolutionary study is the interpretation of this diversity, and the ability to identify and communicate the underlying biological changes that are responsible. In this thesis, I consider the identification of evolutionary lineages using molecular and morphological data. I address the problem of confusing terminology regarding the evolutionary process, focussing on the concepts of anagenesis and cladogenesis, and the challenge of genetic introgression for taxonomic classification.

I investigate molecular and morphological variation in New Zealand true whelks. There are many species of true whelks described, however their taxonomy is mostly restricted to the traditional examination of shell traits. Evolutionary relationships of true whelks inferred from DNA sequences indicate that neither New Zealand nor Southern Hemisphere true whelks are monophyletic, contradicting taxonomic hypotheses and expectations of geographic isolation. I focus on the siphon whelk genus *Penion* Fischer, 1884, a diverse genus with extant species restricted to New Zealand and Australia. All extant species are genetically sampled for phylogenetic and allelic variation analysis. A monophyletic clade is identified for New Zealand *Penion*. Results suggest the existence of a new species and indicate evolutionary relationships for some taxa not captured by the taxonomy.

Shell shape and size are studied using geometric morphometric analyses, confirming that these traits can distinguish taxa divided by deep evolutionary splits under both informed and naïve analyses. Morphometric variation is hierarchical, with closely related taxa being grouped together within large datasets including samples from multiple evolutionary lineages. Overall, morphometric results show reasonably strong concordance with molecular evidence.

Evolutionary lineages in the fossil record are investigated using morphometric analysis within the context of previous molecular and morphometric findings. Results assist with the identification of fossils from two localities and suggest that multiple extinct species of *Penion* are misclassified. Variation in morphometric traits through

time is fitted to models of evolutionary change, and results indicate that the identification and selection of a lineage has a significant impact upon those results.

Keywords

anagenesis; benthic; Buccinidae; Buccinulidae; Buccinioidea; Caenogastropoda; cladogenesis; deep sea; dispersal; developmental biology; endemism; evolution; evolutionary biology; evolutionary lineage; evolutionary rate; divergence; diversity; fossil; gastropod; geometric morphometrics; high-throughput sequencing; hybridisation; introgression; lineage split; marine snail; mitochondrial DNA; mollusc; monophyly; morphology; Neogastropoda; next-generation sequencing; nuclear DNA; palaeontology; paraphyly; phylogenetics; RADseq; ribosomal DNA; sexual dimorphism; shell; siphon whelk; snail; speciation; species; systematics; taxonomy; whelk; zoology

A *Penion* Fischer, 1884 siphon whelk from Tasman Bay.

Preface

The overall aim of this research project, *Evolutionary lineages and the diversity of New Zealand true whelks,* was to investigate the relationship between molecular and morphological variation for the identification of evolutionary lineages. New Zealand true whelks were used as a study system, and I focussed especially on the siphon whelk genus *Penion* Fischer, 1884, which is recognised to be taxonomically diverse. Numerous extant endemic siphon whelk species are recognised in New Zealand, along with a rich fossil record. *Penion* shells exhibit a bewildering level of putative inter- and intraspecific morphological variation. The aim of this project was followed in several stages, which are presented in this thesis as seven independent research chapters (Chapters 1 – 7), with the findings summarised at the end. Most research chapters are followed by supplementary material (including error studies, and additional figures and tables), and taxonomic information is also summarised in Chapter 8 to assist with the interpretation of methods and results.

Research presented in this thesis was produced in collaboration with my supervisors (Mary Morgan-Richards, Steven A. Trewick, and James S. Crampton), but most sampling and laboratory work, and all data analysis and initial drafts of writing were my own work. Within chapters I use the personal pronoun ‘we’, but all work is my own. Mary, Steve and James provided invaluable insight and assistance with conception of the project aims, the design of methods and analyses, discussion of results, editorial guidance, and funding. For writing, I specifically chose many of the topics of research, surveyed the literature and wrote the first drafts of each manuscript with iterative feedback from co-authors. I conducted the majority of molecular sampling, with some assistance from Simon F.K. Hills and Mary. Most DNA extractions, PCR reactions, and necessary clean-up methods were conducted by myself with some assistance from Simon. I worked in cooperation with Michael R. Gemmell to develop the next-generation sequencing method and analytical pipeline. I conducted all shell photography myself. Most specimens were borrowed from museum and university collections acknowledged within chapters, and Mary and I organised the loan of tissue specimens from abroad. High-throughput sequencing was conducted by the Beijing Genomics Institute, Hong Kong or the New Zealand Genomics Limited service. Bruce A. Marshall and Alan G. Beu advised with the taxonomic classification of specimens, as well as the identification of palaeontological provenance and the sex of individual snails.

Chapter 1 is a literature review considering the meaning of the terms ‘anagenesis’ and ‘cladogenesis’ from an evolutionary perspective. These terms are frequently used to discuss speciation and morphological change in the fossil record, and this chapter attempts to clarify the topic. The article was accepted for publication as: Vaux, F., Trewick, S.A., Morgan-Richards, M. (2016). Lineages, splits and divergence challenging the meaning of the terms anagenesis and cladogenesis. *Biological Journal of the Linnean Society* 117, 165 – 176.

Chapter 2 is a reply to a comment written in response to the published version of Chapter 1. The chapter discusses the treatment of species as arbitrary concepts, and it addresses the significance of genetic introgression for the process of biological speciation and taxonomic classification. The chapter was published as: Vaux, F., Trewick, S.A., Morgan-Richards, M. (2016). Speciation through the looking-glass. *Biological Journal of the Linnean Society* (early access).

Chapter 3 is a molecular phylogenetic investigation of true whelks (Neogastropoda: Buccinidae or Buccinulidae) from the Southern Hemisphere. The aim of the chapter was to determine whether true whelks from the Southern Hemisphere, or at least New Zealand, are monophyletic and separate from lineages distributed in the Northern Hemisphere. The findings also provide new insight towards timing of speciation and dispersal in the siphon whelk genera *Antarctoneptunea* Dell, 1972, *Kelletia* Bayle, 1884 and *Penion.* The dataset contains newly sequenced mitochondrial genomes and nuclear ribosomal DNA sequences from numerous species of marine snail. I am hoping to submit an abbreviated version of this chapter to a peer-reviewed journal soon.

Chapter 4 is a molecular phylogenetic and restriction site associated DNA (RAD) sequencing investigation of the siphon whelk genus *Penion.* The aim was to produce a comprehensive hypothesis for the evolutionary relationships of all recognised, extant species of *Penion* from Australia and New Zealand (Chapter 3 contains a subset of species). Analysis of single nucleotide polymorphic (SNP) variation for anonymous nuclear loci was used to investigate species delimitation, and to test phylogenetic concordance between mitochondrial and nuclear DNA. The dataset contains newly sequenced mitochondrial genomes and nuclear ribosomal DNA sequences from all

species of *Penion.* Results from this chapter are intended to be merged with those of Chapter 6, and will be submitted to a peer-reviewed journal for publication.

Chapter 5 is an investigation for evidence of secondary sexual dimorphism in the shells of *Penion chathamensis* (Powell, 1938) using geometric morphometric analysis. Neogastropod molluscs such as *Penion* are dioecious, but sexual dimorphism is an understudied topic of research. Our analysis of shell shape and size variation used a two dimensional, landmark-based geometric morphometric approach with sampling across the entire range of *P. chathamensis.* For comparison I also sampled shells across the entire range of *P. sulcatus* (Lamarck, 1816). This chapter was published as: Vaux, F., Crampton, J.S., Marshall, B.A., Trewick, S.A., Morgan-Richards, M. (2017). Geometric morphometric analysis reveals that the shells of male and female siphon whelksm *Penion chathamenis* are the same size and shape. *Molluscan Research* (early access).

Chapter 6 is an investigation of variation in the shell morphology of all extant species of *Penion.* The aim was to establish if variation in shell morphology in *Penion* is concordant with the evolutionary relationships among species estimated from the molecular results of Chapter 4. The same two dimensional, landmark-based geometric morphometric method as in Chapter 5 was used to analyses shell shape and size. All extant species of *Penion* from Australia and New Zealand were sampled. Results from this chapter are intended to be merged with those of Chapter 4, and will be submitted to a peer-reviewed journal for publication.

Chapter 7 utilises the combined results of Chapters 3 – 6 as a context to analyse the fossil record of *Penion* in Australia and New Zealand. The chapter investigates variation in the shell morphology of fossils classified as extinct and extant species in comparison to modern shell sampling (covered in Chapter 6). The analysis follows the same framework to consider evolutionary lineages and speciation discussed in Chapters 1 and 2, and the method considers the concordance between molecular phylogeny and shell morphological variation in *Penion* (Chapters 3 and 4, 6), and the apparent absence of secondary sexual dimorphism in at least some species (Chapter 5). Since findings from every previous chapter are synthesised, Chapter 7 almost acts as a conclusion of the thesis. The same two dimensional, landmark-based geometric morphometric method as in Chapters 5 and 6 was used to analyses shell shape and size. Shells from all extinct

species of *Penion* from Australia were sampled, as well as a number of fossil species from New Zealand. This chapter has been prepared for publication but will not be submitted until the previous chapters have been published.

Chapter 8 summarises the taxonomy of living and fossil *Antarctoneptunea*, *Kelletia* and *Penion*, which were three genera of key interest for this thesis. Specifically, this section summarises the current, published taxonomy of the group and also suggests revisions based on the results of Chapters 3 – 7. Importantly, this section also specifies the operative taxonomic units (OTUs) used for this thesis. Some taxa were not considered for this study as the examination of shells suggested that numerous fossil taxa were conspecific. These decisions were made independent of geometric morphometric and molecular results. This chapter should be read for reference when the taxonomy and available fossil material for the three genera requires clarification in Chapters 3 – 7. The revisions summarised in this chapter are planned to be converted into a formal taxonomic review that will be submitted for publication.

At the end of this thesis I provide a brief summation of the overall results of Chapters 1 – 8. I also suggest future research topics based on the results of this thesis.

Results from Chapters 1 – 6 were also included within conference presentations listed in Appendix I.

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Thank you to Mary and Steve for being such comprehensive supervisors that have been willing to support and challenge my growth as a researcher during this thesis. It means a great deal that you both trusted me to take this thesis in my own direction, and let me confront controversy. Thank you in particular to Mary for being omnipresent, especially when I've asked "one quick question" in your office doorway that inevitably mutates into a half-hour verbal dysentery. Thank you to James for your tutelage (and patience) with the many topics covered within this thesis that I am still getting to grips with. For having perhaps the most patience, I also grateful to Simon for all of his assistance, from fieldwork to staring at computer errors. This thesis also would not have been possible without the malacological wisdom provided by Alan and Bruce.

I must apologise to my family for spending the last few years on the opposite side of the planet and not visiting home with great frequency (i.e. once), or apt timing (sorry about the wedding Pierre and Emilie!). Thank you Mum and Dad for supporting my career and always encouraging me to seize any opportunities that arise. I am lucky to have you as my parents and love you both. I am forever indebted to Anne Kim for supporting me throughout this thesis. Thank you for making me a better person and for rescuing our whiskered daughter. It was brilliant to grow together over the last few years, and I wish you all luck with your own PhD.

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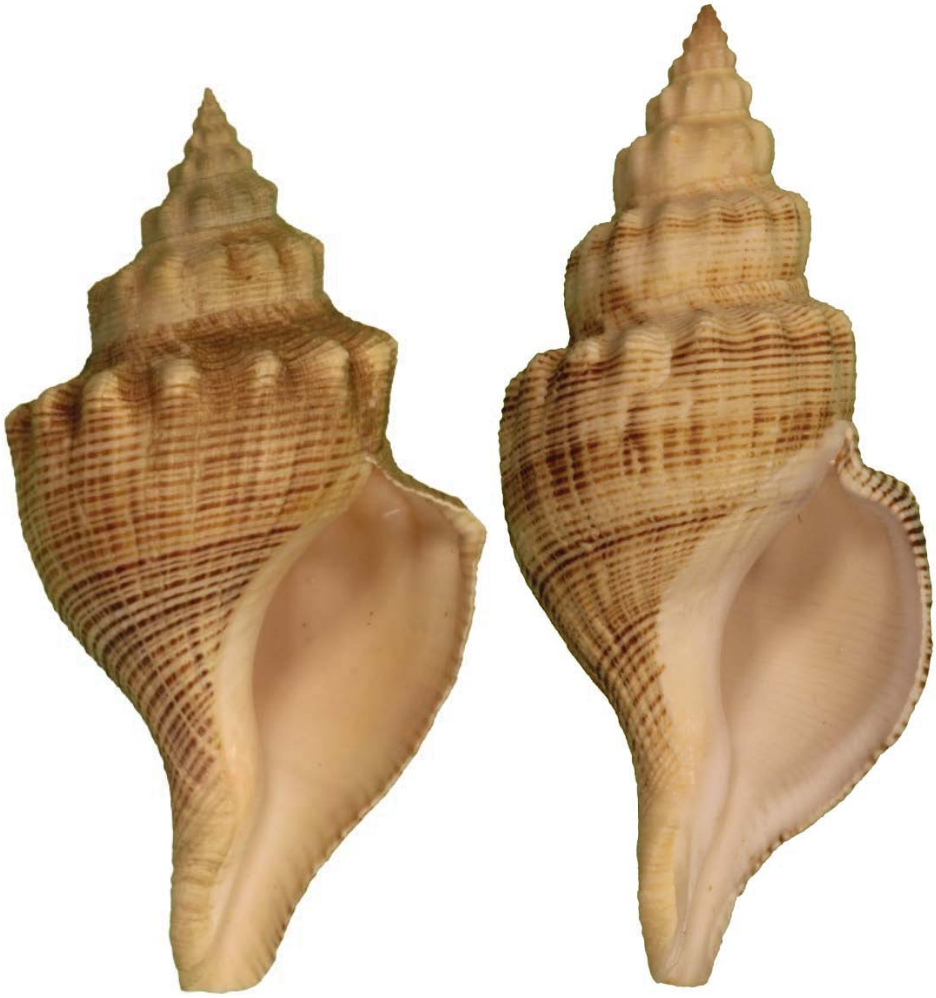

Two shells of *Penion mandarinus* (Duclos, 1832) from waters off of Australia.

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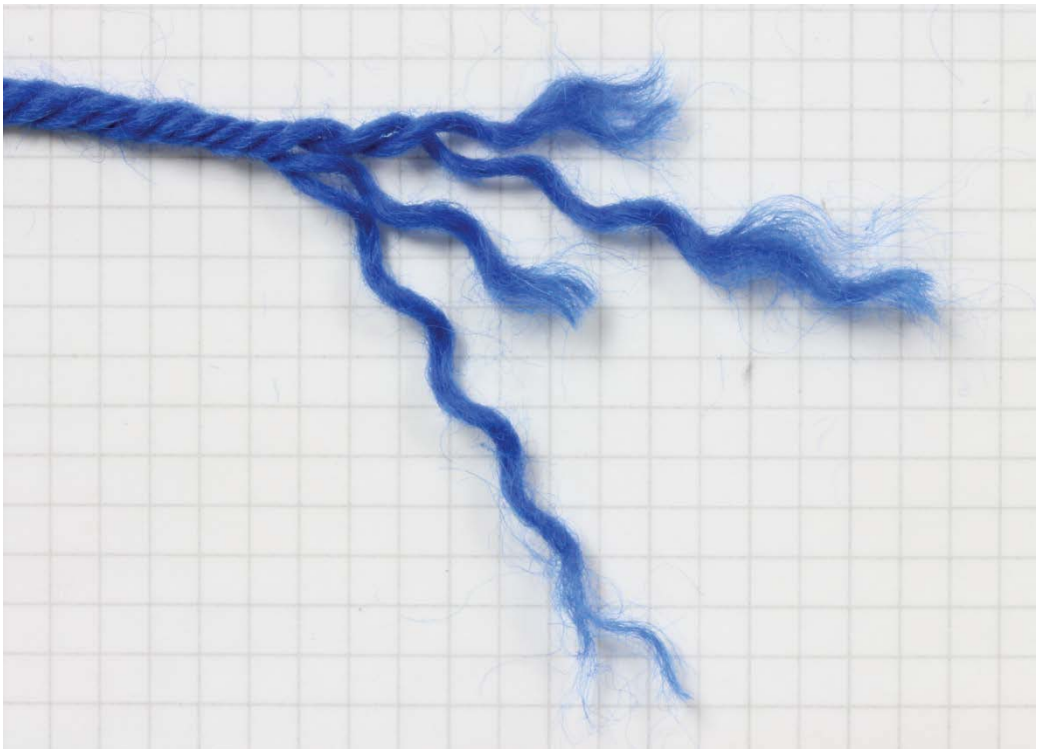
Chapter One

Lineages, splits and divergence challenge whether the terms anagenesis and cladogenesis are necessary



Yarn as a metaphor for evolutionary lineages and taxonomic classification.

The evolutionary process and speciation

In this review, we assess the terms ‘anagenesis’ and ‘cladogenesis’ because they epitomise the barrier to communication that results from the conflation of the process of evolution and our interpretation of life using taxonomy. Opinion may vary regarding the future application of the terms, but we illustrate how the current usage is vague, inconsistent and therefore unhelpful. We conclude that communication across disciplines could be improved by avoiding these terms or acknowledging limitations, and we demonstrate how this can be achieved.

An evolutionary lineage, or line of descent, is the inherent product of evolutionary units replicating in generations over time, and consequently it is a universal feature of all biologically evolving systems (Cutter 2013). A ‘species’ is therefore always a taxonomic description of an arbitrarily delineated segment of an evolutionary lineage in time (de Queiroz 1998, Sites and Marshall 2003, de Queiroz 2007, Podani 2013, White 2013). For different organisms the delineated region will vary in size, scale, and duration in time depending upon the nature of the taxonomic paradigm employed, the availability of data (past and present), and the hypothesis under investigation (de Queiroz 1998, Sites and Marshall 2003, de Queiroz 2007, White 2013). However, although a species is artificial, it remains a hypothesis based on empirical observations of an evolutionary lineage (Barraclough and Nee 2001, de Queiroz 2011, Strotz and Allen 2013, Dynesius and Jansson 2014). See Figure 1.1 for an explanatory metaphor. Philosophically this means that we treat a species as a mental concept based on the material reality of evolutionary lineages (see discussion in Mahner 1993). Recognising the consensus of evolutionary lineages is hugely beneficial as conflicting species concepts, such as those based on reproduction or morphology, become compatible through accommodation of the evolutionary process (Wei 1987, de Queiroz 1998, Cohan 2002, Cutter 2013, Ezard *et al.* 2013, Podani 2013, White 2013).

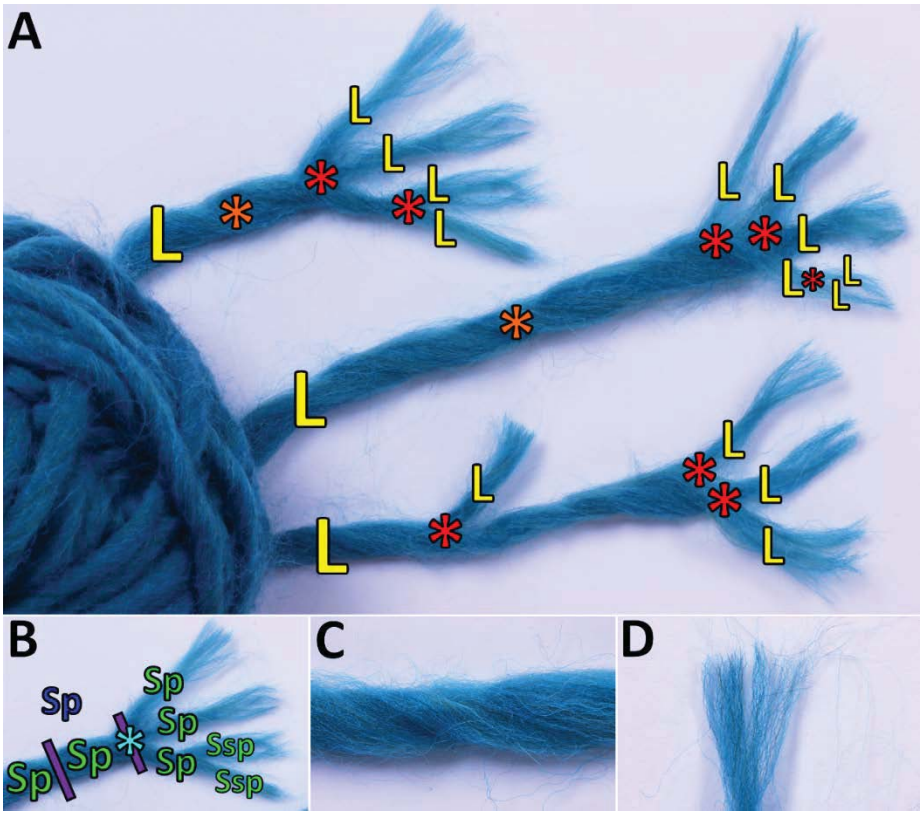
FIGURE 1.1
Yarn as a metaphor for evolutionary lineages: lineage-splitting and splitting hairs.

A) A piece of yarn represents an evolutionary lineage (yellow L). Like evolutionary lineages, yarn is continuous and is comprised of many fibres. In both, splits can be identified (red asterisks). The origin of each piece of yarn in the tangled ball of wool represents the unknown common ancestry of lineages as we move backward in time. Many lineage-splits are also missed due to extinction (orange asterisks).

B) Particular segments of lineages can be classified as species (green Sp, purple lines representing temporal boundaries of segments), relative to the studied organism, the availability of data, and the hypotheses under investigation. Segments of lineages can also be classified as subspecies or varieties (green Ssp), or consolidated as intraspecific variation (unlabelled lineages following the designation of a species). The assignment of these taxonomic categories is arbitrary as the size and scale of segments varies. Not all lineage-splits are classified as speciation (cyan asterisk), and species classifications based on ancestral and derived difference without evidence of lineage-splitting (e.g. chronospecies) do not invoke a discrete speciation event. Species may be described based on limited fossil evidence (blue Sp), because variation is novel or of interest, even when there is limited knowledge of the lineage to which it belongs.

C) Depending upon the scale of observation (limited by the availability of data such as zoom and resolution in photography or sampling in biology) further lineages (fibres) and splits (lineage-splits) can be identified. Many lineages do not persist for a significant length of time and either go extinct or hybridise with the original lineage.

D) Lineages are made up of individuals within populations, and introgression can unite populations (pieces of yarn that split may soon afterward recombine). Differentiating lineages (fibres) is easier when divergence has followed a lineage-split.



Importantly the lineage perspective helps us reconsider the process of evolution over long time periods. For instance, because evolutionary lineages are continuous in time, those lineages represented by taxonomic units such as species can be subdivided into further lineages that reflect classification of subspecies, varieties, or metapopulations that encompass intraspecific variation (Mallet 2008a, Dynesius and Jansson 2014). This perspective emphasises that there is no break in the process of evolution from the lineages studied using population genetics ('microevolution') and the lines of descent studied during 'macroevolutionary' research (Barraclough and Nee 2001, Crampton and Gale 2005, Cutter 2013). The fact that there is no convenient origination point (aside from the origin of life) to which a lineage can be traced, reminds us that our 'start point' for any investigation of a species, population, or a fossil continuum is itself an arbitrary date along a line of descent (Ezard *et al.* 2012).

The evolutionary lineage perspective, with species acknowledged as arbitrary partitions, also allows us to disentangle the different concepts commonly considered under speciation (follow Figure 1.1). 'Divergence' is the accumulation of genetic or phenotypic difference among evolutionary lineages over time that results in distinct variation (Abbott *et al.* 2013, Sætre 2013, Dynesius and Jansson 2014). Divergence is simply a temporal function recording the inevitable change that accrues between partitioned groups of individuals. It results in genetic and phenotypic difference (measured as diversity or distance), typically estimated at the tips of branches in phylogenetic trees. 'Lineage-splitting' (or lineage-branching) is defined by the cessation of gene flow between groups of individuals, and therefore it marks the division of an evolutionary lineage into two or more further lineages (Dynesius and Jansson 2014). Importantly splitting does not guarantee divergence between lineages (Heelemann *et al.* 2014), although increased divergence can be facilitated by reduced gene flow. Divergence is studied using lines of descent through time (lineages), but it is not defined by lineage-splitting. A reproductively isolated population can be a representative sample of the original metapopulation, and likewise a connected population within a metapopulation may be highly divergent. 'Introgression' (or hybridisation, reticulation) is the inverse process of splitting, where gene flow is re-established between lineages intermittently or permanently (see Figure 1.1 D).

'Speciation' like 'species' is an arbitrary taxonomic classification of the evolutionary process. 'Speciation' refers to an arbitrarily selected lineage-split that is deemed to represent the birth of a new species. It is arbitrary because the identification

of a new species depends upon particular diagnostic thresholds (relative to organisms, scale of observation, data etc.), which inherently depends upon divergence rather than splitting. Species origination is an epistemological dilemma – is a species classified when a lineage is distinct (but not necessarily separated by gene flow), or when a lineage is separate (but not necessarily distinct)? If a population is morphologically derived with respect to its ancestral population, should it be classified as separate species based on such difference even if lineage-splitting is not evident? The fact that the answer differs between investigations reflects that the choice is ultimately subjective. So, 'divergence' is an increase in difference among evolutionary lineages, 'splitting' is the cessation of gene flow between lineages, and 'speciation' is the origination of a new species that ideally reflects both divergence and splitting. Divergence and splitting directly describe empirical change among evolutionary lineages, whereas species and speciation are ad hoc classifications applied to interpret the process (see difference between Figure 1.1 i and ii).

The distinction of process and interpretation is advantageous as it recognises, along with traditional splitting and divergent factors such as isolation and niche separation (Barraclough and Nee 2001, Mallet 2008b, Maan and Seehausen 2011), that introgression of lineages below the species-level affects rates of species formation (Abbott *et al.* 2013, Dynesius and Jansson 2014). The distinction also reminds us that there is no inherent reason why an increase in the divergence rate, or the molecular evolutionary rate, should incur an increase in the speciation rate (Pennell *et al.* 2014b). Although there may be a positive correlation between these rates (Webster *et al.* 2003, Lanfear *et al.* 2010, Dowle *et al.* 2013, Venditti and Pagel 2014), a split can only be classified as the generation of a new species when a lineage-segment is assigned to the species-level. This definition of 'speciation' is also preferable as it retains the pure meaning of a new species being generated (de Queiroz 1998), rather than a technical process that reflexively restricts the meaning of 'species.' A review of the lineage framework is presented Figure 1.1 as a metaphor that and Table 1.1 provides a glossary of terms.

TABLE 1.1
A glossary of terms related to anagenesis and cladogenesis.

Term	Definition	Type
Evolutionary lineage	A line of descent of evolutionary units (organisms, replicators). All evolutionary units belong to an evolutionary lineage, but our ability to identify particular evolutionary lineages depends upon the availability and scale of data. Evolutionary lineages are continuous through time and can be subdivided down to the level of individual replicators.	Process and pattern
Species	An arbitrary segment of an evolutionary lineage in time classified as a distinct species. Species can be delineated under many different protocols depending upon divergence-based factors such as the data available, studied organism (species criteria), and the hypotheses under investigation.	Classification
Divergence	The accumulation of genetic or phenotypic difference among evolutionary lineages over time that results in distinct variation. Divergence reflects the genetic or phenotypic diversity among lineages, but it does not necessarily require lineage-splitting. Difference can also be measured through time between ancestor and descendant populations.	Process
Lineage-splitting (or lineage-branching)	The cessation of gene flow between populations that causes an evolutionary lineage to divide into two or more. The point at which an interconnected gene pool splits in two. Lineage-splitting can be reversed via hybridisation.	Process
Introgression (or hybridisation, reticulation)	The re-establishment of gene flow between two evolutionary lineages. The inverse process of lineage-splitting. Introgression can occur between distantly related lineages as well as recently split lineages.	Process
Speciation	Splitting of an evolutionary lineage arbitrarily classified to correspond with the designation of a new species. The origination of a species. The classification of a species often depends upon divergence-based factors such as the data available, studied organism (secondary species criteria), and the hypotheses under investigation. The evidence of divergence, introgression and lineage-splitting itself is often what biologists are interested in when referring to ‘speciation’.	Classification
Stasis	No significant deviation from an evolutionary state (genetic, phenotypic) over a period of time. Described character states are typically a mean as individuals vary. It reflects divergence that is minor, not sustained, or which does not accumulate. It is driven by stabilising selection, frequency-dependent selection, or selection conflict.	Hypothesis regarding process
Gradualism	A slow, continuous rate of evolutionary	Hypothesis

	change. Some rate variation may occur but it is not overall significant. Originally coined by Hutton (1788), referring to the consistency of change in geology. Expanded by Lyell (1833), in response to Whewell (1831), to describe that the laws of nature (physics, biology) are unchanging, but the rates of geological preservation (e.g. sedimentation, erosion) are highly variable. Co-opted by Darwin (1859), to refer to a continuous rate of change during biological evolution rather than abrupt change.	regarding process
Phyletic gradualism	The hypothesis that speciation (and thus divergence and splitting) occurs at a gradualist rate (Eldredge and Gould 1972). Phyletic gradualism is not interchangeable with gradualism itself as it is the rate applied to speciation.	Hypothesis regarding process and classification
Punctuated equilibrium	Originally coined to refer to geologically abrupt allopatric speciation, alternating with extended periods of morphological and speciational stasis or gradualism in the fossil record (Eldredge and Gould 1972). It is not mutually exclusive with phyletic gradualism. Nowadays the term has arguably been corrupted and conflated with numerous other hypotheses (Pennell *et al.* 2014a, Lieberman and Eldredge 2014).	Hypothesis regarding process and classification

Ambiguity of anagenesis and cladogenesis

Although the context of evolutionary lineages clarifies the relationship between the evolutionary process and the classification of species, confusion persists due to the ambiguous usage of some terms. In particular, we observe that the terms of ‘anagenesis’ and ‘cladogenesis’ are frequently used in discussions of evolution and speciation despite the definition and application of the terms varying widely (e.g. Aze *et al.* 2013, Hunt 2013, Strotz and Allen 2013, Patiño *et al.* 2014, Valente *et al.* 2014). The terms have generated criticism (e.g. Dubois 2011), and definition can be vague (e.g. Patiño *et al.* 2014), or even absent (e.g. Drew and Barber 2009). This is alarming as the terms are central to many neo- and palaeobiological studies (e.g. Drew and Barber 2009, Haile-Selassie and Simpson 2013, Hunt 2013, Strotz and Allen 2013, Patiño *et al.* 2014).

Confusion is in part due to the changing usage of anagenesis and cladogenesis over time (Rensch 1929, Rensch 1959), akin to the conceptual evolution of ‘punctuated equilibrium’ (Eldredge and Gould 1972, Lieberman and Eldredge 2014, Pennell *et al.* 2014a). Anagenesis and cladogenesis were originally coined to differentiate between evolutionary change that leads to the classification of higher taxonomic units such as families (called “transspecific evolution”), and ‘narrow-sense’ change at the level of species (“intraspecific evolution”) (Rensch 1929, Glass 1949, Rensch 1959). The terms both considered speciation (assuming divergence as a proxy) and were not differentiated by it, nor were they mutually exclusive (Simpson 1949). Specifically, anagenesis considered a believed trend for increasing complexity in further derived lineages (‘higher evolved organisms’), typically considering morphology (Rensch 1929, Rensch 1959). Cladogenesis was concerned with the evolution of clades – ‘broad’ branches that yielded significant taxonomic diversity (Rensch 1929, Rensch 1959). Cladogenesis was treated as the breadth of an evolutionary tree and anagenesis was the height of branches (where increasing stature was increasing complexity) (Rensch 1929, Rensch 1959). Soon after conception, the terms were applied directly to speciation (Simpson 1949), and later were integrated with the monophyletic clade and grade concepts of Huxley (1957). Afterwards the terms were merged into the framework of ‘cladistics’ as exemplified by Hennig (Mayr 1973). Due to the mixing of terminology, ‘cladogenesis’ was inferred to reference the monophyletic branches used as units in cladistics, even though such ‘clades’ have an independent etymology (Cuénot 1940).

Anagenesis and cladogenesis continue to be used differently by experts among fields. For instance in some biogeography studies, anagenesis is used to refer to founder

effects and the formation of endemic species (Patiño *et al.* 2014, Valente *et al.* 2014). Similarly, some phylogeographic models refer to speciation caused by geographic mechanisms as cladogenetic events (Shaw *et al.* 2015). The discussion of punctuated equilibrium in particular has confused the terms because anagenesis and cladogenesis have been conflated with variation in rates of molecular evolution, speciation, and diversification (Benton and Pearson 2001, Bokma 2008). Mistakenly, anagenesis is connected or synonymised with phyletic gradualism, gradualism or even stasis (Chaline 1977, Bokma 2002, Mattila and Bokma 2008, Pachut and Anstey 2012, Lister 2013, Pearson and Ezard 2014), and cladogenesis for punctuated change (Bokma 2002, Bokma 2008, Lister 2013). Rates of speciation and cladogenesis are also incorrectly assumed to be equal (Pennell *et al.* 2014b). The two terms have even been referred to as 'modes' of evolution, suggesting that fundamental mechanisms are described (Pachut and Anstey 2012, Strotz and Allen 2013).

In palaeontology, usage is fairly consistent, with 'cladogenesis' typically defined as lineage-splitting (branching) (de Queiroz 1998, Jackson and Cheetham 1999, Catley *et al.* 2010. Aze *et al.* 2013, Bapst 2013, Futuyma 2013). Correspondingly, 'anagenesis' (or phyletic change) is treated as evolutionary change that occurs within a lineage (Pachut and Anstey 2012, Johnson *et al.* 2012, Aze *et al.* 2013, Bapst 2013, Futuyma 2013, Strotz and Allen 2013), between lineage-splits (e.g. Hunt 2013, Lister 2013, Van Bocxlaer and Hunt 2013). This means that 'anagenetic change' is used to mean evolutionary change without lineage-splitting (Jackson and Cheetham 1999, Catley *et al.* 2010, Johnson *et al.* 2012, Bapst 2013).

Nevertheless, there is a strong tendency to link anagenesis and cladogenesis to speciation. Cladogenesis is commonly considered to be interchangeable with speciation; lineage-splits are assumed to represent the division of one species into two or more (Benton and Pearson 2001, Mattila and Bokma 2008, Drew and Barber 2009, Lister 2013, Hunt 2013, Strotz and Allen 2013, Dynesius and Jansson 2014, Pearson and Ezard 2014). In contrast, anagenesis generates conflict as to whether it is a form of speciation (e.g. Jackson and Cheetham 1999, Catley *et al.* 2010, Podani 2013), or is not (e.g. Bapst 2013, Ezard *et al.* 2013, Lister 2013, Strotz and Allen 2013, Pennell *et al.* 2014a). Species can be argued to originate without lineage-splitting because the derived genotype or phenotype of a seemingly un-split lineage is taken to be significantly different from the ancestral state (Benton and Pearson 2001, Catley *et al.* 2010, Podani 2013). Such species are often referred to as 'chronospecies' (de Queiroz 1998, Benton

and Pearson 2001, Haile-Selassie and Simpson 2013, White 2013). These anagenetically-produced chronospecies are controversial as they are based on difference of form along a lineage rather than splitting or direct evidence of divergence; therefore they are based on relative character states and particular dates, which can be criticised as an especially arbitrary basis for species delineation (Vanderlaan and Ebach 2014, White 2013, White 2014).

Are anagenesis and cladogenesis necessary terms?

The varied usages of anagenesis and cladogenesis across biological disciplines is not ideal for clarity, and even the seemingly robust definitions used in palaeontology generate ambiguity between the evolutionary process and species classification. However, the context of evolutionary lineages allows us to disentangle the different concepts conflated under anagenesis and cladogenesis. The insight provided prompts us to question whether anagenesis and cladogenesis are necessary.

Not all lineage-splits are informative for studying long-term evolution

While lineage-splits and evolutionary change between them function as identifiably different concepts, the descriptive value of this distinction depends upon observation. Problematically, splits are ubiquitous during evolution but not all splits are fixed, and not all splits are of interest. Breaks in gene flow (splits) result in population structuring (Méndez *et al.* 2011, Abbott *et al.* 2013, Heelemann *et al.* 2014), and can persist for few or many generations (Bhat *et al.* 2014). Breaks in gene flow are not necessarily absolute nor permanent; two allopatric populations may reconnect (Sternkopf *et al.* 2010, Abbott *et al.* 2013), as can so-called incipient species (Bhat *et al.* 2014), and apparently distinct species can successfully hybridise when opportunity arises (Shiga and Kadono 2007, Dubois 2011, Mráz *et al.* 2012, Pruvost *et al.* 2013), even millions of years after lineage-splitting (Mallet 2007, Rothfels *et al.* 2015). Consequently many lineage-splits are masked during evolution and it highlights the importance of introgression during evolution (Mallet 2008a, Abbott *et al.* 2013, Dynesius and Jansson 2014). Ultimately, gene flow and lineage evolution are also terminated by extinction. Depending upon its frequency, population extinction can generate many splits as it prevents interbreeding among family lines and between metapopulations. Lineages-splits are therefore prolific over the course of evolution. However, extinction also erases evidence of lineage-splits because descendants are not

necessarily available for fossil or genetic sampling, which yields long naked branches in molecular phylogenetic reconstructions (Crisp and Cook 2009, Grandcolas *et al.* 2014). With so many splits occurring and being obscured during evolution it is impossible for all to be identified, and therefore the classification of anagenesis and cladogenesis also becomes untenable. Similarly, partitioning events of divergence with lineage-splits (if they correspond at all) faces the same problem of discrimination.

Identifying lineage-splits requires genetic data

The differentiation of anagenesis and cladogenesis via lineage-splitting is popular in palaeontology (e.g. Jackson and Cheetham 1999, Benton and Pearson 2001, Crampton and Gale 2005, Aze *et al.* 2013, Ezard *et al.* 2013, Pearson and Ezard 2014). Using lineage-splits to distinguish anagenesis and cladogenesis creates a quandary for palaeontology however because phylogeny can only be inferred from observations of morphology. Analysis of morphological data alone, with no knowledge of gene flow, means that difference must be used to define cladogenesis instead of lineage-splitting. Sequential changes in morphology along a time series, ideally with constrained geography and sufficient sampling (e.g. Pearson and Ezard 2014), provides a proxy for an evolutionary lineage (path of genetic inheritance and changing phenotype of a lineage). However, the degree of difference (phenotypic or genetic) is an inadequate proxy for the timing or position of lineage-splitting itself (see Figure 1.2 A).

In palaeontology, difference fails to accurately predict the position of lineage-splits because morphology may diverge before or after a true lineage-split (Figure 1.2 B). Consider for example, that many species defined by clear genetic cohesion exhibit differing degrees of morphological variation (Blomster *et al.* 1999, Calsbeek *et al.* 2007, Hopkins and Tolley 2011), and that many of morphologically cryptic species comprise genetically distinct lineages (e.g. Trewick 2000, Feldberg *et al.* 2004, Herbert *et al.* 2004, Heulsken *et al.* 2013). See Figure 1.2 B for an illustration. Simply, without genetic data it is not possible to distinguish within-lineage and between-lineage morphological variation (Van Bocxlaer and Hunt 2013). Increasing evolutionary time can provide confidence that divergent morphology approximates increasingly well with lineage-splitting, but concordance is mostly due to lineage-sorting and extinction. Even when divergence is simultaneous with a geological mechanism such as sea-level change, it cannot be used to precisely estimate when a lineage-split may have occurred as such geographic changes can exist for thousands of years (Page and Hughes 2014). It

remains impossible to be certain of when a split occurred and therefore, it is difficult to precisely estimate periods of anagenetic and cladogenetic change using morphological data alone (Crampton and Gale 2005). We agree with Bapst (2013), that it is important to distinguish lineage-splitting, morphological divergence, and speciation in palaeontology.

FIGURE 1.2

A. Evolutionary lineages and measures of difference.

a) Difference between contemporaneous extant populations (1 and 2) that belong to different lineages.

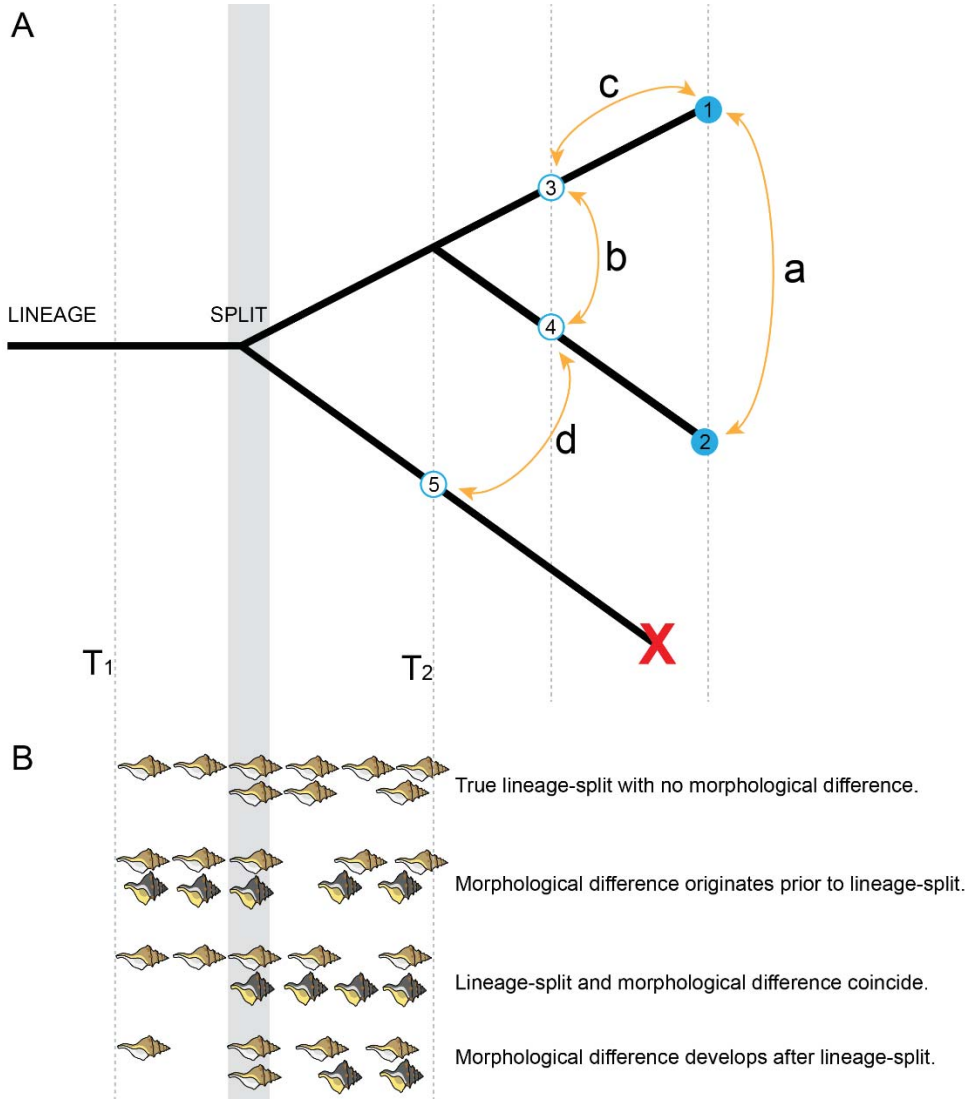
b) Difference between contemporaneous fossil populations. In this example the samples (3 and 4) belong to the same lineages as in a) and are therefore part of the same divergence process.

c) Difference between two populations from different times on the same evolutionary lineage. This is an ancestral/derived relationship. In this example Sample 1 is extant and Sample 3 is fossil, but both samples could be fossil populations. Such populations could be treated as chronospecies, as is common in comparisons between fossil samples of different age that are presumed to belong to the same single lineage.

d) Difference between two populations (4 and 5) from different times on different evolutionary lineages. In contrast to c) these are not ancestral and derived representatives of the same lineage, but in the absence of genetic information it would be impossible to know this.

B. Identifying lineage-splits without genetic data.

Morphological data alone is insufficient to demonstrate lineage-splitting. Morphological difference observed in fossil record between T_1 and T_2 does not necessarily correspond with a lineage-split (although it does provide a testable hypothesis), and divergence may have occurred before or after any existent split within the intermediate time period.



Speciation is arbitrary

Anagenesis and cladogenesis could be distinguished by classifying lineage-splits to be above or below the species-level. This distinction would allow us to ignore the majority of splits that occur during evolution that may not contribute to long-term evolutionary patterns, which would automatically mean that only cladogenesis increases species diversity (Ezard *et al.* 2012, Strotz and Allen 2013), whereas anagenesis results in static species diversity (Ezard *et al.* 2012, Haile-Selassie and Simpson 2013). Unfortunately this strategy is both circular and arbitrary. The distinction of species-level lineages can only be relative as it is dependent upon the studied organism, data available, and hypothesis under investigation (Ezard *et al.* 2013, Haile-Selassie and Simpson 2013). Even within a single genus, where taxonomic species delineation may have reached a consensus that permits a consistent definition of anagenesis, this approach would not lend itself to comparison across the tree of life. It may be helpful for researchers to use the anagenesis and cladogenesis in this relative manner based on the lineage-level or evolutionary persistence, but the limitations must be acknowledged.

Crucially, the distinction is also flawed because it fails to acknowledge that species are arbitrary and that speciation is an artificial concept established on a particular taxonomic paradigm. Since species are arbitrarily classified segments of evolutionary lineages they are not discrete states of evolution. At any point a lineage segment can be revised to occur above or below the species-level, and correspondingly splits could be reclassified from anagenesis to cladogenesis or vice versa. This type of reciprocal illumination means that the criteria used to define the pattern and process are conflated. Likewise, introgression can cause lineages to regress below the species-level (e.g. Abbott *et al.* 2013, Bhat *et al.* 2014, Dynesius and Jansson 2014), and, since the boundaries of a species do not strictly depend upon lineage-splitting (e.g. species with hybridising boundaries, ring species, chronospecies), speciation is not a definitive process with a beginning and end. There is no consistency between organisms for distinguishing particular splits as speciation, and there is no agreed point of complete speciation. This already appears to be recognised by authors who have adopted the terms ‘pseudospeciation’ and ‘pseudoextinction’ to describe divergence in the absence of known splitting (e.g. de Queiroz 1998, Ezard *et al.* 2012, Bapst 2013, Ezard *et al.* 2013, Haile-Selassie and Simpson 2013, Podani 2013). Species-based definitions of anagenesis and cladogenesis are also post hoc and cannot be applied to currently

evolving lineages (i.e. all living lineages) because we cannot predict future splitting, divergence or introgression.

Speciation considers more than lineage-splitting

Some lineage-splits are biologically significant because they represent genuine cases of 'instantaneous speciation.' This process can occur for example via karyotype changes (Moritz and Bi 2011), the evolution of parthenogenesis (Abe 1986), or androgenesis (Scali *et al.* 2012). These scenarios allow neat differentiation of anagenesis and cladogenesis in a species-based manner, but the majority of species appear to have emerged in a less abrupt fashion (Rymer *et al.* 2010, Claramunt *et al.* 2012, Near *et al.* 2012). Most 'speciation' is associated with divergence rather than lineage-splitting – new species are usually characterised by unique, identifiable genetic or phenotypic variation compared to related populations (Bapst 2013). Most often, a particular lineage-split is likely to be merely one step during the change identified as speciation. Focussing on lineage-splitting also distracts from the importance of lineage introgression during speciation (Mallet 2008, Abbott *et al.* 2013, Sætre 2013, Dynesius and Jansson 2014). Even discrete changes related to instantaneous speciation can also be caused by introgession via ploidy changes associated with hybridisation (Mallet 2007, Mráz *et al.* 2012), and the evolution of reproductive systems such as hybridogenesis (Dubois 2011, Pruvost *et al.* 2013).

Even when a lineage-split does represent an abrupt evolutionary change or innovation, many resulting lineages swiftly go extinct. For numerous reasons, systematics generally pays little attention to describing a unique lineage, even if it formed via a single split, unless it persists for a significant length of evolutionary time. The relevance of persistence through time is relative to the studied organism and is dependent upon evolutionary rate estimates that embroil further problems such as gene-tree heterogeneity (McCormack *et al.* 2010, Cutter 2013), and requires accurate estimations of extinction rates that might be intractable (Barraclough and Nee 2001, Quental and Marshall 2010, Morlon *et al.* 2011). For example, a new viral strain may be classified as a species-equivalent within a matter of months, whereas a reproductively isolated group of animals following a karyotype change is unlikely to be classified as a species for thousands or millions of years (Morgan-Richards *et al.* 2001). Species classification is concerned with divergence, introgression, extinction, and informative

value as much as splits and monophyly. Overall, it is unhelpful to synonymise cladogenesis (and lineage-splitting) with speciation.

Understanding evolution without anagenesis and cladogenesis

To illustrate the frequent redundancy of the terms 'anagenesis' and 'cladogenesis', we rephrase excerpts from recent studies using fundamental concepts in evolution. In many cases abandoning the terms improves the clarity of reasoning and expression, making research more accessible, and in a few instances it reveals ambiguity or indicates areas for further research. When we replace the terms anagenesis and cladogenesis from recent publications it reveals differences in current usage that are clearly contradictory.

Palaeontology and phylogenetics

"A model was fit that allows estimation of anagenetic (within-lineage) evolution, cladogenetic (speciational) change and geographic variation within species." (Hunt 2013)

A model was fit that allows estimation of lineage-splitting above and below the species-level, and geographic variation within species.

***

"The signal for anagenetic vs. cladogenetic change is subtle: it hinges upon whether the magnitude of divergence between species is more strongly correlated with elapsed time (as predicted by anagenetic change) or with the number of speciation events (as predicted by cladogenetic change) since their common ancestor." (Hunt 2013)

The signal for phylogenetic gradualism vs punctuated change is subtle: it hinges upon whether the magnitude of divergence between species is more strongly correlated with elapsed time (as predicted by phyletic gradualism) or with the number of speciation events (as predicted by punctuationalism) since their common ancestor.

***

"[They assessed] the relative frequency of anagenesis (evolution within a single evolving lineage) and cladogenesis (lineage branching) in the production of new morphospecies. They conclude that anagenesis is much less prevalent than indicated in our phylogeny." (Aze et al. 2013)

[They assessed] the relative frequency of evolutionary change that does and does not generate a net increase in species diversity during the production of new morphospecies. They concluded that evolutionary change that did not lead to a net increase in species diversity was much less prevalent than indicated in our phylogeny.

***

Biogeography

"The theory of 'punctuated equilibrium' proposes that species change suddenly during short bursts associated with speciation ('cladogenetic change'). It is best exemplified on oceanic islands, where adaptive radiations have led to spectacular cases of endemic speciation… On islands, the gradual evolution of a new species from a founder event has been called 'anagenetic speciation.' This process does not lead to rapid and extensive speciation within lineages, as adaptive radiation may do." (Patiño et al. 2014)

The theory of 'punctuated equilibrium' proposes that species change suddenly during short bursts associated with speciation. It is best exemplified on oceanic islands [sic], where adaptive radiations have led to spectacular cases of endemic speciation… On islands, following a population founder event new species can evolve gradually. This process does not lead to rapid divergence as adaptive radiations may do.

Conclusion

'Anagenesis' and 'cladogenesis' are vague and inconsistently defined terms in current research that have been conflated with other hypotheses (Rensch 1929, Simpson 1949, Jackson and Cheetham 1999, Mattila and Bokma 2008, Patiño *et al.* 2014). The most popular, robust definitions are based on the splitting of evolutionary lineages (Jackson and Cheetham 1999, Catley *et al.* 2010, Bapst 2013, Van Bocxlaer and Hunt 2013), and most authors conflate lineage-splitting with speciation (e.g. Mattila and Bokma 2008, Drew and Barber 2009, Hunt 2013, Dynesius and Jansson 2014). This imprecision is problematic as lineage-splitting is prolific in nature and not all splits are of interest – especially when investigating evolution over long time periods. Splits are common due to population structuring and extinction, and introgression and extinction mask and reduce the consequence of many splits. Species are arbitrary units, and therefore attempts to differentiate between lineage-splits above and below the species-level are relative and dependent upon the studied organism, data available, and hypothesis under investigation.

Studying change in species diversity over time is of immense value and required alongside analysis of the evolutionary process. We emphasise however that conflating descriptions of the evolutionary process (lineages, divergence, splitting, introgression) with taxonomy (species, speciation) does not benefit either line of investigation. Anagenesis and cladogenesis can remain useful terms if future definitions are aware of this separation, even if the terms are accepted to be relative to particular studied organisms. However if this problem is ignored, anagenesis and cladogenesis will remain a barrier to communication across disciplines, and the terms shall remain replaceable with more fundamental, transparent concepts. Studies of evolution do not need to focus solely on identifying species-level change.

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DRC 16

MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Felix Vaux

Name/Title of Principal Supervisor: Mary Morgan-Richards

Name of Published Research Output and full reference:

Vaux, F., Trewick, S.A., Morgan-Richards, M. (2016). Lineages, splits and divergence challenging the meaning of the terms anagenesis and cladogenesis. Biological Journal of the Linnean Society 117, 165 – 176.

In which Chapter is the Published Work: Chapter One

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate:

  and / or

- Describe the contribution that the candidate has made to the Published Work:

  I chose the review topic, read through the literature, wrote the the first manuscript draft, produced figure 1, and processed the iterative feedback from supervisors and reviewers.

Felix Vaux Digitally signed by Felix Vaux Date: 2016.10.10 16:12:44 +13'00'
Candidate's Signature

10/10/2016
Date

Morgan-Richards, Mary
Principal Supervisor's signature

10/10/2016
Date

GRS Version 3– 16 September 2011

Chapter Two

Speciation through the looking-glass

Mary and Steve make connections.

Introduction

In a *BJLS* review (Vaux *et al.* 2016), we considered the usage and meaning of the terms ‘anagenesis’ and ‘cladogenesis.’ We observed that the meaning of these terms has changed over time, and that modern usage is highly varied across disciplines and often ambiguous. We concluded that the terms anagenesis and cladogenesis were not needed to describe evolution or species classification, and that they potentially hamper communication between disciplines. For instance, some authors define ‘anagenesis’ as evolutionary change within a species (e.g. Johnson *et al.* 2012, Hunt 2013, Lister 2013), whereas others consider the term to be synonymous with gradualism (e.g. Ricklefs 2004, Theriot *et al.* 2006, Mattila and Bokma 2008, Pearson and Ezard 2014). Variation in usage between disciplines is obvious. For example many palaeontologists only recognise ‘anagenesis’ when morphospecies do not coexist temporally (e.g. Gould 2001, MacFadden *et al.* 2012); whereas it is common for biogeographers to consider contemporary, but geographically isolated lineages as examples of anagenetic speciation (e.g. Rosindell and Phillimore 2011, Patiño *et al.* 2014, Valente *et al.* 2014).

> “The mode of evolution may be anagenetic if the [first appearance] of the descendant coincides with the [last appearance] of the ancestor within the bounds of the dating precision.” (Strotz and Allen 2013)

> “A common mode of speciation in ocean islands is by anagenesis, wherein an immigrant arrives and through time transforms by mutation, recombination, and drift into a morphologically and genetically distinct species.” (López-Sepúlveda *et al.* 2015)

In a response Allmon (2016), agrees with much of our review, but promotes the treatment of species as being biologically real (Allmon 2016). This view contrasts with our approach of treating species classification as arbitrary segments of an evolutionary lineage (Vaux *et al.* 2016). We welcome the recognition (Allmon 2016) that ‘change’ and ‘branching’ are suitable substitutes for anagenesis and cladogenesis in many discussions of evolution (Simpson 1944, Simpson 1953).

Species and genetic introgression

It is not necessary for us to reiterate thorough exploration of the nature of species and their delimitation (e.g. Darwin 1859, Mayr 1942, Ghiselin 1974, Burger 1975, Mahner 1993, Mallet 1995, de Queiroz 1998, Sites and Marshall 2003, Hey 2006, Konstantinidis *et al.* 2006, Dubois 2011), because it does not actually address our criticism of 'anagenesis' and 'cladogenesis,' or demonstrate the necessity of the terms. Nonetheless, we do favour the acceptance that species are essentially arbitrary constructs, because no concept can be universally and consistently applied to evolving biota (Vaux *et al.* 2016). In doing so we follow the simple and well accepted fact identified by Darwin (1859), that species cannot be immutable whilst also evolving. Specifically, we observe that although some species appear to coincide with in vogue concepts, every species is an arbitrary segment of an evolutionary lineage in time (de Queiroz 1998, de Queiroz 2007, Vaux *et al.* 2016). We agree with Allmon (2016), that species can be established on the biologically real phenomena of evolutionary lineages (a line of descent of evolutionary units (organisms, replicators)), but the delimitation of a segment (especially in time) remains arbitrary (de Queiroz 2011). This is because divergence and lineage-splitting are not always concordant and partitions of variation among evolutionary lineages are ultimately of subjective interest to biologists. Practically, one can rarely identify a discrete origin of a species (if such an event ever occurs), and theoretically speciation is an infinite process referring to change among related evolutionary lineages.

We agree with a source cited by Allmon (2016), that, "a generally applicable concept of a species does not yet exist," (Marie Curie Speciation Network, 2012). The claim that there is a consensus for the definition of a species for, "at least the biparental animal part of [the living world]," (Allmon 2016) is readily falsified (see below) and the need for such a qualifier exposes the inadequacy of the assertion. A unifying concept cannot apply to only a subset of lineages in evolutionary time. Allmon (2016), promotes the view that species are biologically real, and although some taxonomic species are closer representations of evolutionary lineages than others (e.g. Rieseberg *et al.* 2006), problematic organisms remain abundant (Burger 1975, Diamond 1992, Berger and Ogielska 1994, Domingo *et al.* 1995, Konstantinidis *et al.* 2006, Rieseberg *et al.* 2006, Chan *et al.* 2012, Fuchs *et al.* 2015).

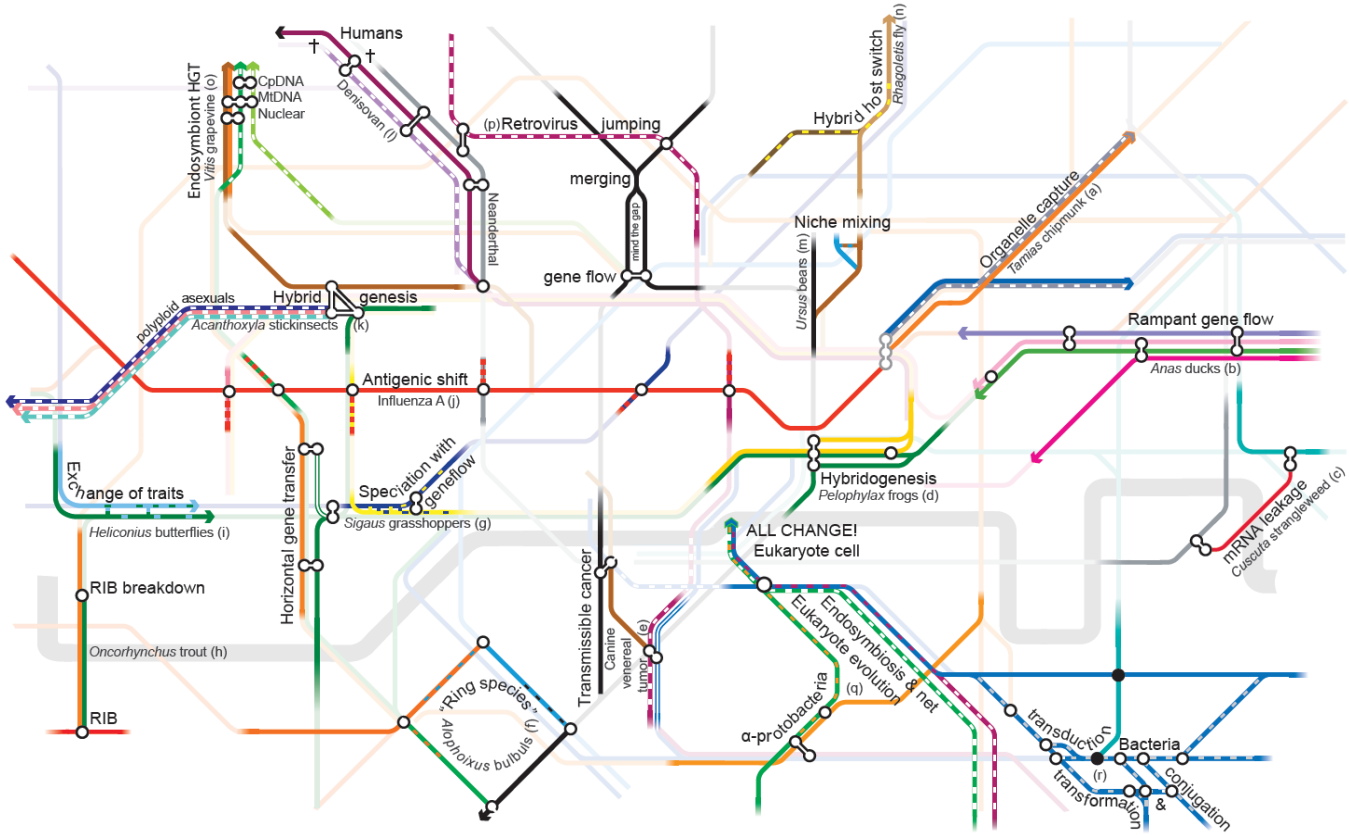
Despite previous reviews (e.g. Anderson and Stebbins 1954, Mallet 2007, Harrison 2012, Abbott *et al.* 2013), it seems that the impact of introgression upon

speciation and taxonomic classification is not fully appreciated. Introgression originates from two sources: reproduction (or vertical gene transfer) and horizontal gene transfer (Figure 2.1). Although hybridisation involving reproduction between members of separate lineages sometimes results in non-viable or infertile offspring (e.g. Wishart *et al.* 1988, Allen and Short 1997, Rieseberg 1997, Davis *et al.* 2015), this is not always the case (e.g. Burger 1975, Rieseberg 1997, Manos *et al.* 1999, Petit *et al.* 2003, Morgan-Richards *et al.* 2004, Trewick *et al.* 2004), even among biparental sexual animals (e.g. Rhymer *et al.* 1994, Derr *et al.* 1991, Schwarz *et al.* 2005, Gelberg 2009, Kraus *et al.* 2012, The *Heliconius* Genome Consortium 2012, Cahill *et al.* 2013, Bull and Sunnucks 2014, Dowle *et al.* 2014, Liu *et al.* 2014, Prüfer *et al.* 2014, Fuchs *et al.* 2015, Good *et al.* 2015, Mckean *et al.* 2016, Morgan-Richards *et al.* 2016; Figure 2.1). Even notoriously infertile first generation hybrids such as mules (*Equus*) can occasionally be fertile (Allen and Short 1997), as are lineages that require sexual stimuli or gametes of another lineage (e.g. Berger and Ogielska 1994, Ragghianti *et al.* 2007), and hybridisation among distantly related organisms is well documented (e.g. Rieseberg and Willis 2007, Rothfels *et al.* 2015). Furthermore, hybrid reproduction can be a source of hybrid vigour and it can transfer highly advantageous traits (e.g. The *Heliconius* Genome Consortium 2012).

Horizontal gene transfer (HGT) has had a significant impact over evolutionary time in all major clades of life. Models for the evolution of the eukaryotic cell rely upon HGT and subsequent genetic introgression (Margulis *et al.* 2000, Georgiades and Raoult 2011, Georgiades and Raoult 2012), and abundant evidence demonstrates that organellar DNA is continuously transferred to the nucleus (Blanchard and Lynch 2000, Stegemann *et al.* 2003), and between organelles (e.g. Goremykin *et al.* 2009). Other prokaryotic endosymbionts (organisms within the cells of another) are also absorbed (e.g. Gonella *et al.* 2015), and undergo HGT (e.g. Kondo *et al.* 2002, Husnik *et al.* 2013, Sloan *et al.* 2014, Wybouw *et al.* 2014), and viruses facilitate HGT between themselves and eukaryotic host genomes (Bejarano *et al.* 1996, Löwer *et al.* 1996, Mallet *et al.* 2004, Carrat and Flahault 2007, Herniou *et al.* 2013, Gasmi *et al.* 2015). Even the most reproductively discrete, biparental, sexual animals are therefore continuously introgressing with DNA of prokaryotic and viral origin. HGT is near-constant in bacteria via direct cell-to-cell exchange, indirect environmental exchange between cells, and indirect exchange between cells via viral infection (e.g. Ochman *et al.* 2000, Krebes *et al.* 2014). In many mutualistic and parasitic situations, non-vectored HGT involves

all combinations of animals, bacteria, fungi and plants, including both nuclear and organellar DNA (e.g. Vaughn *et al.* 1995, Groth *et al.* 1999, Davis and Wurdack 2004, Woloszynska *et al.* 2004, Hall *et al.* 2005, Moran and Jarvik 2010, Yoshida *et al.* 2010, Acuña *et al.* 2012, Kim *et al.* 2014, Nikolaidis *et al.* 2014, Wybouw *et al.* 2014). HGT is observed between animal hosts and transmissible cancers (Metzger *et al.* 2016, Strakova *et al.* 2016), and syncytial growth (nuclei sharing among cells) in fungi also provides the potential for HGT and viable interspecies genetic mosaics (*in sensu* Roper *et al.* 2013). These genetic exchanges often produce functional genes (e.g. Mallet *et al.* 2004, Nikolaidis *et al.* 2014), and associated traits often have the potential to be significantly advantageous and are of clear taxonomic interest (Bock 2010, Moran and Jarvik 2010, Herniou *et al.* 2013, Nikolaidis *et al.* 2014, Crisp *et al.* 2015, Gasmi *et al.* 2015). Resulting changes in the evolutionary trajectory of a lineage affect the overall pattern of lineage-splitting and divergence among populations, meaning that introgression does not merely result in gene-tree heterogeneity. A plethora of examples illustrate how reproduction and HGT maintain introgression and unclear boundaries for species classification (Figure 2.1); species do not, "maintain their separateness," (Allmon 2016).

FIGURE 2.1
Hybrid reproduction (vertical gene transfer) and horizontal gene transfer (HGT) result in frequent introgression among evolutionary lineages, which reveals that putative taxonomic species do not remain separate and that their delimitation is ultimately subjective. Here we illustrate some of the range of processes that have so far been identified, citing examples in primary and review literature (letter codes a – r refer to references listed in Supplementary Table 2.1, and many other examples are provided therein). Re-purposing an unrelated network demonstrates that modes of introgression are so prolific and diverse that almost any example can be illustrated by an arbitrary selection of intersecting lines. Note that the network relationships demonstrate gene flow, but not phylogeny or a scaled representation of change through time.



In some ways we and Allmon are speaking past one another, as perceptions of the status of species are sensitive to the resolution at which they are observed. At the scale typically used to investigate trends in biodiversity, species can appear coherent and separate. Most taxonomic work depends on arbitrary distinctions made by experts with the primary objective of defining distinct units. However, at a closer range where lineage-splitting and divergence are studied in detail, it often becomes apparent that such coherence is superficial. Under most definitions (e.g. Aze *et al.* 2013, Lister 2013, Strotz and Allen 2013), it is this scale of lineage-splitting at which periods of anagenesis and cladogenesis are defined, and thus where problems arise. Similar scale differences also affect the study of topics such as evolutionary stasis, where a trait can appear morphologically static over long periods of time, but less so over a shorter time period with more frequent sampling intervals (Hunt 2012).

"'When I use a word,' Humpty Dumpty said in a rather scornful tone, 'it means just what I choose it to mean -- neither more nor less.'"
(Lewis Carroll, 1871 in *Through The Looking Glass*)
[Also aptly quoted in Harrison 2012]

Allmon (2016) conflates species classification (and delimitation) with speciation by suggesting that we are not interested in studying speciation. Though seemingly an arid enterprise, clarification of terms used in evolutionary biology is needed for the intelligent exploration of biology. We explicitly stated that the classification (and observation) of a species depends upon divergence-based factors and the hypothesis of interest (Vaux *et al.* 2016). What this means is that the origination of species as a classified taxon is arbitrary, but the process of lineage-splitting and divergence that creates the diversity used to describe it is biologically real (and interesting). When most evolutionary biologists refer to 'speciation' we believe that they mean the latter process, and not the pedantic and arbitrary delimitation of a taxon. The process is of interest as it considers the biological evidence available (genetic variation, phenotypic variation, selection), whereas taxonomy is deciding when and how to assign names based, usually, on a subset of that evidence. The fact that we treat a species as an arbitrary concept does not prevent hypothesis testing, the study of lineage-splitting, divergence or diversification rates, or investigation of the fossil record (e.g. Darwin 1859).

Anagenesis and Cladogenesis

There are many instances where palaeontological evidence provides estimates of when lineage-splits must have occurred (e.g. Strotz and Allen 2013, Pearson and Ezard 2014, Kimura *et al.* 2016), and we also agree that palaeontologically recognised species can be comparable to living taxa (even if this is difficult to demonstrate) on a lineage divided into segments in time (de Queiroz 1998, Kimura *et al.* 2016). However, morphological crypsis leading to underestimation of diversity is not the only problem for the morphological identification of extinct species. The treatment of, "estimates of species and speciation rates [as] *minimum* estimates," (Allmon 2016), is flawed as there are also cases of taxonomic over-splitting in palaeontology that leads to overestimation of diversity (e.g. Hills *et al.* 2012, Aze *et al.* 2013).

Despite lengthy discussion of species classification in the fossil record Allmon (2016), does not define the terms or address the actual concern of our review: the ability to consistently define (and delineate in time) anagenesis (phyletic change) and cladogenesis (divergence concurrent with lineage-splitting) based on morphological evidence alone. Morphological divergence and lineage-splitting are not necessarily concordant. Even in palaeontological studies incorporating genetic data, estimates that utilise independent loci within a lineage will provide a range of dates (rather than a single estimate) for a lineage-split. This conflation is problematic for the delimitation of anagenesis and cladogenesis as most palaeontological definitions assume their mutual exclusivity (e.g. Aze *et al.* 2013, Lister, 2013).

"Never use a long word when a diminutive one will do."
(William Safire, 1979 in *On Language, New York Times Magazine*)

The claim that we have only demonstrated, "disparate usage by a few modern authors," (Allmon 2016), is inaccurate as our review cited many recent papers that vary in the meaning given to anagenesis and cladogenesis (e.g. Mattila and Bokma 2008, Drew and Barber 2009, Catley *et al.* 2010, Dubois 2011, Johnson *et al.* 2012, Pachut and Anstey 2012, Aze *et al.* 2013, Bapst 2013, Futuyma 2013, Hunt 2013, Podani 2013, Strotz and Allen 2013, Dynesius and Jansson 2014, Pearson and Ezard 2014, Patiño *et al.* 2014, Valente *et al.* 2014). For this contemporary variation to exist, the terms cannot have remained consistent, "for more than half a century," as Allmon (2016), suggests. We do not think this variation should be ignored as previous authors have also

discussed the problematic meaning of the terms (Benton and Pearson 2001, Dubois 2011), and because textbooks and educational research demonstrate that definitions vary (e.g. Catley *et al.* 2010, Johnson *et al.* 2012, Futuyma 2013), indicating that this ambiguity may be inherited by future scientists. If we follow the definition used by Simpson (1944), as suggested (Allmon, 2016), why do we need multiple words for 'branching' and 'phyletic change'? What is the necessity of redundant terminology (likewise with 'tokogenesis' for gene flow (Allmon 2016))?

Conclusion

Ultimately, the necessity of terms such as anagenesis and cladogenesis reflects a wider problem in academic communication. Researchers will decide whether to use complex terminology (giving each term the meaning they choose), or longer sentences with simple words. Biological evolution fundamentally operates under the basic principles of variation, selection, and heritability, which can be effectively modelled using even simple descriptions such as the univariate breeder's equation ($R = Sh^2$). Although this process generates rich complexity in nature, we consider that descriptions of biological evolution need not require complex and alienating language. We do not expect all readers to agree with our views on anagenesis and cladogenesis, but we hope it can at least be agreed that the terms in their current state are problematic for the communication of science, and in future authors should clearly express their definition of the terms or otherwise avoid them.

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Supplementary Data for Chapter Two

Supplementary Table

SUPPLEMENTARY TABLE 2.1
A table of primary and review references for evidence of particular modes of genetic introgression via reproduction (vertical gene transfer (VGT)) and horizontal gene transfer (HGT), most of which are illustrated by single examples in Figure 2.1. Only a small amount of the available literature is listed, and we deliberately focus upon examples from biparental sexual animals.

Figure Position	Mode of Introgression and Examples for VGT	Comments and Examples for HGT	References
	Reproduction leading to fertile hybrids		
l	Humans (*Homo*)		Prüfer *et al.* 2014
	Felinae (*Felis, Prionailurus, Leptailurus)*	Most F1 males infertile	Davis *et al.* 2015 Gelberg 2009
m	Bears (*Ursus*)	Niche mixing	Cahill *et al.* 2013 Liu *et al.* 2014
	Equidae (*Equus*)	Typically sterile, occasionally fertile	Allen and Short, 1997
	Deer (*Odocoileus*)		Derr *et al.* 1991
a	Chipmunks (*Tamias*)	Includes mitochondrial capture	Good *et al.* 2015
d	Edible frogs (*Pelophylax*)	Hybridogenesis with a klepton	Berger and Ogielska 1994 Ragghianti *et al.* 2007
h	Trout (*Oncorhynchus*)	Breakdown of reproductive isolation barrier	Heath *et al.* 2009
b	Ducks (*Anas*)		Rhymer *et al.* 1994 Kraus *et al.* 2012
f	Bulbuls (*Alophoixus*)	'Ring species'	Fuchs *et al.* 2015
g	Grasshoppers (*Sigaus*)	Speciation with gene flow	Dowle *et al.* 2014
k	Stick insects (*Clitarchus, Acanthoxyla*)		Morgan-Richards *et al.* 2016
i	Butterflies (*Heliconius*)		The *Heliconius* Genome Consortium 2012
n	Fruitflies (*Rhagoletis*)		Schwarz *et al.* 2005
	Velvet worms (*Euperipatoides*)		Bull and Sunnucks 2014
	Oaks (*Quercus*)	Includes cytoplasmic capture	Burger 1975 Petit *et al.* 2003
	Ferns (*Cystopteris*, *Gymnocarpium*)	Includes hybridisation among distant relatives	Rothfels *et al.* 2015

	Hawkweed (*Hieracium*)		Morgan-Richards *et al.* 2004 Trewick *et al.* 2004
		Endosymbiotic Horizontal Gene Transfer	
q	Evolution of the eukaryotic cell	Proto-Eukaryote, Rhizobiales, Rickettsiales, α-Proteobacteria	Margulis *et al.* 2000 Georgiades and Raoult 2011, 2012
	Endosymbiont transmission	*Cardinium* through plants to *Scaphoideus titanus, Macrosteles quadripunctulatus, Empoasca vitis*	Gonella *et al.* 2015
o	mtDNA to nDNA	Near-ubiquitous	Blanchard and Lynch 2000 Stegemann *et al.* 2003
o	CpDNA to mtDNA	Grapevine (*Vitis vinifera)*	Goremykin *et al.* 2009
	Prokaryote endosymbiont DNA to host Eukaryote nDNA	*Wolbachia* to *Callosobruchus chinensis* Pyllidae (*Carsonella* and *Pachypyslla venusta*) Mealybugs (*Tremlaya princeps* to *Planococcus citri*) α- or β-Proteobacteria and Arthropoda	Kondo *et al.* 2012 Husnik *et al.* 2013 Sloan *et al.* 2014 Wybouw *et al.* 2014
		Viral Horizontal Gene Transfer	
j	Viral to Viral DNA via Eukaryote host	Includes antigenic shift *Influenzavirus A*	Carrat and Flahault 2007
p	Viral DNA to host Eukaryote nDNA	Retroviridae and humans Geminiviridae to tobacco (*Nicotiana tabacum*)	Bejarano *et al.* 1996 Löwer *et al.* 1996 Mallet *et al.* 2004
	Virus to Eukaryote nDNA to Eukaryote nDNA	*Bracovirus, Ichnivirus*, Braconidae wasps, Lepidoptera	Herniou *et al.* 2013 Gasmi *et al.* 2015
r	Transduction in bacteria		Ochman *et al.* 2000
		Non-Vectored Horizontal Gene Transfer	
r	Transformation and conjugation in bacteria	Bacteria	Krebes *et al.* 2014
	Prokaryote DNA to Eukaryote nDNA	Lactobacillales to *Saccharomyces* α- or β-Proteobacteria DNA to Arthropoda Bacteria to *Hypothenemus hampei*	Hall *et al.* 2005 Wybouw *et al.* 2014 Acuña *et al.* 2012
	Eukaryote nDNA to Prokaryote DNA	Plant to Bacteria	Nikolaidis *et al.* 2014
	Eukaryote nDNA to Eukaryote nDNA	*Saccharomyces* complex Fungi and *Acyrthsiphon pisum* Plant to Fungi	Groth *et al.* 1999 Moran and Jarvik 2010 Nikolaidis *et al.* 2014
c	Eukaryote mRNA to other Eukaryote	*Cuscuta* to *Arabidopsis*	Kim *et al.* 2014

e	Eukaryote mtDNA to Eukaryote mtDNA	Fungi to *Peperomia*	Vaughn *et al.* 1995
		Saccharomyces complex	Groth *et al.* 1999
		Rafflesiaceae endophytic parasite and hosts	Davis and Wurdack 2004
		Striga hermonthica parasite and hosts	Yoshida *et al.* 2010
		Transmissible cancers	Strakova *et al.* 2016
	Plant CpDNA to plant mtDNA	Eudicots to *Phaseolus*	Woloszynska *et al.* 2004

MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Felix Vaux

Name/Title of Principal Supervisor: Mary Morgan-Richards

Name of Published Research Output and full reference:

Vaux, F., Trewick, S.A., Morgan-Richards, M. (2016). Speciation through the looking-glass. Biological Journal of the Linnean Society (in press).

In which Chapter is the Published Work: Chapter Two

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate:

  and / or

- Describe the contribution that the candidate has made to the Published Work:

  I read through the literature, wrote the the first manuscript draft, helped produce the figure and found key research examples, and processed the iterative feedback from supervisors and reviewers.

Felix Vaux — Digitally signed by Felix Vaux Date: 2016.10.10 16:12:18 +13'00'
Candidate's Signature

10/10/2016
Date

Morgan-Richards, Mary
Principal Supervisor's signature

10th Oct 2016
Date

Chapter Three

Paraphyly of Southern Hemisphere true whelks and the concordance of a dated phylogeny with the fossil record

Shells of buccinulid true whelks.

Introduction

Taxonomy faces the simultaneous challenge of proposing and revising evolutionary hypotheses. We cannot easily interpret the bewildering diversity of life without taxonomy tentatively identifying groups, but we also expect taxonomy to be accurate and updated based on the best evidence available. Taxonomy is always therefore a working hypothesis that is testable and capable of being disproved. However, although not intended to be flawless, taxonomy can be seriously misled by biogeographic hypotheses that are inferred from current distributions. A famous example is the Old World and New World divide, which correctly predicts shared ancestry and separate evolutionary radiations of monkeys (Catarrhini and Platyrrhini) (Perelman *et al.* 2011), but conversely is incorrect for the paraphyletic and convergent clades of vultures (Gypaetinae, Aegypiinae and Cathartidae) (Wink 1995, Gibb *et al.* 2007).

In this study, we investigate a similar biogeographic hypothesis in taxonomy regarding marine snails. Under current taxonomy, true whelks in the Southern Hemisphere are hypothesised to be the product of geographic isolation from the Northern Hemisphere followed by an evolutionary radiation (Powell 1951, Harasewych and Kantor 1999, Hayashi 2005). This taxonomic hypothesis is based on biogeographic patterns and soft-body morphology (Powell 1929, Powell 1951, Harasewych and Kantor 1999), which we aim to test using molecular phylogenetics. We focus especially on true whelks from New Zealand as the initial Southern Hemisphere hypothesis was based on endemic taxa (Finlay 1928, Powell 1929), and because the region exhibits high species diversity (Powell 1979, Willan *et al.* 2010). The taxonomy prompts the key question: are true whelks in the Southern Hemisphere monophyletic? If not, at approximately what time did lineages disperse across the globe and can a dated phylogeny help with the interpretation of the fossil record for the Southern Hemisphere taxa.

True whelks are a diverse group of Neogastropod marine and freshwater snails that are typically carnivores or scavengers (Strong *et al.* 2008, Spencer *et al.* 2009, Willan *et al.* 2010). The overall taxonomy of Neogastropoda (Colgan *et al.* 2007, Cunha *et al.* 2009), and Mollusca itself remains uncertain (Wagner 2001, Kocot *et al.* 2011). However, neogastropod species are frequently sampled for phylogenetic and biogeographic studies as taxa are diverse, widely distributed, and frequently occur within easily accessible shallow water habitats (Harasewych *et al.* 1997, Colgan *et al.* 2007, Cunha *et al.* 2009). New Zealand hosts a high diversity of endemic

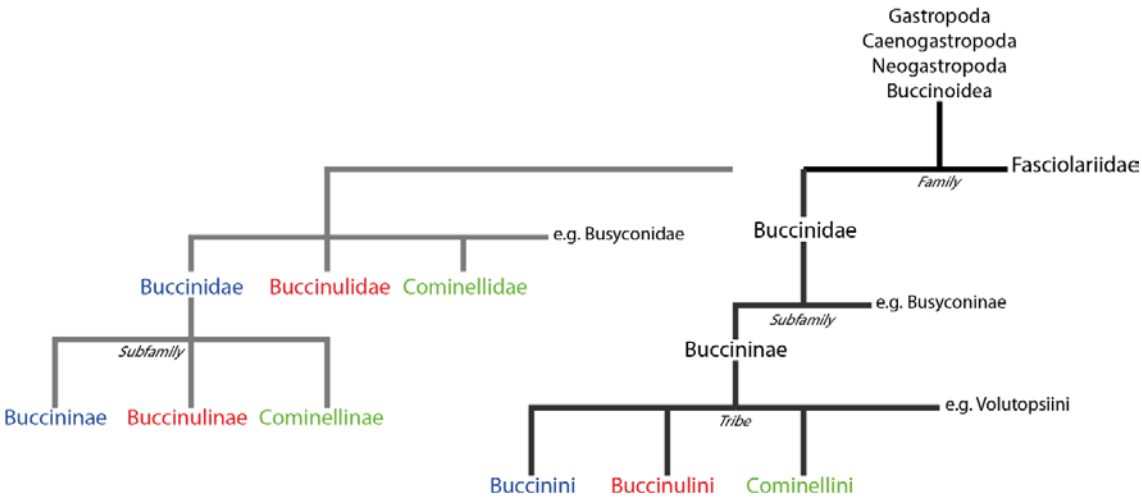
Neogastropoda (Powell 1979, Spencer *et al.* 2009, Willan *et al.* 2010, Spencer *et al.* 2017), of which true whelks represent a significant proportion (Powell 1951, Powell 1979, Willan *et al.* 2010), with an abundant fossil record (Beu and Maxwell 1990). New Zealand true whelks occupy an unusual variety of niches compared to other regions (Powell 1929, Dell 1956, Beu *et al.* 1976, Willan 1978, Powell 1979, Willan *et al.* 2010), and they exhibit significant morphological variation (Powell 1927, Powell 1947, Dell 1956, Ponder 1973, Powell 1979).

For comparisons of a dated phylogeny to fossil record evidence, we focus especially on the genera of siphon whelks *Penion* Fischer, 1884 and Kellet's whelks *Kelletia* Bayle, 1884. These large, predator-scavenger true whelks are considered to be taxonomically diverse; numerous extant, endemic *Penion* species are recognised from waters off Australia (Ponder 1973), and New Zealand (Powell 1979, Spencer *et al.* 2017), and two separate species of *Kelletia* are recognised from North America (Zacherl *et al.* 2003a, Vendetii 2009), and South Korea and Japan (Zacherl *et al.* 2003b, Hayashi 2005, Kim *et al.* 2012, Hwang *et al.* 2014). *Penion* and *Kelletia* are hypothesised to be closely related based on shell morphology and limited DNA sequence data (Ponder 1973, Hayashi 2005), which we shall test using mitochondrial genomic and nuclear DNA sequence data. The fossil record for both genera is rich: 17 extinct fossil *Penion* species are recognised from New Zealand (Beu and Maxwell 1990), along with 4 from Australia (Ponder 1973), 11 from Argentina and Chile (Frassinetti 2000, Nielsen 2003, Parras and Griffin 2009, Reichler 2010), and one species from Antarctica (Beu 2009). Similarly, 5 extinct species of *Kelletia* are recorded from North America (Arnold 1910, Anderson and Martin 1914, Kanakoff 1954, Addicott 1970, Hertlein 1970), in addition to 2 from Ecuador (Olsson 1964), and one from Japan (Ozaki 1954). Although specimens have been extensively collected, no interpretation or analysis of the overall fossil record has been made. Using fossil calibrations from independent neogastropod lineages, we aim to compare the estimated divergence dates among extant *Penion* and *Kelletia* species with the fossil record and geographic distribution of the group.

Traditionally, all true whelks are classified as the single monophyletic family Buccinidae (Caenogastropoda: Neogastropoda: Buccinoidea) (Thiele 1912, Powell 1951, Harasewych and Kantor 1999, Donald *et al.* 2015). However, an alternative paradigm moves many species into the additional, sister family of Buccinulidae (Finlay 1928, Powell 1929, Powell 1951, Bouchet *et al.* 2005), which under different taxonomic hypotheses may be rendered as the subfamily Buccinulinae or tribe Buccinulini instead

(Bouchet *et al.* 2005, Hayashi 2005; Figure 3.1). The basis of this hypothetical separation is that Buccinulidae represents a Southern Hemisphere radiation, in isolation of the Northern Hemisphere dominated Buccinidae (Powell 1951, Powell 1965). New Zealand true whelks dominate the proposed Buccinulidae clade (Finlay 1928, Powell 1929, Powell 1951), likely due to the high rate of endemism (Spencer *et al.* 2009, Willan *et al.* 2010, Spencer *et al.* 2017). The classification of true whelks and distinction of the two families depends upon morphological differences in opercula and radulae (Powell 1951, Harasewych and Kantor 1999). However, radula morphology is often incapable of discriminating species and it is possible that variation for the trait reflects environmental plasticity (Dell 1956, Dell 1972, Willan 1978). Furthermore, stomach anatomy struggles to distinguish Buccinidae and Buccinulidae, despite this trait allowing other neogastropod families to be separated (Kantor 2003).

FIGURE 3.1
A simplified illustration of current taxonomic hypotheses for Southern Hemisphere whelks. Whelks from the Southern Hemisphere (red) can be classified as a clade independent from Northern Hemisphere whelks (blue), either as a family, subfamily or tribe dependent upon the wider taxonomic hypothesis for Neogastropoda. *Cominella* Gray, 1850, a group of Southern Hemisphere whelks, can also be classified within a separate clade (green), which also fluctuates in classification from family to tribe. Not all related clades are shown. Fasciolariidae is likely to be a monophyletic sister taxon to the buccinid/buccinulid whelks.



The monophyly of Southern Hemisphere true whelks and the taxonomic hypothesis of Buccinulidae has not yet been tested thoroughly using molecular data. Only a few previous phylogenetic studies have sequenced true whelks from the Southern Hemisphere (Hayashi 2005, Oliverio and Modica 2010, Donald *et al.* 2015). Hayashi (2005) produced a phylogeny using mitochondrial 16S rRNA gene sequences from a selection of worldwide true whelks, which included four species in three genera from New Zealand. Results found mixed support for the monophyly of Buccinulidae (Hayashi 2005). A second study by Donald *et al.* (2015) produced a phylogeny of *Cominella* Gray, 1850 in New Zealand and Australia, sequencing 21 species from three true whelk genera. However no Northern Hemisphere species were sequenced and the monophyly of Buccinulidae was not addressed.

The original Buccinulidae classification was based on New Zealand taxa (Finlay 1928, Powell 1929), and the traditional assumption of biogeographic isolation for the islands likely influenced the hypothesis of isolation in the Southern Hemisphere. Perhaps because of its very late colonisation by humans (McGlone and Wilmshurst 1999, Wilmshurst *et al.* 2008), New Zealand is often assumed to be biogeographically isolated. This view has led to the perennial popularity of vicariance-based hypotheses for the evolution of New Zealand taxa (especially terrestrial), typically involving former Gondwanan landmasses (Craw *et al.* 1999, Cooper and Millener 1993, Gibbs 2006, Trewick *et al.* 2007). However many recent studies of extant populations have demonstrated that migration to and from New Zealand is common (e.g. Battley 1997, Hermandez *et al.* 2015). Phylogenetic evidence indicates that dispersal events are frequent (e.g. Trewick 2000, Winkworth *et al.* 2002, Knapp *et al.* 2005, Goldberg *et al.* 2008), and paraphyly has been demonstrated for some putative endemic radiations (e.g. Phillips *et al.* 2010). It is important to remember that the present geographic remoteness of New Zealand has existed for less than 85 Ma (final split of Zealandia from Gondwana; Tulloch *et al.* 2009). Furthermore, the accuracy of geological reconstructions affects likelihood of vicariant mechanisms (e.g. Turner 1991, Knapp *et al.* 2005, Goldberg *et al.* 2008), and routes of dispersal (e.g. Winkworth *et al.* 2015). Overall, we should not assume that New Zealand taxa are biogeographically isolated, and therefore it is prudent to investigate the monophyly of Buccinulidae.

Despite New Zealand being an oceanic archipelago, the phylogeny and dispersal ability of native marine invertebrates has only been investigated in a small number of species (e.g. Sponer and Roy 2002, Donald *et al.* 2005, Hills *et al.* 2011, Donald *et al.*

2015). Like terrestrial species, aquatic organisms can undergo both vicariance and dispersal. Ocean currents provide a means of dispersal across large distances (e.g. Turner 1991, Dutton *et al.* 2014), but they change through time (Rahmstorf 2002), and species vary in their ability to transgress the widest regions of deep water (e.g. Lessios *et al.* 1998, Parsons 1998, Baums *et al.* 2012, Dutton *et al.* 2014, Hermandez *et al.* 2015). Land formations can represent long-lasting barriers to dispersal (Bacon *et al.* 2015), but they form gradually in a complex manner (Bacon *et al.* 2015, Ingley *et al.* 2015), and can be circumvented (e.g. Miura *et al.* 2012).

The developmental biology of marine snails is likely to have an effect upon dispersal ability. Species can exhibit direct development, where offspring hatch from eggs as small versions of benthic adults, or indirect (planktonic) development where larvae emerge with a different phenotype to adults that is adapted for planktonic dispersal (Thorson 1950, Jablonksi and Lutz 1983, Hendricks 2012). Indirect developing larvae can acquire nutrition from yolk in egg sacs (lecithotrophy) or feed while suspended in water column as plankton (planktotrophy; Nützel 2014). Direct development is often predcicted to result in a lower potential for dispersal than indirect development (Jablonski and Lutz 1983, Johannesson and Johannesson 1995, Hendricks 2012), resulting in reduced gene flow and increased partitioning of genetic variation among populations (e.g. Keeney *et al.* 2013, Ellingson and Krug 2016). This prediction is not always true however (e.g. Cumming *et al.* 2014). As with other benthic marine invertebrates, direct development in marine snails has been argued to be more frequent at polar latitudes and at deeper sea depths (Jablonksi and Lutz 1983).

Northern Hemisphere true whelk lineages exhibit both direct and indirect development (e.g. Zheng *et al.* 2005, Smith *et al.* 2013). Many true whelks from New Zealand appear to undergo direct development (e.g. Ponder 1973, Pilkington 1974, Powell 1979, Morley 2013, Donald *et al.* 2015), which may have contributed to the concept of biological isolation for these islands and the Southern Hemisphere. Extant New Zealand *Penion* are all believed to undergo direct development (Powell 1979), although this hypothesis has not been tested experimentally, whereas living taxa from Australia exhibit protoconch (larval shell) morphology suggestive of indirect development (Ponder 1973). In contrast, extant *Kelletia* have been demonstrated to undergo indirect development, with larvae that can feed directly when egg yolk resources are depleted (facultative planktotrophy; Rosenthal 1970, Zacherl *et al.* 2003b, Vendetti 2009). By comparing developmental strategies with estimated diverge dates

and fossil record evidence, we aim to determine if the evolution of *Penion* and *Kelletia* fits the prediction of limited dispersal and geographic isolation for buccinulid true whelks.

Methods

Taxonomy and Sampling

As discussed above, the majority of Southern Hemisphere true whelks can be classified as Buccinidae (Thiele 1912, Powell 1951, Harasewych and Kantor 1999, Bouchet *et al.* 2005, Hayashi 2005), or Buccinulidae (Finlay 1928, Powell 1929, Powell 1951, Bouchet *et al.* 2005, Hayashi 2005). Depending on the taxonomic hypothesis for Gastropoda overall, Buccinulidae can also be referred to as a subfamily Buccinulinae or tribe Buccinulini (Bouchet *et al.* 2005, Hayashi 2005; Figure 3.1). *Cominella* is also sometimes classified within Cominellidae (or Cominellinae or Cominellini), but this genus has also been classified within Buccinulidae alongside other Southern Hemisphere true whelks (Powell 1951, Hayashi 2005, Donald *et al.* 2015). The majority of species classification is based on traditional morphological analysis of conchology and soft-body tissues such as the radula, operculum, stomach, and gonads (Powell 1951, Dell 1956, Dell 1972, Ponder 1973, Powell 1979, Harasewych and Kantor 1999, Kantor 2003, Spencer *et al.* 2009, Willan *et al.* 2010, Spencer *et al.* 2017). See Chapter 8 for a full summary of the taxonomy of *Antarctoneptunea* Dell, 1972, *Kelletia* and *Penion.*

The majority of specimens were borrowed from museum and university collections (acknowledged below), although some individuals were collected in the field for this study (Tables 3.1 and 3.2). Specimens were collected either via trawling (20 – 500 m depth for most sampling) or by hand from the intertidal zone. Some specimens were caught as trawling fishery bycatch. Captured individuals were swiftly frozen, thawed and removed from shells, and then preserved in 95% ethanol. All sampled specimens were identified by experienced molluscan taxonomists: Bruce A. Marshall (Collection Manager Sciences, Museum of New Zealand Te Papa Tongarewa) and Alan G. Beu (Palaeontologist, GNS). We used a public database (GenBank) to retrieve sequence data from other Northern Hemisphere taxa (from Claremont *et al.* 2008, Vendetti 2009, Barco *et al.* 2010, Oliverio and Modica 2010, Zou *et al.* 2011a, Zou *et al.* 2011b, Kim *et al.* 2012; see Tables 3.1 and 3.2). We did not sample any specimens from the putative Buccinulidae genera *Antarctodomus* A. Adams, 1863 and *Euthrenopsis* Powell, 1929 from southern New Zealand and the subantarctic.

We sampled all species of the Buccinulidae genera *Antarctoneptunea* and *Kelletia*, and selected representatives of *Aeneator* Finlay, 1926, *Austrofusus* Kobelt, 1879, *Buccinulum* Deshayes, 1830, *Cominella*, *Pareuthria* Strebel, 1905, and *Penion* (Tables 3.1 and 3.2). These genera are dominated by New Zealand taxa, with the exception of *Antarctoneptunea*, *Kelletia* and *Pareuthria*. *Kelletia* is restricted to the Sea of Japan and the Pacific coast of Honshu (Zacherl *et al.* 2003b, Hayashi 2005, Kim *et al.* 2012, Hwang *et al.* 2014), and the waters surrounding southern California, USA and Baja California, Mexico (Zacherl *et al.* 2003a, Vendetii 2009). Species of *Pareuthria* and *Antarctoneptunea aurora* (Hedley, 1916), the type species of this genus, are restricted to the polar circle of Antarctica (Dell 1972; Oliverio and Modica 2010). Species of *Penion* occur off the coast of New Zealand and Australia (Ponder 1973), and a number of extant whelks off the coast of Chile are classified as *Aeneator* (McLean and Andrade 1982, Araya 2013). We sampled representatives of four Buccinidae genera restricted to the Northern Hemisphere; *Buccinum* Linnaeus, 1758, *Colus* Röding, 1798, *Neptunea* Röding, 1798, and *Volutopsius* Mörch, 1857 (Tables 3.1 and 3.2).

As outgroup taxa we sampled the fasciolariid species *Glaphyrina caudata* Quoy & Gaimard, 1833, *Pararetifusus carinatus* Ponder, 1970 (here newly referred to *Pararetifusus* Kosuge, 1967), and *Taron dubius* (Hutton, 1878), which are all endemic to New Zealand. In addition we also included whole mitochondrial genome sequences for the nassariid species *Tritia obsoleta* (Say, 1822) and *T. reticulata* (Linnaeus, 1758) generated by previous studies (Simison et al. 2006; Cunha et al. 2009). Both of these species are restricted to the Atlantic Ocean. We use Fasciolariidae as the primary outgroup for our buccinulid phylogeny as the group is widely accepted to be the sister group to Buccinidae/Buccinulidae (Harasewych et al. 1997; Hayashi 2005; Kosyan et al. 2009), and appears to be monophyletic (Couto et al. 2016). Nassariidae is also considered to be sister to Buccinidae/Buccinulidae (Harasewych et al. 1997; Hayashi 2005; Cunha et al. 2009), although some nassariid groups are difficult to distinguish from buccinid whelks based on shell and soft-part morphology (Haasl 2000), and the taxon appears to be paraphyletic (Hayashi 2005; Kosyan et al. 2009; Oliverio and Modica 2010; Galindo et al. 2016).

DNA Extraction and Sequencing

50 mg sections of foot or columella muscle tissue were cut from preserved specimens using a sterile scalpel blade. These sections were pressed and dried to

remove ethanol and were then diced into a dozen pieces, and sometimes also crushed by a sterile pestle. Tissue was transferred to a clean 2 ml Eppendorf microtube and placed in 300 µl CTAB buffer (2% hexadecyl-trimethyl-ammonium bromide, 100 mM Tris-HCl pH 8.0, 1.4 M NaCl, 20 mM EDTA). Tissue was digested using 15 µl of 1/10 Proteinase K and incubated overnight (15 – 20 hours) at 57 °C. To reduce RNA contamination, 4 µl of 1/10 RNase A was added to each sample following digestion and then incubated for a further 15 minutes. DNA was isolated using high-salt precipitation, following purificiation using chloroform (24:1 chloroform-isoamyl alcohol), sodium acetate (3 M NaOAc), and -20 °C chilled 70% ethanol, which is a modification of previous molluscan DNA extraction methods (Thomaz *et al.* 1996, Trewick *et al.* 2009). This extraction method has been found to be the most successful for attaining high molecular weight DNA while avoiding the potential problem of mucopolysaccharide contamination interfering with enzymatic reactions using neogastropod tissue (Winnepenninckx *et al.* 1993). Samples were re-suspended in 50 µl of TE buffer (10 mM Tris, 0.1 mM EDTA), or 100 µl for larger yields of DNA. DNA was quantified using the Qubit Fluorometric Quantitation kit (Life Technologies, Thermo Fisher Scientific Inc.).

Total DNA extracts from 32 individuals of 29 putative species were processed for high-throughput sequencing using the ThruPLEX DNA-seq Kit (Rubicon Genomics). Fragmented genomic DNA was paired-end sequenced on an Illumina HiSeq 2500 (Table 3.1). Reads for each of the 32 individuals were de-multiplexed using standard indexes incorporated in the library-preparation kit. Resulting Illumina short-sequence reads that passed standard quality filters had adapter sequences removed using cutadapt 1.11 (Martin 2011). Geneious 9.1.3 (Kearse *et al.* 2012), was used to pair sequence reads and to edit, assemble and align sequences. The whole mitochondrial genome and 45S nuclear ribosomal cassette (18S, ITS1, 5.8S, ITS2, 28S) were both constructed by mapping paired reads to reference annotated molluscan mitochondrial genomes/gene regions. A new target sequence, using only reads from the sequenced individual was generated. Reads were then iteratively re-mapped to the target sequence in order to extend coverage of each genomic region.

The mitochondrial genes cytochrome oxidase I (*cox1*) and 16S rRNA, as well as the nuclear ribomsal RNA 28S gene from additional individuals of each species were also amplified using PCR and Sanger sequencing (Table 3.2). Alignments used for regions of these genes were assembled with reference to the whole genome sequences

produced from the high-throughput sequencing above. This smaller scale sequence data was used to investigate relationships among species with greater sampling.

TABLE 3.1
A list of individuals that were Illumina sequenced to yield mitochondrial genome and nuclear ribosomal sequence data. Specimen with origins marked with an asterisk (*) were obtained from aquaria or fish markets (Simison *et al.* 2006, Vendetti 2009), and therefore precise localities are unknown. Highlighted taxa are Buccinulidae genera, grey coloured taxa are Buccinidae, or Fasciolaridae and Nassariidae species used as outgroups. Colours used for each Buccinulidae genus correspond to the highlighting of lineages in phylogenetic trees (Figures 3.2 – 3.4, Supplementary Figure 3.7). 'P' indicates partial success of sequencing and 'Y' total success.

Taxon	rDNA cassette	mtDNA genome	Voucher ID	Location	GenBank Accession	Source
Putative 'Southern' true whelks (Neogastropoda: Buccinoidea: Buccinulidae)						
Aeneator benthicolus	Y	Y	M.274111	Cape Palliser		This thesis
Aeneator elegans	Y	Y	SFKH-TMP015	Chatham Rise		This thesis
Aeneator otagoensis	Y	Y	M.279437	Tasman Bay		This thesis
Aeneator recens	Y	Y	M.190119	Cape Turnagain, Manawatu-Wanganui		This thesis
Aeneator valedictus	P	Y	SFKH-TMP013	TAN 616/83		This thesis
Austrofusus glans	Y	Y	SFKH-TMP014	Island Bay, Wellington		This thesis
Buccinulum linea	Y		SFKH-TMP016	Nelson, Nelson		This thesis
Buccinulum fuscozonatum	Y	Y	M.302907/2	Ariel Bank, Gisborne		This thesis
Buccinulum pallidum	Y	Y	M.258277/6	Stewart Island		This thesis
Buccinulum p. finlayi	Y	Y	M.302870/2	Point Gibson, Canterbury		This thesis
Buccinulum robustum	Y	Y	M.314755/1	Oneroa Bay, Bay of Islands		This thesis
Buccinulum v. littorinoides	Y		SFKH-TMP011	Mahia Peninsula		This thesis
Buccinulum v. vittatum	Y	Y	SFKH-TMP012	Hicks Bay, Gisborne		This thesis
Cominella adspersa	Y	Y	SFKH-TMP009	Urupukapuka Bay, Bay of Islands		This thesis
Cominella v. brookesi	Y	Y	SFKH-TMP010	Spirits Bay, Northland		This thesis
Kelletia kelletii	Y	Y	KK12	Santa Barbara, California, USA*		This thesis
Kelletia lischkei	Y	Y	KL2	Kansai, Mie Prefecture, Japan		This thesis
Penion benthicolus	Y	Y	M.183832	Chatham Rise		This thesis
Penion chathamensis	Y	Y	M.190082/2	Chatham Rise		This thesis
Penion chathamensis	Y	Y	M.190085	Chatham Rise		This thesis
Penion c. cuvierianus	Y	P	M.183792/1	Red Mercury Island		This thesis
Penion c. cuvierianus	Y	Y	M.183927	Coromandel		This thesis
Penion mandarinus	Y	Y	C.456980	Gabo Island, Victoria, Australia		This thesis
Penion maximus	Y	Y	C.487648	Terrigal, New South Wales, Australia		This thesis

Penion sulcatus	Y	Y	Phoenix1	Tauranga, Bay of Islands		This thesis
Penion sulcatus	Y	Y	Phoenix9	Auckland		This thesis
Putative 'Northern' true whelks (Neogastropoda: Buccinoidea: Buccinidae)						
Buccinum undatum	Y	Y	20140783	Reykjanesskagi, Iceland		This thesis
Colus sp.	Y	Y	20140782	Moray Firth, Scotland, UK		This thesis
Volutopsius norwegicus	Y	Y	20140781	Hornsund Fjord, Svalbard, Norway		This thesis
Tulip and spindle snails (Neogastropoda: Buccinoidea: Fasciolariidae)						
Glaphyrina caudata	Y	Y	SFKH-TMP004	Off Farewell Spit, Golden Bay		This thesis
Pararetifusus carinatus	Y	Y	SFKH-TMP005	Chatham Rise		This thesis
Taron dubius	Y	Y	SFKH-TMP006	Hot Water Beach, Coromandel		This thesis
Dog whelks (Neogastropoda: Buccinoidea: Nassariidae)						
Tritia obsoleta		Y		California, USA*	NC_007781	Simison *et al.* 2006
Tritia reticulata		Y		California, USA*	NC_013248	Cunha *et al.* 2009

TABLE 3.2
A list of individuals that were PCR amplified and sequenced for the mitochondrial *cox1*, 16S or nuclear ribosomal 28S genes. Specimen with origins marked with an asterisk (*) were obtained from aquaria or fish markets (Simison et al. 2006, Vendetti 2009, Barco et al. 2010), and therefore precise localities are unknown. Colours used for each Buccinulidae genus correspond to the highlighting of lineages in phylogenetic trees (Figure 3.5, Supplementary Figures 3.4, 3.6).

Taxon	mtDNA *cox1*	mtDNA 16S	rDNA 28S	Voucher ID	Location	GenBank Accession	Source
Antarctoneptunea aurora	Y			MNA0095	Adare Peninsula, Ross Sea		This paper
Antarctoneptunea aurora	Y			MNA0096	Hallet Peninsula, Ross Sea		This paper
Buccinulum linea		Y		NUGB-G2011	Leigh Harbour, Auckland, NZ	AB044256	Hayashi 2005
Buccinulum pertinax pertinax				M.285278/1	Deep Bay, Stewart Island, NZ		This paper
Buccinulum vittatum vittatum				M.285285/3	Ringaringa, Stewart Island, NZ		This paper
Cominella adspersa		Y		NUGB-G2012	Orewa, Auckland, NZ	AB044265	Hayashi 2005
Kelletia kelletii			Y	UCMP-557057	Monterey Bay, California, USA	FJ710099	Vendetti 2009
Kelletia kelletii		Y		NUGB-G2051	Santa Barbara Island, California, USA	AB121037	Hayashi 2005
Kelletia lischkei	Y			KL1	Kansai, Mie Prefecture, Japan		This paper
Kelletia lischkei	Y			KL3	Kansai, Mie Prefecture, Japan		This paper
Kelletia lischkei		Y		NUGB-G2031	Wakasa Bay, Fukui Prefecture, Japan	AB044263	Hayashi 2005
Kelletia lischkei	Y				Yeosu, South Jeolla, South Korea	HM180632	Kim et al. 2012
Kelletia lischkei	Y				Yeosu, South Jeolla, South Korea	HM180633	Kim et al. 2012
Kelletia lischkei	Y				Yeosu, South Jeolla, South Korea	HM180634	Kim et al. 2012
Kelletia lischkei	Y				Yeosu, South Jeolla, South Korea	HM180635	Kim et al. 2012
Kelletia lischkei	Y				Yeosu, South Jeolla, South Korea	HM180636	Kim et al. 2012
Pareuthria campbelli	Y			M.317715	Campbell Island, NZ	KP694137	Donald et al. 2015
Pareuthria campbelli	Y			M.317715	Campbell Island, NZ	KP694138	Donald et al. 2015
Pareuthria campbelli	Y			M.317716	Campbell Island, NZ	KP694139	Donald et al. 2015
Pareuthria fuscata	Y	Y		IM-2009-4613	Ushuaia, Tierra del Fuego, Argentina	FM999174 FM999126	Oliverio and Modica 2010
Penion benthicolus	Y			M.274268	Cape Kidnappers, Hawkes Bay, NZ		This paper
Penion chathamensis		Y		NUGB-G2009	Chatham Rise, NZ	AB044266	Hayashi 2005
Penion sulcatus		Y		NUGB-G2016	New Zealand	AB044267	Hayashi 2005
Buccinum bayani	Y			UCMP-556091	Tokyo, Kantō, Japan*	FJ710068	Vendetti 2009
Buccinum bayani	Y				Tokyo, Kantō, Japan*	FJ710069	Vendetti 2009
Buccinum middendorfi	Y			UCMP-556105	Nagoya, Japan*	FJ710071	Vendetti 2009
Buccinum middendorfi	Y				Nagoya, Japan*	FJ710072	Vendetti 2009
Buccinum opisoplectum		Y		NUGB-G2029	Japan	AB044257	Hayashi 2005

Buccinum pemphigus	Y			LSGB2320301	Bohai Strait, China	HQ834057	Zou et al. 2011a
Buccinum pemphigus	Y			LSGB2320303	Shaungtaizi River Estuary, China	HQ834059	Zou et al. 2011a
Buccinum shenshumaruae			Y	UCMP-556095	Jōetsu, Niigata Prefecture, Japan*	FJ710095	Vendetti 2009
Buccinum tenuissimum			Y	UCMP-556096	Joetsu, Japan*	FJ710096	Vendetti 2009
Buccinum tsubai			Y	UCMP-556097	Jōetsu, Niigata Prefecture, Japan*	FJ712705	Vendetti 2009
Buccinum undatum			Y	BAU-2008004	London, UK*	FN677456	Barco et al. 2010
Buccinum undatum			Y	BMNH-20070640	Rekjanes, Iceland	EU391567	Claremont et al. 2008
Buccinum yokomaruae	Y				Yellow Sea, China	JN052995	Zou et al. 2011b
Buccinum yokomaruae	Y				Yellow Sea, China	JN052996	Zou et al. 2011b
Buccinum yokomaruae	Y				Yellow Sea, China	JN052997	Zou et al. 2011b
Neobuccinum eatoni			Y	IM-2009-4614	Terra Nova Bay, Antarctica	FM999149	Oliverio and Modica 2010
Neptunea arthitica			Y	UCMP-556104	Nagoya, Aichi Prefecture, Japan*	FJ710101	Vendetti 2009
Neptunea constricta			Y	UCMP-556094	Joetsu, Japan*	FJ710102	Vendetti 2009
Neptunea eulimata			Y	UCMP-556098	Wakkanai, Hokkaido, Japan	FJ710103	Vendetti 2009
Neptunea frater			Y	UCMP-556110	Sōma, Fukushima Prefecture, Japan	FJ710104	Vendetti 2009
Neptunea intersculpta		Y		NUGB-G2032	Hokkaido, Japan	AB044265	Hayashi 2005
Neptunea kuroshio			Y	UCMP-556093	Awa-gun, Japan	FJ710101	Vendetti 2009
Neptunea polycostata			Y	UCMP-556108	Sendai, Miyagi Prefecture, Japan*	FJ710107	Vendetti 2009

Molecular phylogenetic analysis and divergence date estimation

All sequence alignments used for phylogenetic analyses were concatenated to remove missing regions and sequence ambiguities. Gblocks 0.91b (Castresana 2000), operating under standard settings was used to eliminate poorly aligned positions and regions with low homology from DNA alignments used for phylogenetic reconstruction. SplitsTree 4 (Huson and Bryant 2006), was used to investigate the unrooted phylogenetic network derived from the DNA sequence alignments used to produce phylogenies in order to examine the structure of the phylogenetic signal. Partitions in sequence data were investigated for protein-encoding, tRNA and rRNA genes. jModelTest 2.1.6 (Guindon and Gascuel 2003; Darriba et al. 2012), was used to statistically identify the best fitting nucleotide substitution model for each gene partition. The generalised time-reversible substitution model (GTR + I + G) (Tavaré 1986), was found to be most appropriate for substitution model for the mtDNA protein-encoding, rRNA and nuclear rDNA sequences, whereas the HKY + I + G model (Hasegawa et al. 1985), was most suitable for the mtDNA tRNA regions. When sequence data were partitioned, these models were applied for unlinked substitution models. Molecular phylogenies were estimated using Bayesian MCMC inference via MrBayes 3.2 (Ronquist et al. 2012), and BEAST 1.8.3 (Drummond *et al.* 2012). Tracer 1.6 (Rambaut *et al.* 2014) was used to evaluate posterior statistics for Bayesian MCMC parameters. Maximum-likelihood phylogenetic trees were also estimated using RAxML 8.2.8 (Stamatakis 2014). Figtree 1.4.2 (Figtree 2016), was used to graphically view and edit tree outputs, and support for phylogenetic nodes was inferred using posterior probability. All phylogenetic reconstruction was processed using CIPRES Science Gateway (Miller *et al.* 2010).

We wanted to estimate the timing of genetic divergences among the putative Buccinulidae taxa, and in particular investigate estimated divergence dates among lineages of *Penion*. Using BEAST 1.8.3, both a sequence alignment of mtDNA from 29 individuals and mtDNA and nuclear rDNA from 27 individuals, were fossil calibrated and used to phylogenetically estimate divergence dates among taxa. Due to the assumptions of model used, for these fossil-calibrated phylogenies only one individual of each putative species was included. The time calibrated phylogenetic analysis was carried out using the lognormal-relaxed clock model (Drummond *et al.* 2006), and the speciation birth-death process tree prior (Gernhard 2008). Priors for calibrations based on fossil data outside of New Zealand were fitted with a normal distribution. Based on

the occurrence of the earliest known buccinoid fossils (Kaim and Beisel 2005), the mean tree root height was estimated to be 165 Ma (SD = 4.0 Ma). Likewise, based on earliest known fossil occurrences of Fasciolariidae (Allison 1955; Tracey *et al.* 1993), we estimated the earliest mean convergence date to be 139.8 Ma (SD = 3.0 Ma).

A recent divergence is also calibrated for our phylogeny, incorporating the earliest known fossil occurrence of the extant species *Buccinulum vittatum vittatum* Quoy & Gaimard, 1833 (3.0 Ma; Beu and Maxwell 1990). This calibration sets a minimum divergence time between the sampled living species *B. v. vittatum* and *B. robustum* in the resulting phylogeny. Following the method of Hills (2010), the prior for this calibration was fitted with a lognormal distribution modelled on estimates of sampling biases in the New Zealand geological record (Crampton *et al.* 2003). This method means that our date estimates for these lineages incorporates measured uncertainty in the fossil record (i.e. whether fossils of a species may occur earlier in time than known under current sampling). Crucially to avoid circularity, no fossil calibrations were used from *Penion* or its immediate sister clades (*Kelletia*, *Antarctoneptunea*, see results). The divergence dates estimated from our phylogenetic trees (using Fasciolariidae and *B. v. vittatum*) are therefore independent of the fossil record of *Penion* (and allies) during subsequent comparisons. The maximum clade credibility tree was generated from BEAST MCMC sampling using TreeAnnotator 1.7.5, and visualised in FigTree 1.4.2.

Results

Sequence data

We assembled new mitochondrial genome sequences from 29 individuals belonging to 27 putative species (Table 3.1). We also assembled new nuclear rDNA sequences (18S, 5.8S, 28S rRNA genes) for 31 individuals belonging to 28 putative species (Table 3.1). In addition, sequences from 15 further individuals for the mtDNA 16S rRNA and *cox1* genes were amplified and Sanger sequenced (Table 3.2). All sequenced mtDNA genomes contained the standard gene complement and order described for previously sequenced neogastropod species (Simison *et al.* 2006, Cunha *et al.* 2009, Hills *et al.* 2011). Mitochondrial genome sequences varied between 15,104 to 15,264 bp in length, and nuclear rDNA sequences varied between 5334 to 5340 bp in length. Statistics concerning sequence length and nucleotide ratios are summarised in Supplementary Tables 3.1 and 3.2.

Most individuals yielded complete mtDNA and rDNA sequence data, however 3 out of 29 specimens had low sequence coverage for regions of mtDNA or rDNA, and therefore the set of taxa and number of individuals varies slightly for trees based on marker (see Table 3.1). One specimen of *P. c. cuvierianus* (Powell, 1927), *B. linea* (Martyn, 1784) and *B. vittatum littorinoides* (Suter, 1913) had low sequencing read coverage for the mitochondrial genome, but all three specimens provided nuclear ribosomal cassette sequences (Table 3.1). The 3' end of the 28S rRNA gene was poorly covered for *Aeneator valedictus* (Watson, 1886) (Table 3.1). Although our estimated sequence scaffolds for the nuclear ribosomal data include internal spacer region 1 (ITS1) and ITS2, these regions were excluded from phylogenetic analysis as individuals contain multiple ITS sequence variants. A third of the nuclear rDNA 18S gene was removed from the 5' end for phylogenetic analyses, as all high-throughput sequenced specimens had reduced read coverage at this region.

Mean pair-wise mtDNA variability across all true whelks (Buccinidae and Buccinulidae) was 22.5%, whereas values within putative Buccinidae and Buccinulidae were 16.6% and 22.6% respectively. This suggests that the sampled, putative Buccinulidae have (on average) more divergent mtDNA genomes than Buccinidae taxa sampled in this study. The three sampled Fasciolariidae species had a mean pair-wise mtDNA variability of 17.5%. At the generic-level, mtDNA mean pair-wise variability was 7.8%, 29.6% and 21.2% for *Aeneator*, *Buccinulum* and *Penion* respectively. Pair-wise mtDNA variability for *Cominella* and *Kelletia* (both n = 2), was 13.9% and 10.7% respectively. Based on the proportion of variable sites per gene, some genes such as ND2 and ND5 convey more phylogenetic information than others such as 16S rRNA at different levels of phylogenetic investigation (see Supplementary Figure 3.1), which agrees with previous results from true whelks (e.g. *Cominella*, Donald *et al.* 2015). Compared to the mtDNA, variation among rDNA sequences was very low (Supplementary Figure 3.1).

Phylogenetic reconstruction

Sequence alignments used for phylogenetic reconstruction had gaps and ambiguous nucleotides manually removed for the regions and specimens mentioned above. For mtDNA sequences, gblocks retained 97% of the original mtDNA protein-encoding nucleotide positions, and 61% and 76% of the mtDNA tRNA and rRNA positions respectively. This analysis resulted in sequence lengths of 9251, 983 and 894

bp respectively for mtDNA protein-encoding, tRNA and rRNA sequence regions. 99% of the nuclear rDNA nucleotide positions were also retained, leaving an alignment sequence length of 4667 bp available for phylogenetic reconstruction.

The phylogenetic relationships inferred from mitochondrial and nuclear ribosomal markers are broadly similar, and both reveal that Southern Hemisphere whelks (Buccinulidae) are paraphyletic with Northern Hemisphere (Buccinidae) taxa (Figures 3.2 and 3.3). Results also indicated that New Zealand true whelks are not monophyletic (Figures 3.2 and 3.3). Bayesian and maximum-likelihood derived phylogenies were similar (Figures 3.2 and 3.3, Supplementary Figures 3.4 and 3.5). Phylogenies did exhibit a significant difference for the evolutionary relationship of *Aeneator* and *Buccinulum* (Figures 3.2 and 3.3); the mitochondrial data suggested a sister relationship with *Penion,* whereas nuclear markers suggested a sister relationship with a clade of southern and northern true whelk genera. Relationships between some closely related taxa also differed between phylogenies (e.g. *P. c. cuvierianus* and *P. chathamensis* (Powell, 1938); Figures 3.2 and 3.3).

A likely explanation for the difference between mtDNA and nuclear rDNA phylogenetic trees is that there is less phylogenetic information available from the nuclear rDNA sequence data. Based on the proportion of variable sites per gene, sequence variation exhibited for the nuclear rDNA 18S, 5.8S and 28S rRNA genes was small (see Supplementary Figure 3.1). The final sequence length used for rDNA (4773 bp) is much shorter than the total length of sequence alignments used for mtDNA phylogenetic reconstruction (11,363 bp), and rDNA is more conserved. When the mtDNA and nuclear rDNA sequence alignments are investigated as a splits network to investigate all possible phylogenetic relationships among specimens (Supplementary Figures 3.2 and 3.3), it is apparent that the phylogenetic signal is much more constrained within the mtDNA than rDNA sequence data. Specifically, the shorter branch lengths in the rDNA data (Supplementary Figure 3.3) indicate smaller genetic distances among specimens, and the box structures shown between many taxa (especially *Aeneator* and *Buccinulum*; Supplementary Figure 3.3) for rDNA indicate that many alternative relationships are possible. In contrast for the mtDNA sequence data, most relationships are similar, almost every genus can be separated with a single incompatible split, and the branch lengths between individuals are large (Supplementary Figure 3.2). The area with the most possible splits for the mtDNA sequence alignment focussed on our sampling of Nassariidae and Fasciolariidae (Supplementary Figure 3.2),

where there is low Bayesian posterior probability support on our phylogenetic tree (Figure 3.2). When a phylogeny was produced using both mtDNA and nuclear rDNA sequence data (Figure 3.4), the inferences were dominated by the phylogenetic signal present in the mitochondrial genomic data.

Three additional phylogenetic trees were inferred from short-length sequence data from the rDNA 28S (Supplementary Figure 3.6), mtDNA *cox1* (Figure 3.5), and 16S rRNA (Supplementary Figure 3.8) fragments. Sequences were concatenated to remove ambiguous bases. Aligned sequence lengths were 1486, 502 and 261 bp for 28S, *cox1* and the 16S respectively. The *cox1* and 16S rRNA genes present similar relationships to the overall mtDNA tree (Figures 3.3, 3.5, Supplementary Figure 3.8), whereas the rDNA 28S rRNA tree exhibits a similar topology to the overall rDNA tree (Figure 3.4, Supplementary Figure 3.6). These trees indicate that neither New Zealand nor Buccinulidae true whelks are monophyletic, and the mtDNA *cox1* tree indicated that *P. benthicolus* Dell, 1956 is sister to *A. aurora* (Figure 3.5).

FIGURE 3.2
An mtDNA phylogeny demonstrating paraphyletic relationships of Northern and Southern Hemisphere whelks. The Bayesian phylogeny is based on an alignment of 31 concatenated mitochondrial genome sequences (incorporating protein-encoding, tRNA and rRNA genes). Two sequence partitions were used: 1) protein-encoding and rRNA genes (10,145 bp), and 2) tRNA genes (983 bp) using the GTR + I + G and HKY + I + G substitution models respectively. The phylogeny was generated using BEAST 1.8.3 with an MCMC length of 100 million generations, sampling every 1000 with a 10% burn-in. Node posterior support values are given, but only if support was less than 1.0. Genera putatively belonging to Buccinulidae are shown in different colours, and the geographic origin of specimens between the Northern and Southern hemispheres is listed on the right.

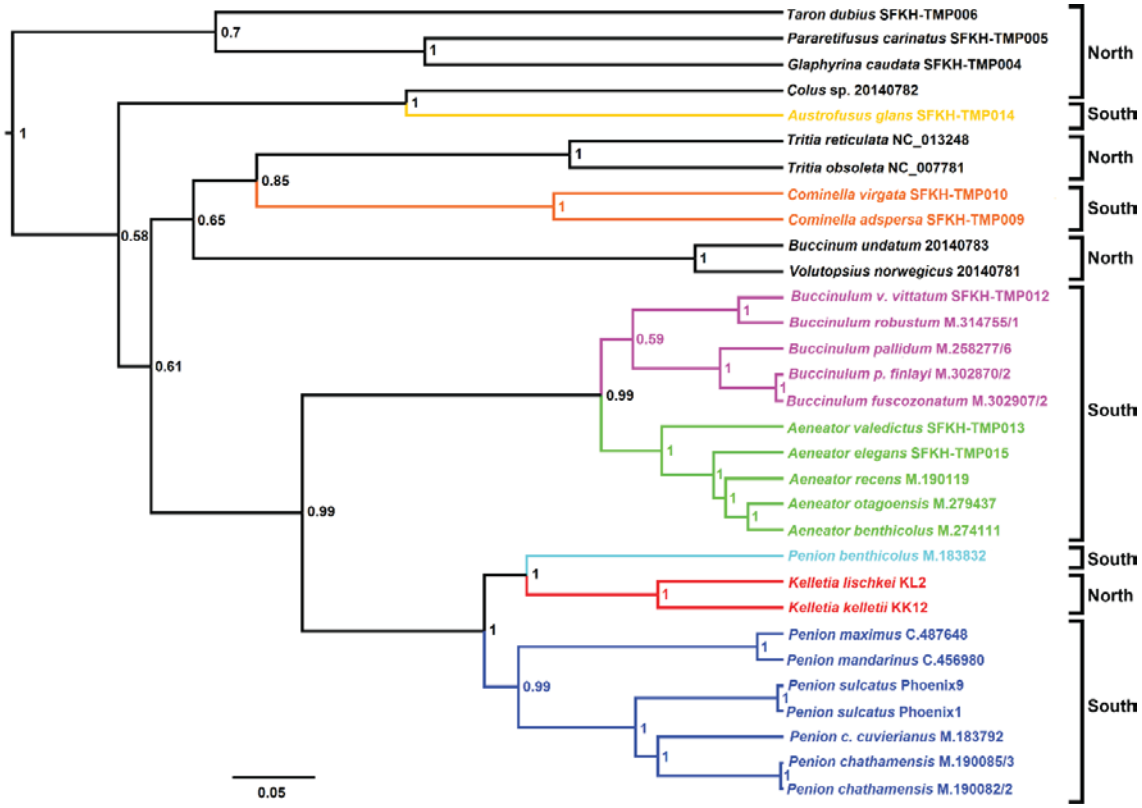


FIGURE 3.3
A nuclear 45S rDNA phylogeny demonstrating paraphyletic relationships of Northern and Southern Hemisphere whelks. The Bayesian phylogeny is based on a 4667 bp alignment of 31 concatenated nuclear rDNA gene sequences (18S, 5.8S, 28S rRNA). Sequence data was not partitioned and the GTR + I + G substitution model was used. Reciprocal monophyly was enforced for the Fasciolariidae (*Glaphyrina caudata, Pararetifusus carinatus, Taron dubius*) and for the Buccinidae/Buccinulidae taxa. BEAST 1.8.3 using and MCMC length of 100 million, 1000 sample frequency and a 10% burn-in was used to generate this phylogeny. Posterior support values are also shown at nodes, but only if support was less than 1.0. Genera putatively belonging to Buccinulidae are shown in different colours, and the geographic origin of specimens between the Northern and Southern hemispheres is listed on the right.

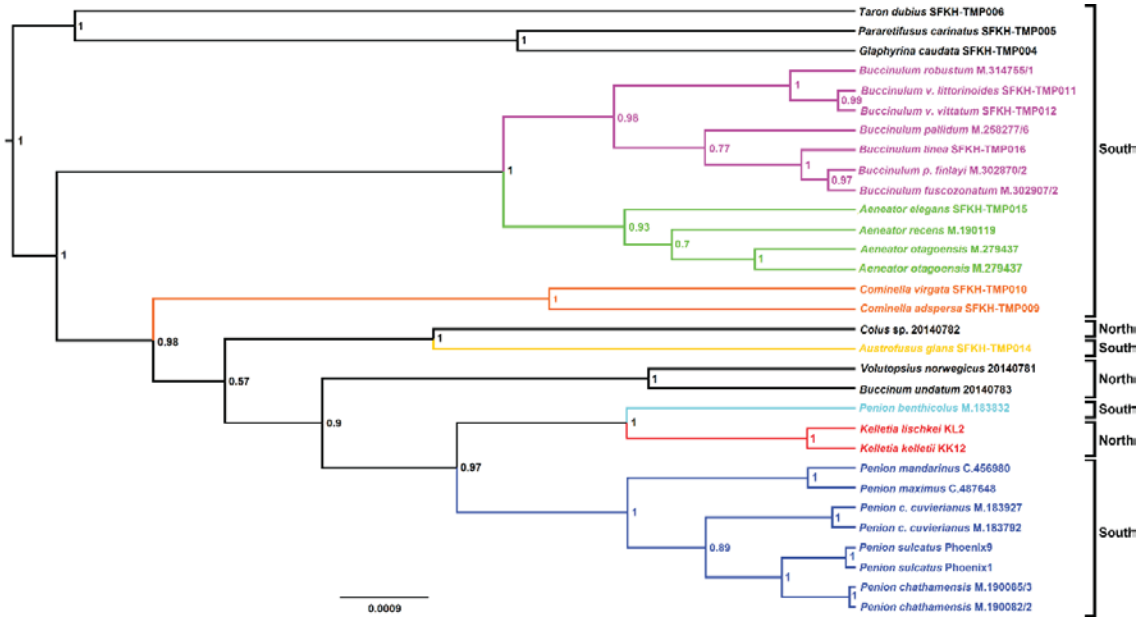


FIGURE 3.4a

A Bayesian phylogeny based on an alignment of 27 concatenated mitochondrial genome (incorporating protein-encoding, tRNA and rRNA genes) and nuclear ribosomal rRNA 18S, 5.8S and 28S sequences, which has been fossil calibrated to estimate divergence dates among the whelk lineages. The entire phylogeny is shown in A), whereas B) focusses on the divergence dates estimated for *Penion* and *Kelletia*, with comparison to the partial fossil record of the clade (shading shows estimated time range for referenced fossil taxa), with photos of extant shells and fossils for illustration. Black stars indicate splits that fossil calibrated. Two sequence partitions were used: 1) mtDNA protein-encoding and rRNA genes and nuclear rDNA genes (14,812 bp), and 2) tRNA genes (894 bp) using the GTR + I + G and HKY + I + G substitution models respectively. Fossil dates used to calibrate the tree originated from the earliest known buccinoid fossils (tree root height), earliest Fasciolariidae (un-enforced outgroup), and the earliest known occurrence of the tip branch *Buccinulum vittatum*. BEAST 1.8.3 using and MCMC length of 100 million, 1000 sample frequency and a 10% burn-in was used to generate this phylogeny. Node labels are estimated median divergence dates with the 95% highest posterior density (HPD) range shown as a horizontal bar (grey in a), yellow in b)). Posterior support values are also shown at nodes, but only if support was less than 1.0. Genera of putative Buccinulidae are shown in different colours.

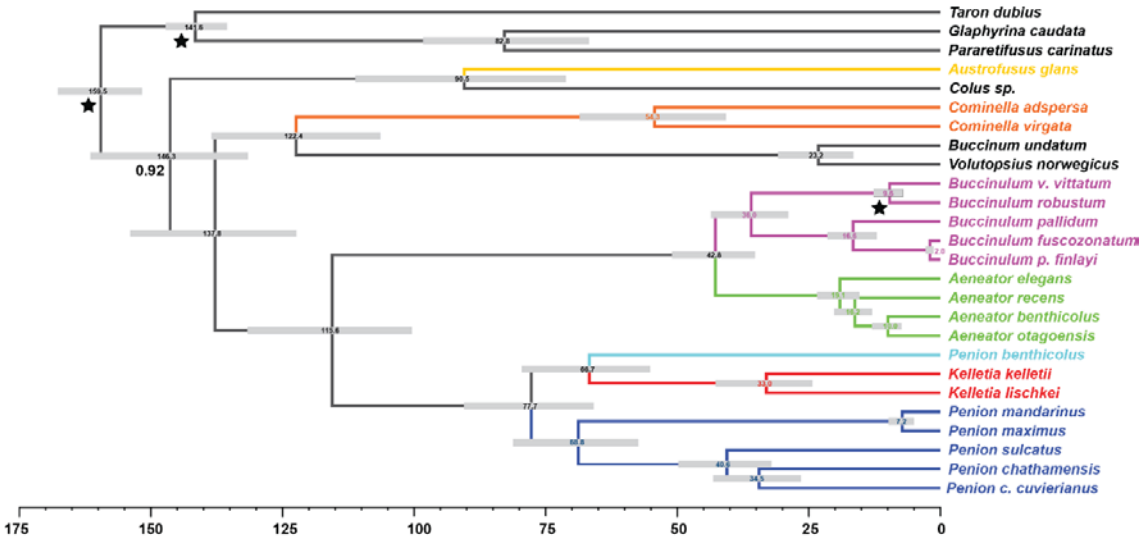


FIGURE 3.4b

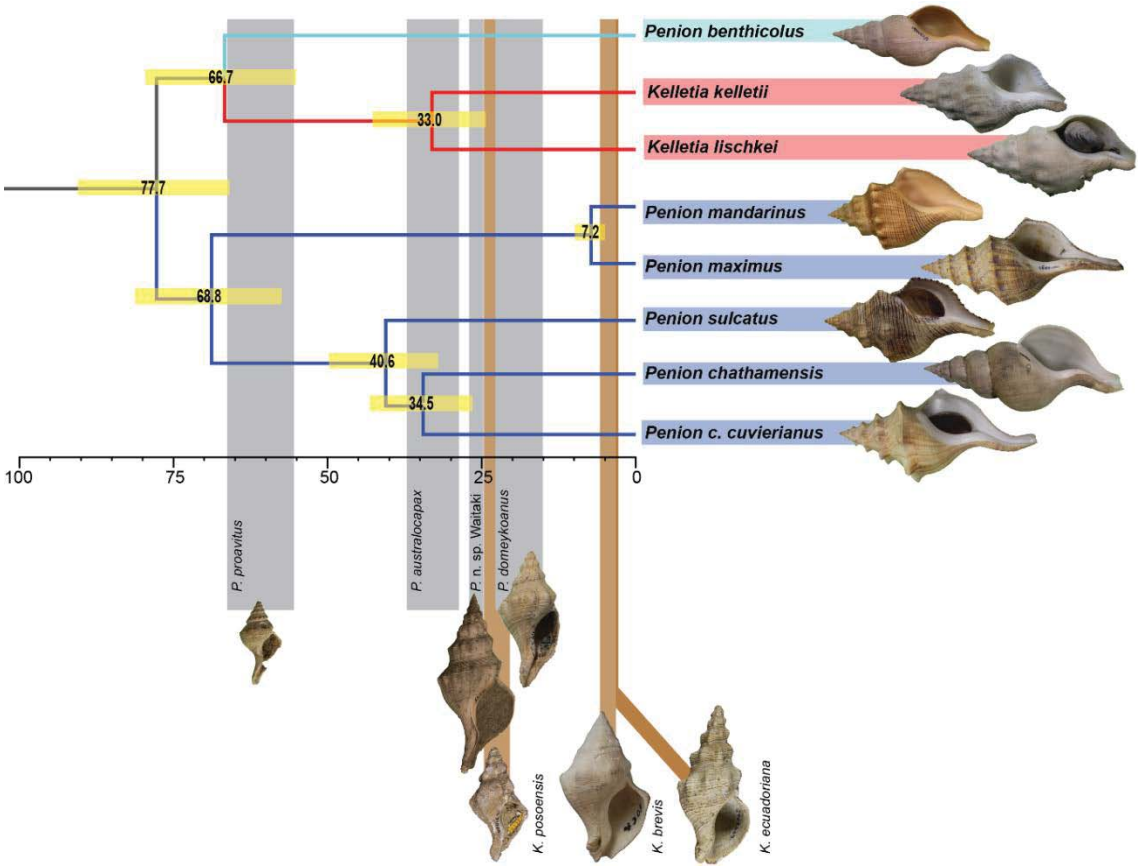
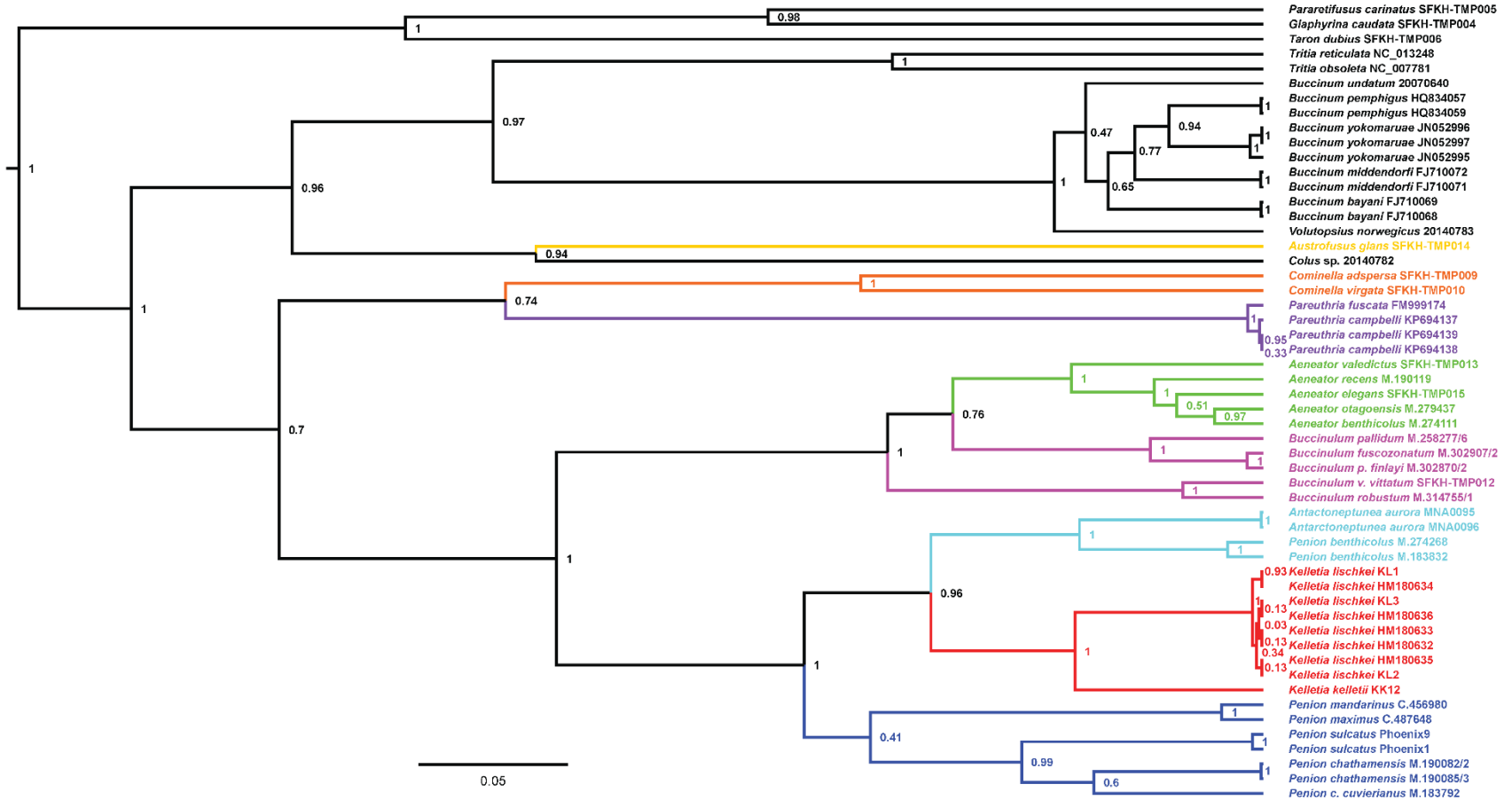

Penion benthicolus
Kelletia kelletii
Kelletia lischkei
Penion mandarinus
Penion maximus
Penion sulcatus
Penion chathamensis
Penion c. cuvierianus
66.7
33.0
77.7
7.2
68.8
40.6
34.5
100
75
50
25
0
P. proavitus
P. australocapax
P. n. sp. Waitaki
P. domeykoanus
K. posoensis
K. brevis
K. ecuadoriana

FIGURE 3.5
A Bayesian phylogeny based on a 439 bp alignment of mitochondrial *cox1* gene sequences obtained from 54 individual marine snails. The GTR + I + G substitution model was used. The phylogeny was produced using a Bayesian method (100 million MCMC, 10% burn-in, 1000 sample frequency, node labels are posterior support values), via BEAST 1.8.3. For this tree no outgroup was specified explicitly but reciprocal monophyly was enforced for the Fasciolariidae and Buccinidae/Nassariidae. Genera putatively belonging to Buccinulidae are shown in different colours.



Divergence date estimation

We estimated divergence dates among extant taxa by fossil calibrating a combined mtDNA and rDNA sequence phylogeny (Figure 3.4), and an mtDNA sequence phylogeny (Supplementary Figure 3.7). The mtDNA and rDNA phylogeny was based on an alignment of 27 sequences, with two partitions: 1) mtDNA protein-encoding and rRNA genes and nuclear rDNA genes (15,891 bp), and 2) tRNA genes (1065 bp) using the GTR + I + G and HKY + I + G substitution models respectively. The mtDNA only phylogeny used 25 sequences, again with two partitions: 1) protein-encoding and tRNA genes (10,635 bp), and 2) tRNA genes (1065 bp) using the GTR + I + G and HKY + I + G substitution models respectively. Based on posterior outputs, we were able to successfully calibrate these trees using earliest known fossil occurrences for *Buccinulum v. vittatum*, Fasciolariidae and the earliest known buccinoidean fossil. 95% highest posterior density ranges for estimated divergence dates do not differ substantially between the two phylogenetic trees (Figure 3.4, Supplementary Figure 3.7), likely due to the dominance of phylogenetic information from mtDNA sequence data.

Posterior results also indicated that the inclusion of a calibration for the earliest occurrence of Nassariidae (estimated at 66.0 Ma, SD = 5.0 Ma; Palmer and Brann 1965, Haasl 2000, Sessa and Patzkowsky 2009), did not have a significant impact upon our results. This calibration may not have had a significant impact because only two mtDNA sequences from *Tritia reticulata* and *Tritia obsoleta* were sampled. Alternatively, this calibration may have little impact because our phylogenies find Nassariidae to be paraphyletic with Buccinidae/Buccinulidae (Figure 2, Supplementary Figure 3.7). This finding corroborates previous molecular (Hayashi 2005, Galindo *et al.* 2016), and morphological findings (Haasl 2000).

Discussion

Evolution of Southern Hemisphere and New Zealand true whelks

All phylogenies in this study imply paraphyly for both Southern Hemisphere (putative Buccinulidae) and New Zealand true whelks (Figures 3.2 – 3.5, Supplementary Figures 3.4 – 3.6). Although we have not sampled all New Zealand true whelks, and only a small proportion of species classified within Buccinulidae, it is apparent that neither group is monophyletic as taxa form clades with Buccinidae species. For long-length sequence data, the closest sampled relatives of *Cominella* appear to be the Northern Hemisphere taxa *Buccinum undatum* Linnaeus, 1785 and *Volutopsius*

norwegicus (Gmelin, 1791), and likewise *Austrofusus glans* (Röding, 1798) is more closely related to the *Colus* specimen sampled from the North Sea than to any of the sampled Southern Hemisphere taxa (Figures 3.2 and 3.3). Short-length sequence data implies that *Cominella* and *Pareuthria* are sister (Figure 3.5 and Supplementary Figure 3.8), however most phylogenetic results indicate that this clade is more closely related to Northern Hemisphere taxa than putative Buccinulidae (Figures 3.2 and 3.3, Supplementary Figure 3.8).

Only a subgroup of the sampled, putative Buccinulidae are monophyletic, with *Aeneator, Antarctoneptunea, Buccinulum, Kelletia* and *Penion* appearing to be closely related (based on mtDNA and nuclear rDNA evidence). However, it is entirely possible than an unsampled Northern Hemisphere snail lineage may also be nested within this clade (in addition to *Kelletia* north of the equator). If the short DNA fragments provide correct phylogenetic relationshops (Supplementary Figure 3.6), the Northern Hemisphere genus *Neptunea* is not closely related, despite previous studies noting the morphological and ecological similarities with *Penion* and *Antarctoneptunea* (Ponder 1973, Dell 1972). If it was desired to retain the Buccinulidae classification, purely as a taxonomic rank – then this group of species appears most appropriate, however any description of the clade would be wise to avoid biogeographic reasoning. As noted above, *Kelletia* is distributed in the Northern Hemsiphere and might represent a dispersal event from this otherwise Southern Hemsiphere restricted group (Powell 1929, Powell 1951, Ponder 1973). This group therefore inherently challenges an assumption of isolation in the Southern Hemisphere. Likewise, the extant distribution of these taxa also does not support the assumption of biogeographic isolation for New Zealand true whelks, as *Antarctoneptunea* is restricted to the polar circle of Antarctica, and extant species of both *Penion* (Ponder 1973), and *Aeneator* (McLean and Andrade 1982, Araya 2013), occur outside of New Zealand. Fossils of *Penion* also are documented from Australia (Ponder 1973), Chile and Argentina (Ponder 1973, Frassinetti 2000, Nielsen 2003, Parras and Griffin 2009, Reichler 2010), and Antarctica (Beu 2009), and similarly fossil species of *Kelletia* are known from Ecuador (Olsson 1964), as well as from the extant locations of the USA (Arnold 1910, Anderson and Martin 1914, Kanakoff 1954, Addicott 1970, Hertlein 1970), and Japan (Ozaki 1954).

The key implication of this phylogenetic analysis therefore is that the assumptions of geographic isolation and a separate evolutionary radiation in the Southern Hemisphere are not valid for true whelks. The occurrence of multiple, separate

lineages in New Zealand implies that true whelks do not find it difficult to transgress large distances over evolutionary time. As in other marine molluscs, these findings indicate that dispersal is common on an evolutionary timescale, even in lineages that undergo direct development (e.g. Donald *et al.* 2005, Huelsken *et al.* 2013, Cumming *et al.* 2014, Donald *et al.* 2015). New Zealand may be geographically remote enough to cause an increased rate of endemism in benthic marine snails, but on an evolutionary time-scale over millions of years the islands are clearly not so isolated as to prevent migration. This finding corresponds with many studies of terrestrial fauna (e.g. Battley 1997, Trewick 2000, Goldberg *et al.* 2008). Studies of other marine molluscs have demonstrated that a high rate of endemism, as observed in genera such as *Aeneator*, *Cominella* and *Penion*, is not mutually exclusive with dispersal ability (e.g. Huelsken *et al.* 2013). It is recommended that Buccinulidae (and alternative representations) is retired as an alternative classification of many true whelks, and instead Buccinidae should be retained as the family classification.

Observations regarding Buccinidae and Fasciolariidae

Recent taxonomic summaries of Buccinidae (e.g. Bouchet *et al.* 2005), have suggested that *Buccinum* and *Volutopsius* reside within the separate tribes of Buccinini and Volutopsini respectively. However, the relatively small genetic distance (only 0.44% and 2.30% pair-wise variability for rDNA and mtDNA respectively) and recent divergence time suggested by our phylogenetic analysis suggests otherwise (Figure 3.4, Supplementary Figure 3.7). A previous assessment of soft-body and radula morphology hypothesised that *Penion* represent an early split among Buccinidae (Harasewych 1990), but this instead may be example of plesiomorphy or convergence.

The sampled Fasciolariidae taxa used in our phylogenies (*Glaphyrina caudata, Pararetifusus carinatus, Taron dubius*), are consistently monophyletic and sister to all other sampled taxa (Figure 2, 4, Supplementary Figure 3.7). This monophyly agrees with recent research that samples most subclades of the family (Couto *et al.* 2016). Fasciolariidae was also indicated to be sister clade of Buccinidae/Nassariidae, again in concordance with morphological data (Kosyan *et al.* 2009).

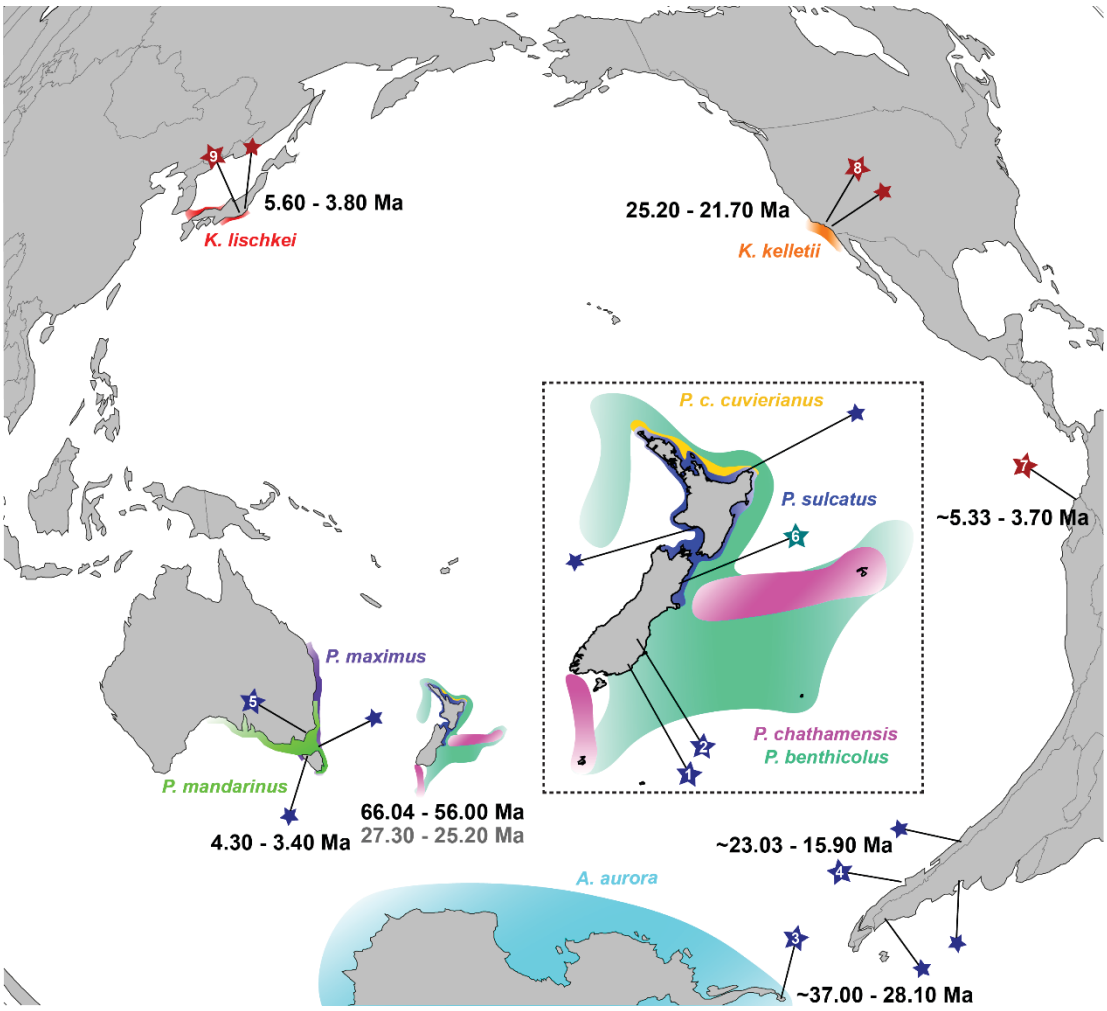
Penion benthicolus and *Antarctoneptunea*

Our molecular phylogeny indicates that *Penion* and *Kelletia* are closely related (Figures 3.2 – 3.5, Supplementary Figures 3.4 – 3.6). This result agrees with the

previous mitochondrial 16S rRNA gene phylogeny produced by Hayashi (2005), and it concurs with earlier hypotheses based on shell morphology and soft-body anatomy (Powell 1929, Wenz 1941, Ponder 1973, Stilwell and Zinsmeister 1992), and previous taxonomic confusion of the genera (Palmer and Brann 1965). In addition, our phylogenetic evidence also indicates that *P. benthicolus* is paraphyletic to other *Penion*, forming a clade with *Antarctoneptunea aurora* (Figure 3.5). Since its discovery, the evolution and classification of *A. aurora* has puzzled molluscan taxonomists (Dell 1972). Morphological comparisons have been made to *Penion* (Dell 1972). Conversely the radula morphology (Dell 1956), and small shell size of *P. benthicolus* has been noted to be unusual within *Penion* (Powell 1979). A comparison of the shells of *P. benthicolus* and *A. aurora* clearly demonstrates the similarity of the two species (Figure 3.6). Of note, both taxa exhibit a proportionately large, beehive-shaped protoconch (Figure 3.6), and occur at deep water depths in subantarctic waters (Dell 1956, Dell 1972; Figure 3.7). Although genetic evidence is limited (477 bp of *cox1* from two individuals of *A. aurora*), we recommend that the species are treated as sister and that *P. benthicolus* is reclassified as *A. benthicola* (Dell, 1956) (henceforth referred to as such).

FIGURE 3.6
A comparison of *P. benthicolus* and *Antarctoneptunea aurora* shells. A: M.274268 [MNZ] *P. benthicolus* from off Cape Kidnappers, 815 m, it should be noted that the last teleoconch whorl is broken; B: M.118756 [MNZ] *P. benthicolus* from east of Auckland Islands, 390 – 400 m; C: M.242882 [MNZ] *A. aurora* from the Ross Sea, 494 – 498 m; D: M.059741 [MNZ] *P. benthicolus* from Hikurangi Trench, 1549 – 1723 m.

FIGURE 3.7
A map showing the extant distributions of *Antarctoneptunea* (*A. aurora* in cyan, *P. benthicolus* in mint green), *Kelletia* (*K. lischkei* Kuroda, 1938 in red, *K. kelletii* (Forbes, 1850) in orange), and *Penion* (*P. chathamensis* in pink, *P. c. cuvierianus* in yellow, *P. mandarinus* (Duclos, 1832) in green, *P. maximus* (Tryon, 1881) in purple, *P. sulcatus* (Lamarck, 1816) in blue). The map also marks the location of key fossils referred to within the discussion: 1) *P. proavitus* from Wangaloa, Otago (66.04 – 56.00 Ma); 2) *P.* n. sp. Waitaki from Lake Waitaki, Canterbury (27.3 – 25.2 Ma); 3) *P. australocapax* from Seymour Island, Antarctic Peninsula (approximately 37.0 – 28.1 Ma); 4) *Penion* spp. from numerous locations in Chile and Argentina (approximately 23.03 – 15.90 Ma); 5) *P. mandarinus* from Kalimna, Victoria (4.3 – 3.4 Ma); 6) *P. benthicolus* from Oaro, Canterbury (2.40 – 1.63 Ma); 7) *K. ecuadoriana* Olsson, 1964 and *K. rugosa* Olsson, 1964 from Esmeraldas, Ecuador (approximately 5.33 – 3.70 Ma); 8) *K. posoensis* from San Luis Obispo County, California (25.2 – 21.7 Ma); 9) *K. brevis* from Cape Inuwaka, Chiba Prefecture (5.6 – 3.8 Ma). The colour of fossil markers reflects putative classification (*P. benthicolus* in dark green, *Kelletia* in burgundy, *Penion* in navy blue). Markers without numbers show the location of further fossil sites not discussed within the text. The age estimates shown are the earliest known fossil occurrences of the clade within each region (Antarctica, Argentina and Chile, Australia, Japan, New Zealand, USA).



Concordance of molecular derived dates and fossil evidence, and the evolution of *Antarctoneptunea*, *Kelletia* and *Penion*

It seems likely that the common ancestor of the monophyletic *Antarctoneptunea*, *Kelletia* and *Penion* clade evolved in the Southern Hemisphere, most likely on the Zealandian continental shelf or in Southern Ocean around 76 million years ago (based on occurrences discussed below and the occurrence of the related taxa *Aeneator* and *Buccinulum* in New Zealand). The divergence dates estimated from molecular phylogenies using fossil calibrations from independent lineages (*Buccinulum*, Fasciolariidae; Figure 3.4, Supplementary Figure 3.7) show close concordance with the documented fossil record of *Penion* and *Kelletia*. The earliest occurrences within regions also hint at the possible route of dispersal for the clade. We hypothesise that the repeated loss of the planktonic larval phase has shaped the observed phylogeny and affected dispersal ability, biogeography, and the potential for gene flow and speciation for the clade.

The earliest known fossil belonging to the clade is *P. proavitus* (Finlay & Marwick, 1937) from 66.0 – 55.80 Ma in New Zealand (Figure 3.7 label 1; Beu and Maxwell 1990, Beu *et al.* 1997), but the type specimens of the species are juveniles and the only known adult specimen is poorly preserved (Finlay and Marwick 1937). Based on the molecular phylogenetic divergence dates estimated (Figure 3.4), we suggest that this fossil species may represent a crown lineage of either the entire clade (median divergence date 77.77 Ma; Figure 3.4) or monophyletic *Penion* (median 68.84 Ma; Figure 3.4). The next-earliest known Australasian fossils are *P.* n. sp. Waitaki and *P.* n. sp. Waimumu from 27.3 – 25.2 Ma, again from New Zealand (Figure 3.7 label 2; pers. comm. Alan G. Beu, GNS Science 2016). These fossils occur later the estimated period of divergence for New Zealand and Australian *Penion*, and occur within the range estimated for the split of *P. chathamensis* and *P. c. cuvierianus* (median 34.51 Ma; 95% HPD 43.30 – 26.78 Ma; Figure 3.4). The Antarctic fossil species *P. australocapax* (Stilwell & Zinsmeister, 1992) occurs slightly earlier, dated to approximately 37.0 – 28.1 Ma, but the chronostratigraphy for the region is also less certain (Figure 3.7 label 3; Stilwell and Zinsmeister 1992, Beu 2009). This fossil range does however overlap with the estimated period of divergence for speciation among the genetically sampled New Zealand *Penion* (Figure 3.4). Afterwards, numerous fossils classified as *Penion* are documented from Argentina and Chile (Frassinetti 2000, Nielsen 2003), the earliest of which are dated approximately to 23.03 – 15.9 Ma (stratigraphy uncertain; Figure 3.7

label 4), or potentially to 20.43 – 15.97 Ma (more reliable stratigraphy; Reichler 2010). These fossils occur later than the period of divergence estimated for New Zealand *Penion* (median 40.62 Ma; 95% HPD 50.04 – 32.53 Ma; Figure 3.4). The earliest, reliable fossils of Australian *Penion* first occur 4.3 – 3.4 Ma (Figure 3.7 label 5; Ponder 1973), which is also close to (but not technically within) the date range predicted from the phylogeny (median 7.25 Ma; 95% HPD 9.86 – 5.05 Ma; Figure 3.4). Other Australian taxa that have been classified as *Penion* do occur much earlier, but these fossils are highly divergent in shell morphology, and likely represent unrelated Buccinidae or Fasciolariidae (Ponder 1973; Chapters 7 and 8).

The earliest known fossils of *Kelletia* belong to *K. posoensis* (Anderson & Martin 1914), dated to 25.2 – 21.7 Ma from California within the distribution of extant *K. kelletii* (Forbes, 1850) (Figure 3.7 label 8; Anderson and Martin 1914, Addicott 1970), which occurs within the estimated period of divergence for the split between *K. lischkei* Kuroda, 1938 and *K. kelletii* (median 33.08 Ma; Figure 3.4). In addition, later fossil species of *Kelletia* are also known from Ecuador, dated approximately to the 5.33 – 3.7 Ma (stratigraphy uncertain; Figure 3.7 label 7; Olsson 1964). Previously these fossils were hypothesised to represent a southward migration of *Kelletia* from California (Lindberg 1991), but instead it now seems plausible that these species descended from lineages that migrated northward from the Southern Hemisphere. A similar dispersal route is hypothesised for *Haliotis* Linnaeus, 1758 abalone (Bester-van der Merwe *et al.* 2012). The earliest known fossils of *Kelletia* in Japan belong to *K. brevis* Ozaki, 1954 from 5.6 – 3.8 Ma (Figure 3.7 label 9; Ogasawara 2002, Wade *et al.* 2011, Shiba *et al.* 2012), which is compatible with the estimated period of divergence between the two extant *Kelletia* lineages (Figure 3.4). It is possible though that the fossil record for *K. lischkei* and presumed close, extinct relatives is incomplete as modern populations occur on rocky substrates within coastal waters (Hwang *et al.* 2014), an environment that is variably represented in the marine fossil record (Crampton *et al.* 2003), with preservation rates affected by lithology (Foote *et al.* 2015). This scenario seems likely, given that the earliest fossil occurrence of *K. lischkei* itself is from only 0.13 Ma (Ogasawara 2002).

Antarctoneptunea aurora has no documented fossil record, but *A. benthicola* is represented in the New Zealand fossil record from 2.4 Ma (Figure 3.7 label 6; Beu and Maxwell 1990). However, the fossil record of this species is unlikely to represent the origin of *Antarctoneptunea* as deep-water localities are sparsely represented in the New

Zealand fossil record (Crampton *et al.* 2003; e.g. Beu 1979). It has been suggested though that *P. australocapax* from the Antarctic Peninsula (within the range of extant *A. aurora*) may be a misclassified species of *Antarctoneptunea* (Beu 2009).

Given the wide distribution of extant species and fossils (Figure 3.7), the evolution of *Antarctoneptunea*, *Kelletia* and *Penion* implies that this group of true whelks has been able to disperse over very large distances. The Buccinulidae hypothesis of geographic isolation is clearly incorrect for this clade. It also seems incorrect to interpret the fossil record of *Kelletia* in isolation of *Penion* and *Antarctoneptunea*, and the previous prediction of migration of *Kelletia* from the Northern Hemisphere is unlikely (Lindberg 1991). Considering the rich fossil record for this clade across the Pacific (e.g. Ozaki 1954, Olsson 1964, Addicott 1970, Ponder 1973, Beu and Maxwell 1990, Nielsen 2003, Beu 2009), *Antarctonetpunea*, *Kelletia* and *Penion* represent a useful system for future investigations of speciation and long-distance dispersal in marine invertebrates.

Development and dispersal of *Antarctoneptunea*, *Kelletia* and *Penion*

The *Antarctoneptunea*, *Kelletia* and *Penion* clade appears to exhibit a mixture of developmental strategies (Supplementary Figure 3.9). *Kelletia* exhibit indirect development with facultative planktotrophic larvae (Rosenthal 1970, Ponder 1975, Zacherl *et al.* 2003a; Vendetti 2009), whereas *A. benthicola* and *A. aurora* are believed to be direct developers based on their very large protoconchs (Dell 1956, Dell 1972; Supplementary Figure 3.9). In the monophyletic *Penion* clade, developmental biology is less certain and experimental studies are required. Modern New Zealand *Penion* all exhibit the size and morphology of protoconchs and eggs that is consistent with direct development (Ponder 1973, Powell 1979, Beu *et al.* 1997; Supplementary Figure 3.9), however Australian species exhibit small protoconchs, leading to speculation that at least *P. mandarinus* (Duclos, 1832) may undergo indirect development (Ponder 1973; Supplementary Figure 3.9). In addition, some New Zealand fossil species (Beu *et al.* 1997; Supplementary Figure 3.9), and all fossil *Penion* from Chile and Argentina exhibit small protoconchs akin to those possessed by modern Australian taxa (Beu *et al.* 1997, Nielsen 2003).

Indirect development with lecithotrophic larvae is believed to be the ancestral state of Gastropoda (Ponder and Lindberg 1997, Nützel 2014), but following the innovation of internal fertilisation, most Caenogastropoda have subsequently evolved

planktotrophic larvae or direct development (Nützel 2014). The transition from indirect to direct development is likely to be one-way due to the physiological connection to internal fertilisation and brooding (Nützel 2014), analogous to the technical challenge that would be faced to revert from placental development to egg-laying in mammals. It is therefore likely that the ancestral state of the *Penion, Kelletia* and *Antarctoneptunea* clade was also indirect development with planktotrophic development, and that independent lineages of *Antarctoneptunea* and monophyletic, modern New Zealand *Penion* have transitioned to direct development. If Australian *Penion* do in fact exhibit direct development, *Penion* could still have dispersed across the Tasman Sea via egg rafting, even despite the counter-current of the Tasman Front (see Figure 3.7). This scenario is very similar to the hypothesised dispersal of direct-developing *Cominella* from waters off Zealandia to Australia (Donald *et al.* 2015). Similarly the broad distribution of *Antarctoneptunea aurora*, despite the species likely being a direct developer, could be facilitated via egg rafting in the Antarctic Circumpolar Current within recent geological time (see Figure 3.7).

The two extant species of *Kelletia* occur a vast distance apart, from the Sea of Japan to the coast of Baja California, Mexico (Hayashi 2005). This distribution closely aligns to the North Pacific Gyre (see Figure 3.7), and both regions provide similar environments for molluscs (Hall 1964). Before even switching to planktotrophy, *K. kelletii* larvae can feed on yolk reserves for 18 days (Vendetti 2009). Such an adaptation could well have allowed a common ancestor to disperse over such a large distance, and notably *K. kelletii* been capable of swift range extension due to its developmental strategy (Zacherl *et al.* 2003b). In all cases above, the survival of larvae would probably be exceptional with or without relevant adaptations, but the occurrence is not necessarily rare on the timescale of biological evolution.

Overall, as with other true whelks in New Zealand, the relationship of *Antarctoneptunea, Kelletia* and *Penion* indicates that we should not ignore the potential for long-distance dispersal in benthic, marine gastropods. It seems likely that direct- as well as indirect-developing lineages have been able to disperse across significant distances, challenging the traditional assumption of limited potential for dispersal in direct-developing snails (see discussion in Johannesson and Johannesson 1995, Hendricks 2012, Donald *et al.* 2015). Although phenotypic convergence remains most probable to explain similarities in the shell morphology of snails divided by large distances, relatedness is a possibility. The classification of *A. aurora* puzzled

taxonomists (Dell 1972), arguably because a broader geographic scale was not considered. Perhaps the classification of some fossils in the North Atlantic as *Penion* or *Kelletia* (sometimes *Boreokelletia* Anderson, 1964; e.g. Palmer and Bran 1965, Anderson 1973, Gilbert 1973, Kollmann and Peel 1983, CoBabe and Allmon 1994, Moths and Albrecht 2010), is not as incongruous as it first appears.

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Supplementary Data for Chapter Three

Supplementary Tables

SUPPLEMENTARY TABLE 3.1
A summary of statistics for the length and nucleotide composition for the concatenated DNA sequences for the nuclear ribosomal RNA genes 18S, 5.8S and 28S (the internal transcribed spacer regions are not included). All listed specimens were newly sequenced for this study.

		Concatenated nuclear rDNA 18S, 5.8S, 28S					
Species	**Museum ID**	**Length (bp)**	**% A**	**% C**	**% G**	**% T**	**GC bias**
Pararetifusus carinatus	SFKH-TMP005	5337	23.3	24.5	30.0	22.2	54.5
Glaphyrina caudata	SFKH-TMP004	5339	23.3	24.5	30.0	22.2	54.5
Taron dubius	SFKH-TMP006	5339	23.3	24.7	30.1	22.0	54.8
Austrofusus glans	SFKH-TMP014	5338	23.4	24.4	30.0	22.2	54.4
Colus sp.	20140782	5334	23.4	24.5	30.0	22.2	54.5
Volutopsius norwegicus	20140781	5338	23.4	24.4	30.0	22.3	54.4
Buccinum undatum	20140783	5339	23.4	24.3	30.0	22.3	54.3
Cominella adspersa	SFKH-TMP009	5339	23.3	24.6	30.0	22.0	54.6
Cominella v. brookesi	SFKH-TMP010	5339	23.1	24.9	30.3	21.7	55.2
Buccinulum fuscozonatum	M.302907/2	5340	23.2	24.8	30.1	22.0	54.9
Buccinulum linea	SFKH-TMP016	5340	23.2	24.8	30.1	22.0	54.9
Buccinulum v. littorinoides	SFKH-TMP011	5340	23.2	24.7	30.1	22.0	54.8
Buccinulum pallidum	M.258277/6	5340	23.2	24.7	30.2	21.9	54.9
Buccinulum per. finlayi	M.302870/2	5340	23.2	24.7	30.1	22.0	54.8
Buccinulum robustum	M.314755/1	5340	23.2	24.8	30.1	21.9	54.9
Buccinulum v. vittatum	SFKH-TMP004	5340	23.2	24.7	30.1	22.0	54.8
Aeneator benthicolus	M.274111	5340	23.2	24.6	30.1	22.1	54.7

Aeneator elegans	SFKH-TMP015	5340	23.2	24.7	30.1	22.0	54.8
Aeneator otagoensis	M.279437	5340	23.2	24.7	30.2	21.9	54.9
Aeneator recens	M.190119	5340	23.2	24.6	30.1	22.1	54.7
Penion benthicolus	M.183832	5337	23.3	24.4	30.0	22.3	54.4
Kelletia kelletii	KK12	5337	23.4	24.4	29.9	22.3	54.3
Kelletia lischkei	KL2	5337	23.4	24.3	29.9	22.4	54.2
Penion maximus	C.487648	5339	23.5	24.4	29.9	22.2	54.3
Penion mandarinus	C.456980	5339	23.4	24.4	29.9	22.2	54.3
Penion sulcatus	Phoenix9	5339	23.3	24.4	30.0	22.3	54.4
Penion sulcatus	Phoenix1	5339	23.3	24.4	30.0	22.3	54.4
Penion chathamensis	M.190085/3	5339	23.3	24.4	29.9	22.3	54.3
Penion chathamensis	M.190082/2	5339	23.3	24.4	29.9	22.3	54.3
Penion c. cuvierianus	M.183792	5339	23.3	24.3	30.0	22.3	54.3
Penion c. cuvierianus	M.183927	5339	23.3	24.3	30.0	22.4	54.3

SUPPLEMENTARY TABLE 3.2

A summary of the statistics for the length and nucleotide composition for the mitochondrial genomes newly sequenced as part of this study. Specimens marked with one asterisk (*) exhibit drops in read coverage for some small regions, for example *K. kelletii* has 54 bp missing from *cox1*. Specimens marked with two asterisks (**) have genomes with large gaps in genome coverage for some regions, such as *B. v. vittatum* that has 266, 151 and 64 bp missing from the ATP6, *cox1* and ND2 genes respectively.

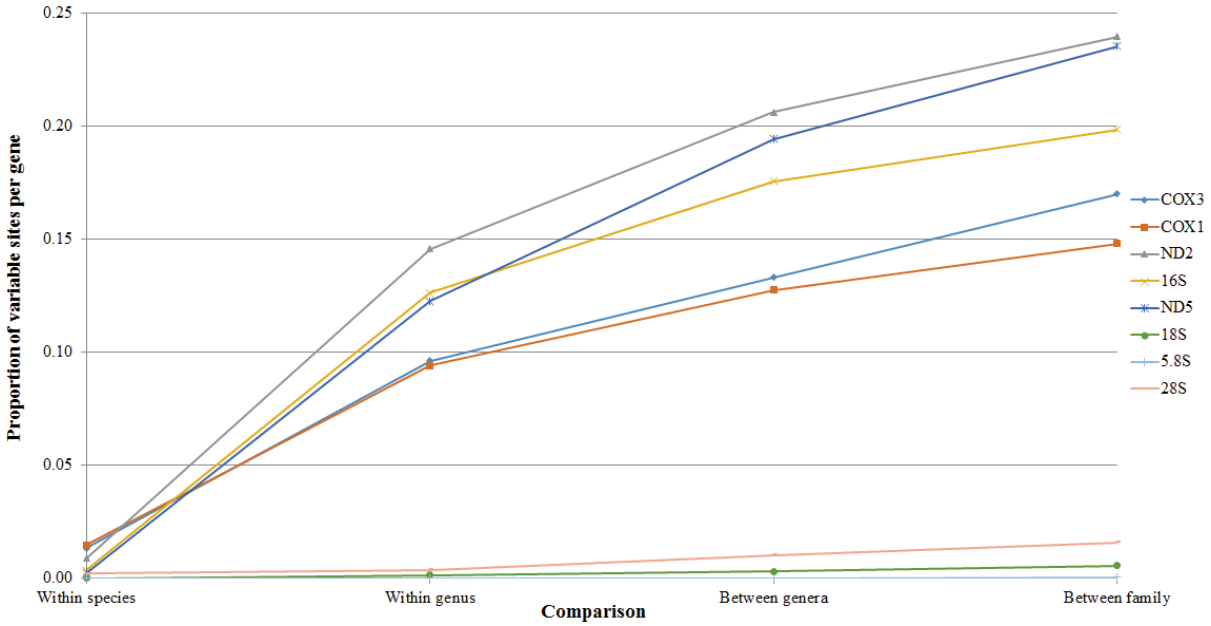
		mtDNA genome						
Species	**Museum ID**	**Length (bp)**	**% A**	**% C**	**% G**	**% T**	**GC bias**	
Pararetifusus carinatus	SFKH-TMP005	15204	31.5	13.4	15.0	40.1	28.4	
Glaphyrina caudata	SFKH-TMP004	15235	31.5	13.3	14.6	40.7	27.9	
Taron dubius	SFKH-TMP006	15189	29.3	15.7	17.0	38.0	32.7	
Austrofusus glans	SFKH-TMP014	15195	31.1	14.5	15.3	39.1	29.8	
Colus sp.	20140782	15158	30.5	14.9	15.8	38.7	30.7	
Volutopsius norwegicus	20140781	15232	29.3	15.7	16.5	38.4	32.2	
Buccinum undatum	20140783	15231	29.5	15.6	16.3	38.7	31.9	
Cominella adspersa	SFKH-TMP009	15251	30.4	15.7	16.0	38.0	31.7	
Cominella v. brookesi	SFKH-TMP010	15263	29.6	15.9	16.7	37.8	32.6	
Buccinulum fuscozonatum	M.302907/2	15246	30.2	14.8	15.8	39.1	30.6	
Buccinulum pallidum	M.258277/6	15247	30.9	14.1	15.2	39.7	29.3	
Buccinulum per. finlayi	M.302870/2	15247	30.1	14.8	15.9	39.1	30.7	
Buccinulum robustum	M.314755/1	15244	29.6	15.2	16.1	39.0	31.3	**
Buccinulum v. vittatum	SFKH-TMP012	15244	29.6	15.1	16.4	38.9	31.5	**
Aeneator benthicolus	M.274111	15254	30.4	14.7	15.7	39.2	30.4	
Aeneator elegans	SFKH-TMP015	15254	30.3	14.6	15.8	39.3	30.4	
Aeneator otagoensis	M.279437	15249	30.3	14.7	15.5	39.5	30.2	*
Aeneator recens	M.190119	15264	30.0	14.9	16.0	39.1	30.9	
Aeneator valedictus	SFKH-TMP013	15258	29.3	15.8	16.7	38.2	32.5	
Penion benthicolus	M.183832	15229	29.5	16.2	17.0	37.3	33.2	

Kelletia kelletii	KK12	15104	29.3	16.0	17.1	37.6	33.1	*
Kelletia lischkei	KL2	15225	29.6	16.1	16.8	37.5	32.9	
Penion maximus	C.487648	15249	30.6	15.1	16.0	38.2	31.1	
Penion mandarinus	C.456980	15250	30.4	15.1	16.2	38.3	31.3	
Penion sulcatus	Phoenix9	15227	29.2	16.0	17.2	37.5	33.2	
Penion sulcatus	Phoenix1	15227	29.2	16.1	17.2	37.4	33.3	
Penion chathamensis	M.190085/3	15227	28.6	16.8	18.0	36.7	34.8	
Penion chathamensis	M.190082/2	15228	28.5	16.8	18.0	36.7	34.8	
Penion c. cuvierianus	M.183792	15235	28.6	16.9	17.8	36.7	34.7	
Penion c. cuvierianus	M.183927	15241	28.3	17.1	18.0	36.6	35.1	**

Supplementary Figures

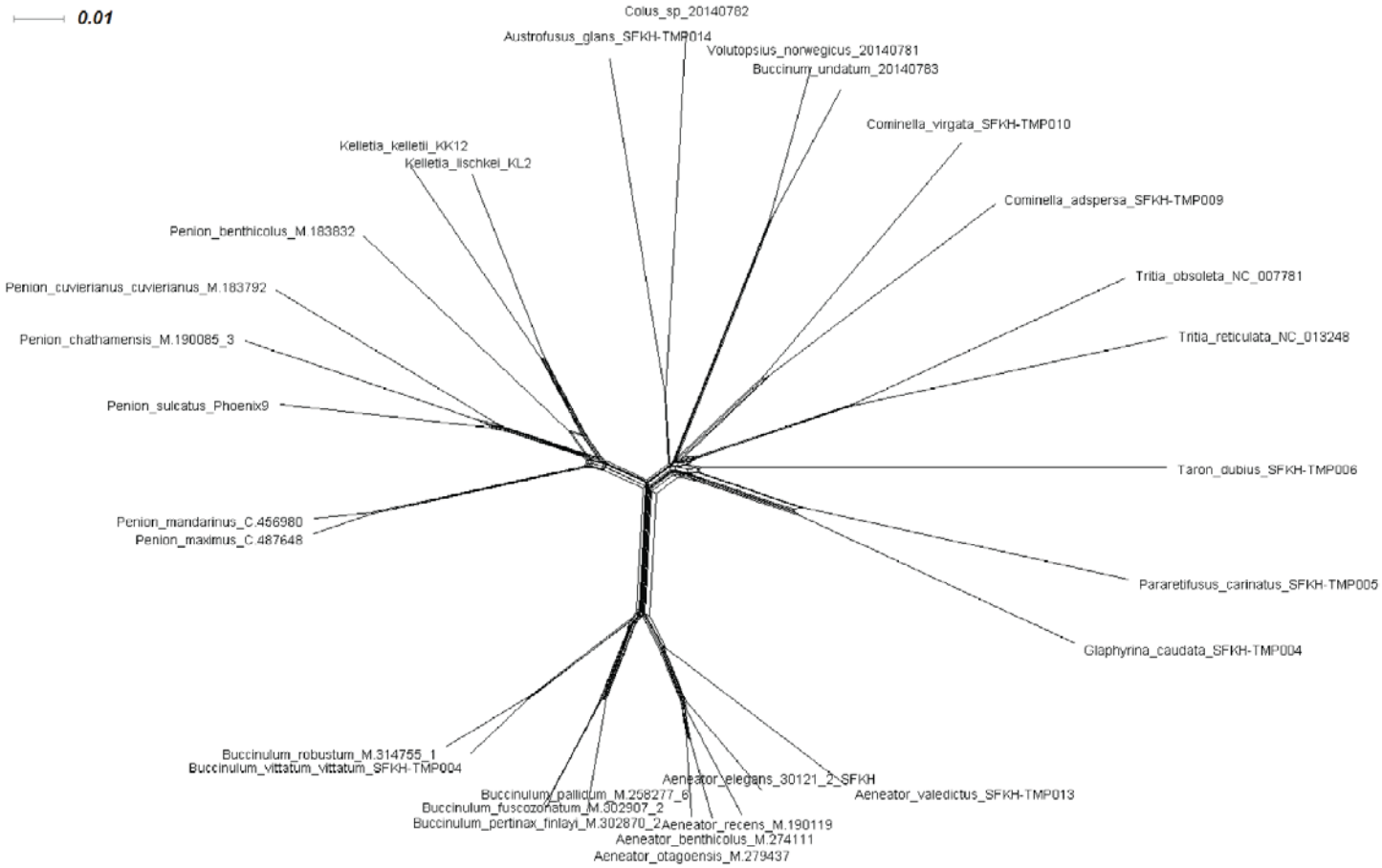
SUPPLEMENTARY FIGURE 3.1

The proportion of variable sites per sequence length (bp) for a selection of mtDNA and nuclear rDNA genes reflects different rates of DNA substitution. The trends plotted effectively represent change in the phylogenetic information provided by each gene for different levels of investigation. Average numbers of variable sites were used for groups for genus- and family-level comparisons. For example, we used the average number of differences for all sampled whelk (Buccinidae/Buccinulidae) taxa from all sampled Fasciolariidae taxa. Sampling from *Aeneator*, *Buccinulum* and *Penion* was used to estimate generic-level differences as these groups contained more than two specimens. Likewise, only *P. sulcatus*, *P. chathamensis*, and *P. c. cuvierianus* were used for within-species estimates as these taxa were sampled twice. Since read coverage varies for some genes, not all individuals were included for estimates made for each gene.

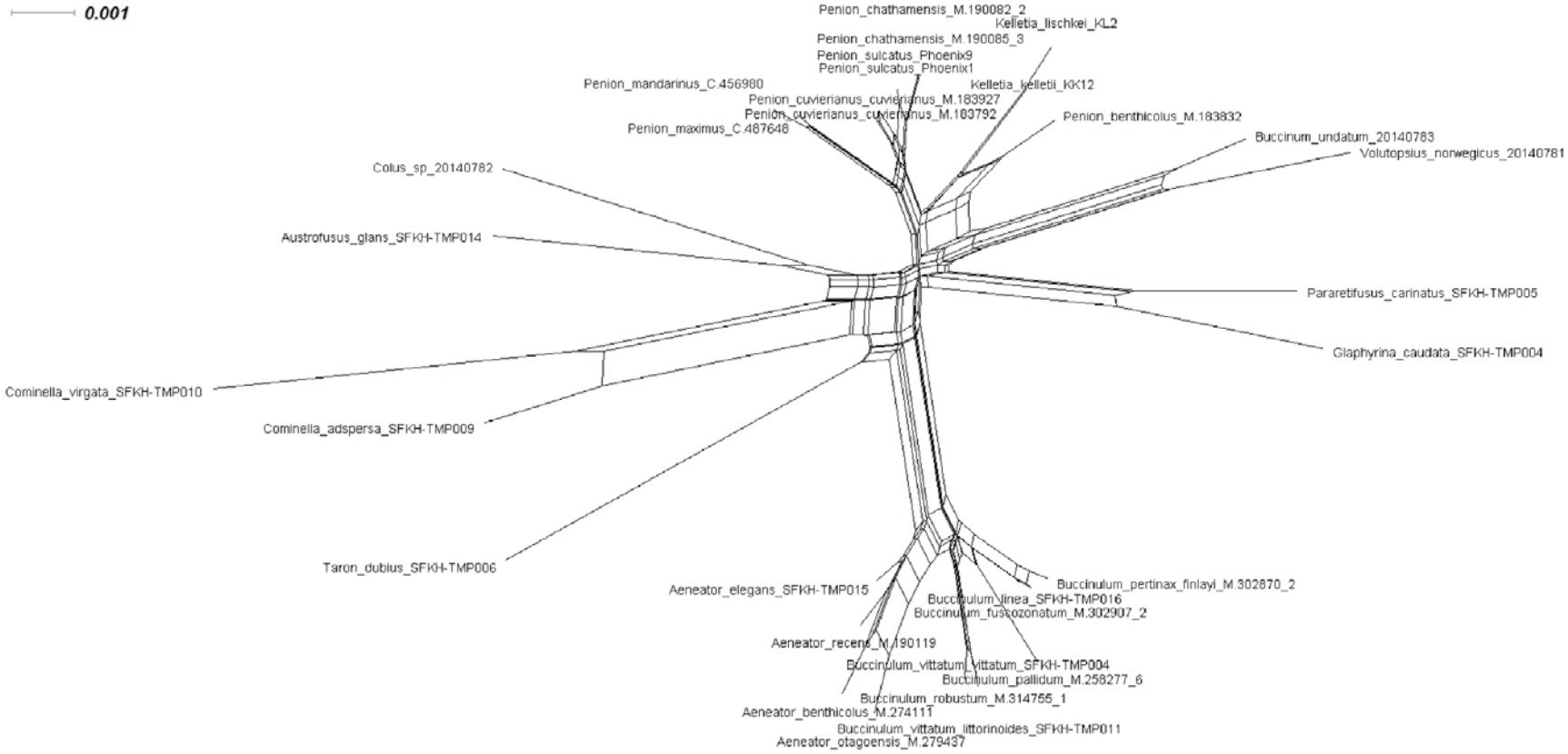


SUPPLEMENTARY FIGURE 3.2

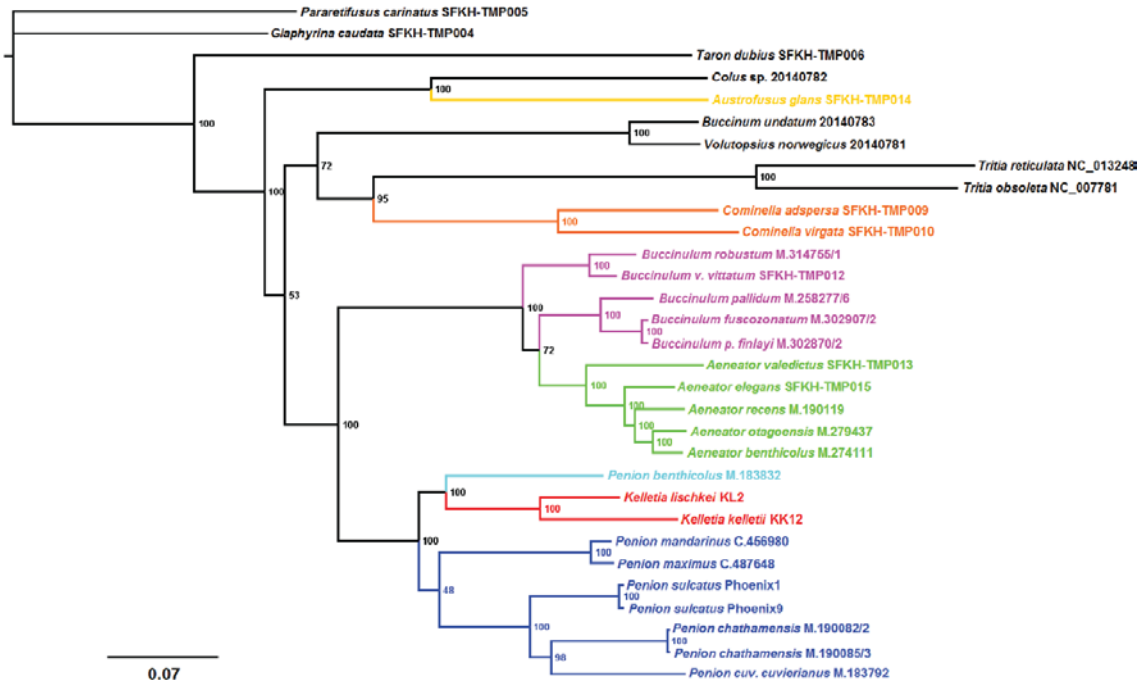
The splits network of based on an alignment of 31 concatenated mitochondrial genome sequences (incorporating protein-encoding, tRNA and rRNA genes; 11,128 bp). Splits were generated using the Neighbor-Net algorithm in SplitsTree 4. The splits network presents a generalisation of all of possible topological solutions for the phylogenetic signal contained in the underlying sequence data, but it does not quantify the likelihood of alternative phylogenetic relationships. Edge length is proportional to split weight, and box structures within the network indicate signal for alternative topologies in the underlying sequence data.



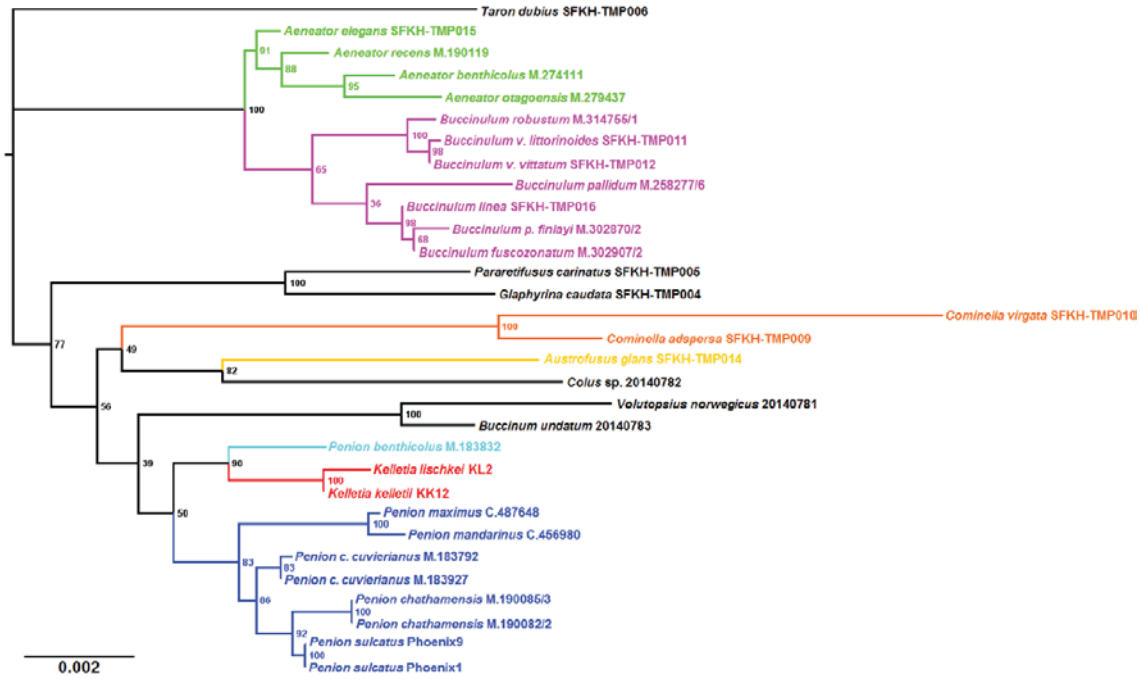
SUPPLEMENTARY FIGURE 3.3
The splits network of based on a 4667 bp alignment of 31 concatenated nuclear rDNA gene sequences (18S, 5.8S, 28S rRNA genes). Splits were generated using the Neighbor-Net algorithm in SplitsTree 4. The splits network presents a generalisation of all of possible topological solutions for the phylogenetic signal contained in the underlying sequence data, but it does not quantify the likelihood of alternative phylogenetic relationships. Edge length is proportional to split weight, and box structures within the network indicate signal for alternative topologies in the underlying sequence data.).



SUPPLEMENTARY FIGURE 3.4
A maximum-likelihood derived phylogeny (generated using RAxML 8.2.8) based an alignment of 31 concatenated mitochondrial genome sequences (incorporating protein-encoding, tRNA and rRNA genes; 11,128 bp). No partitions were used. No outgroup or monophyly was enforced for this tree. Genera putatively belonging to Buccinulidae are shown in different colours.

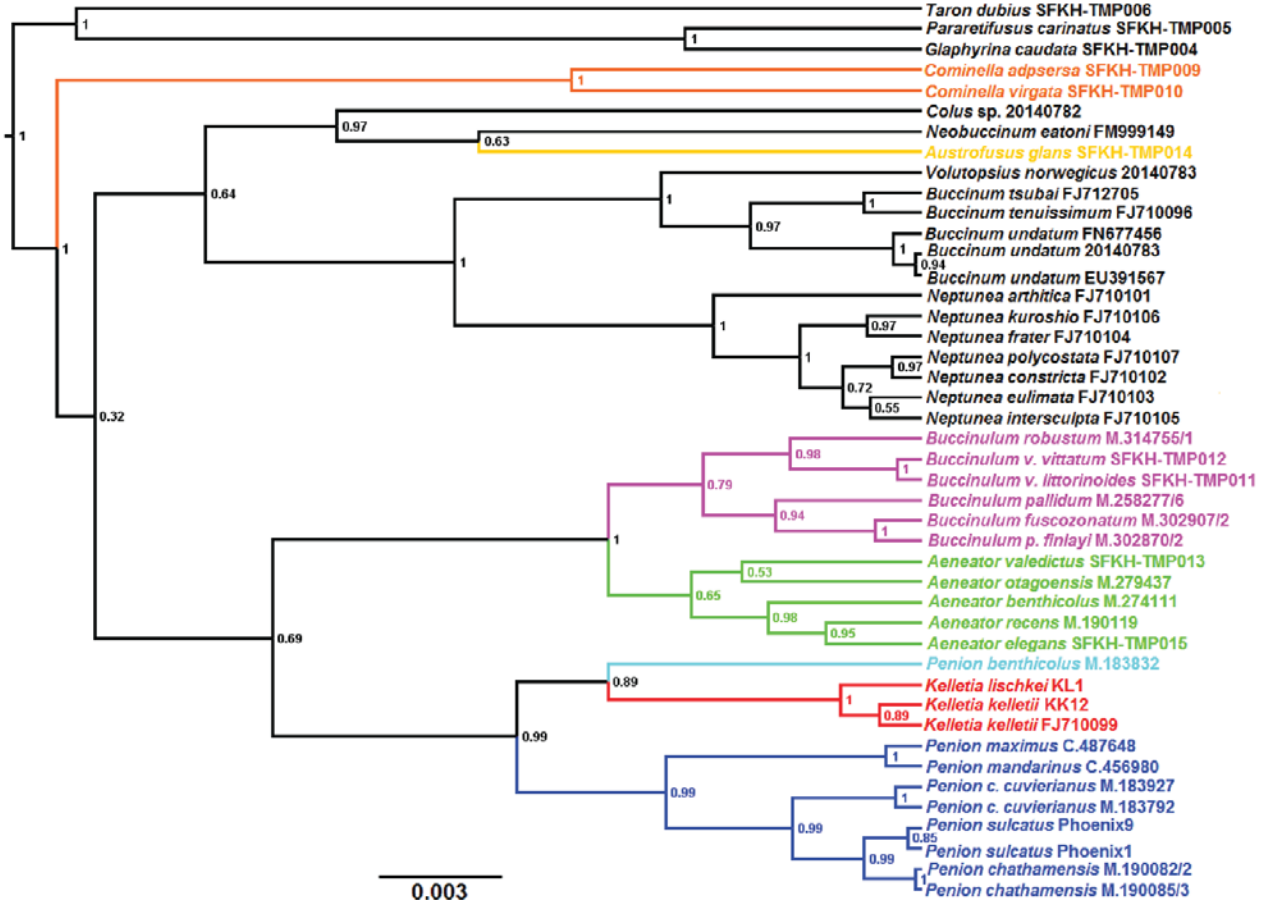


SUPPLEMENTARY FIGURE 3.5
A maximum-likelihood derived phylogeny (generated using RAxML 8.2.8) based on a 4667 bp alignment of 31 concatenated nuclear rDNA gene sequences (18S, 5.8S, 28S rRNA). No partitions were used. No outgroup or monophyly was enforced for this tree. Genera putatively belonging to Buccinulidae are shown in different colours.



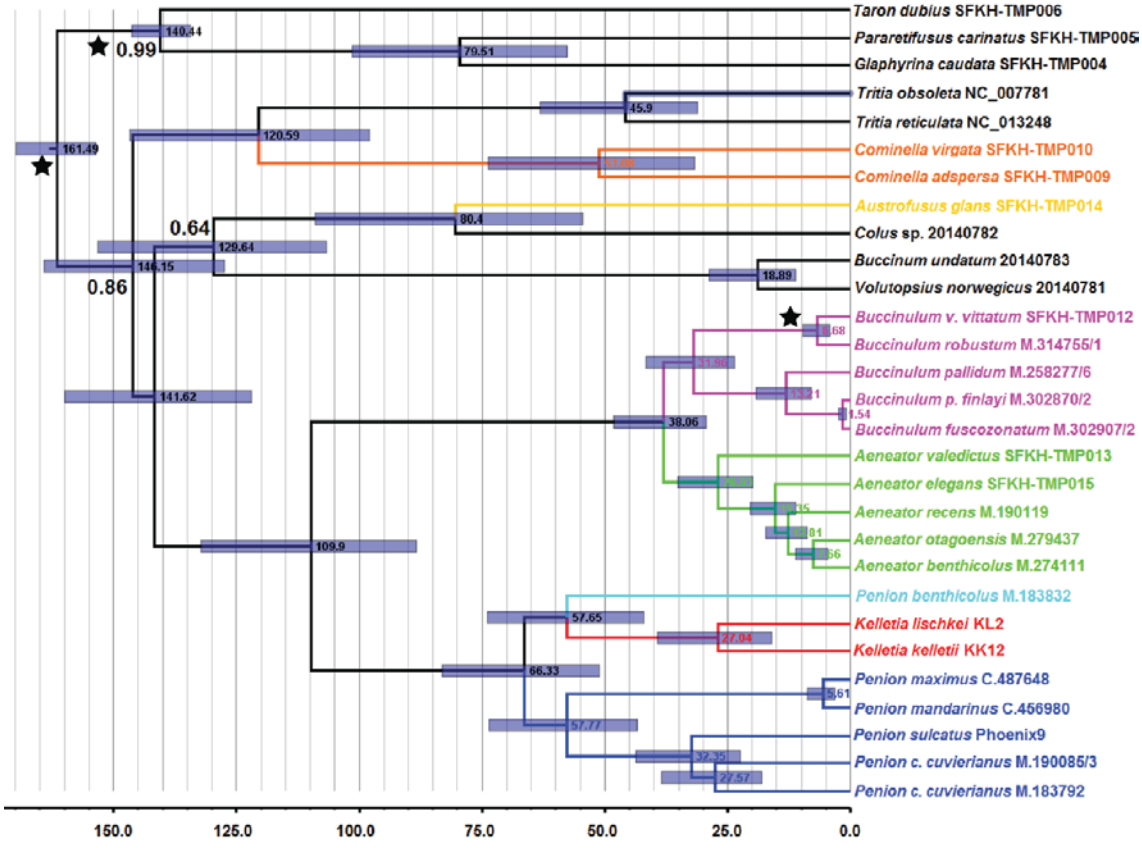
SUPPLEMENTARY FIGURE 3.6

A Bayesian phylogeny based on an alignment of nuclear ribosomal 28S RNA gene sequences obtained from 44 individual marine snails (1486 bp). The GTR + I + G substitution model was used. The phylogeny was produced using a Bayesian method (100 million MCMC, 10% burn-in, 1000 sample frequency, node labels are posterior support values), via BEAST 1.8. For this tree no outgroup was specified explicitly but reciprocal monophyly was enforced for the Fasciolariidae and Buccinidae/Nassariidae. Genera putatively belonging to Buccinulidae are shown in different colours.



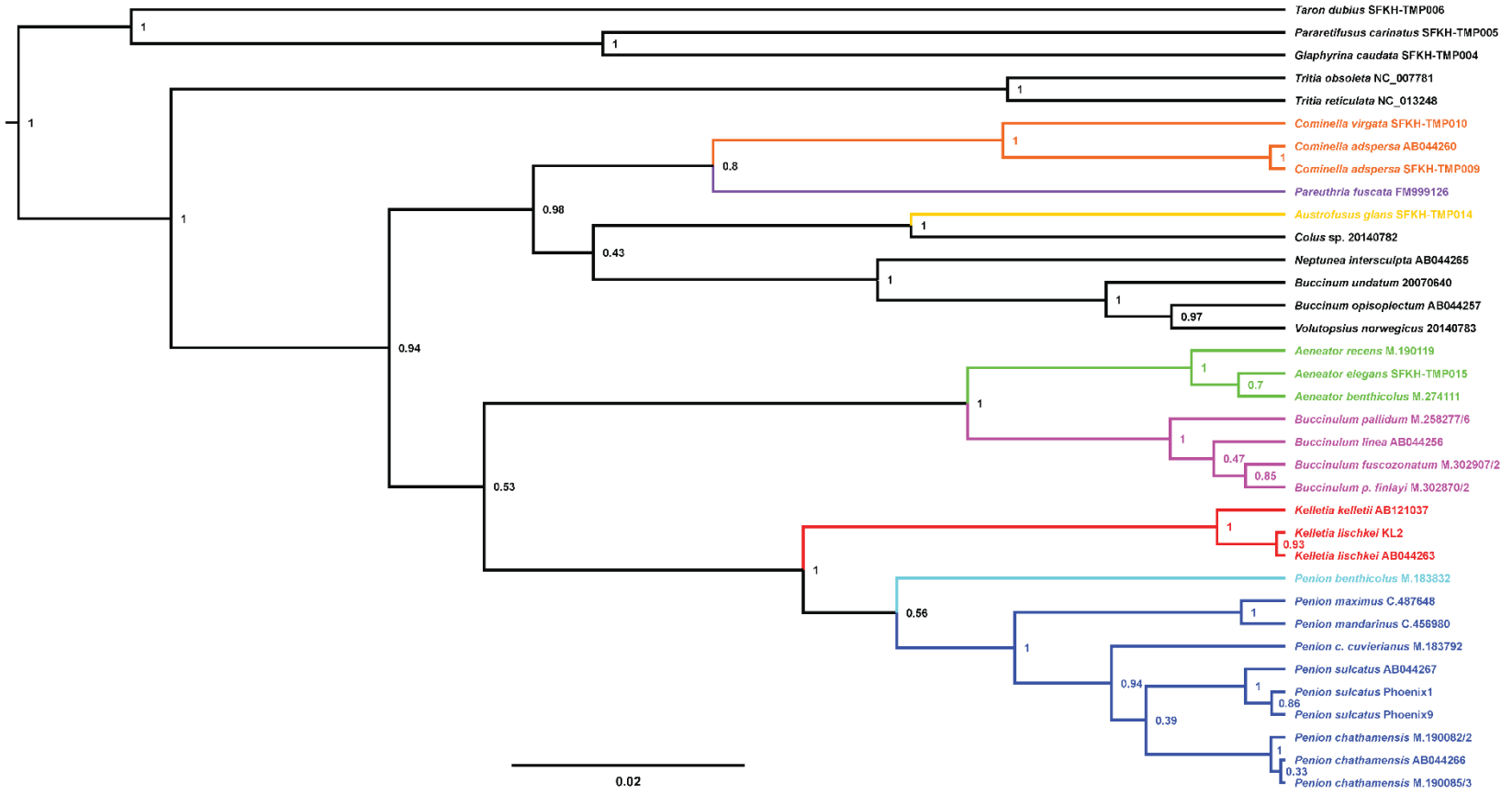
SUPPLEMENTARY FIGURE 3.7

A Bayesian phylogeny based on an alignment of 25 concatenated mitochondrial genome sequences (incorporating protein-encoding, tRNA and rRNA genes), which has been fossil calibrated to estimate divergence dates among the whelk lineages. Two sequence partitions were used: 1) protein-encoding and tRNA genes (10,635 bp), and 2) tRNA genes (1065 bp) using the GTR + I + G and HKY + I + G substitution models respectively. Black stars indicate splits that fossil calibrated. Fossil dates used to calibrate the tree originated from the earliest known buccinoid fossils (tree root height), earliest Fasciolariidae (un-enforced outgroup), and the earliest known occurrence of the tip branch *Buccinulum v. vittatum.* BEAST 1.8.3 using and MCMC length of 100 million, 1000 sample frequency and a 10% burn-in was used to generate this phylogeny. Node labels are estimated median divergence dates with the 95% highest posterior density (HPD) range shown as a blue bar. Posterior support values are also shown at nodes, but only if support was less than 1.0. Putative buccinulid genera are shown in different colours.

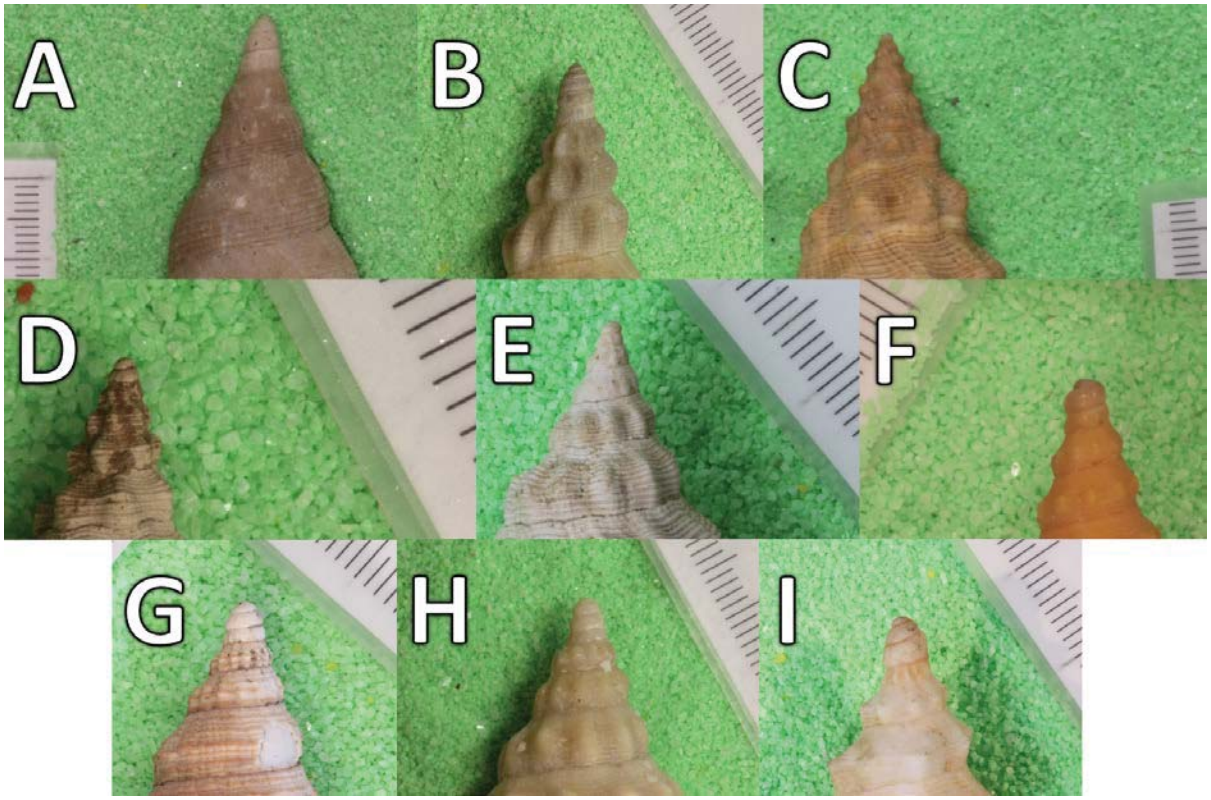


SUPPLEMENTARY FIGURE 3.8

A Bayesian phylogeny based on an alignment of mitochondrial 16S RNA gene sequences obtained from 35 individual marine snails (868 bp). The GTR + I + G substitution model was used. The phylogeny was produced using a Bayesian method (100 million MCMC, 10% burn-in, 1000 sample frequency, node labels are posterior support values), via BEAST 1.8. For this tree no outgroup was specified explicitly but reciprocal monophyly was enforced for the Fasciolariidae and Buccinidae/Nassariidae. Genera putatively belonging to Buccinulidae are shown in different colours.



SUPPLEMENTARY FIGURE 3.9
Protoconchs of various species of *Antarctonetpunea, Kelletia* and *Penion.* The size proportionate to the teleoconch and number of whorls exhibited by a protoconch can indicate development, with indirect developing species often exhibiting small protoconchs and direct developing taxa typically presenting large protoconchs with more than two whorls. A: M.242882 [MNZ] *Antarctoneptunea aurora* from the Ross Sea; B: M.070957 [MNZ] *Penion benthicolus* from off Cape Maria van Diemen, Northland; C: SYD6 [AUS] *Kelletia kelletii* from Balboa Bay, California, only bottom protoconch whorl is preserved but small size can be observed; D: TM1288 [GNS] *Penion bartrumi* (Laws, 1941) fossil from Pakurangi Point, Kaipara, dated to the Altonian, 18.7 – 15.9 Ma; E: 1021 [GNS] *Penion mandarinus* fossil from Strathdowne, Victoria, dated to Werrikooian, 1.81 – 1.00 Ma; F: F221248 [VIC] *Penion maximus* from off Eden, New South Wales; G: MA73478 [AM] *Penion sulcatus* fossil from Te Piki, Bay of Plenty, dated to the Nukumaruan, 2.40 – 1.63 Ma; H: M.314708 [MNZ] *Penion chathamensis* from off Tairoa Head, Otago; I: RM5335 [GNS] *Penion c. cuvierianus* from the Hauraki Gulf, Auckland.



Chapter Four

Molecular phylogenetics and and RAD sequencing of New Zealand siphon whelks (*Penion*)

Fieldwork sites (clockwise, top right: Castlepoint, Doubtless Bay, Wellington, Golden Bay).

Introduction

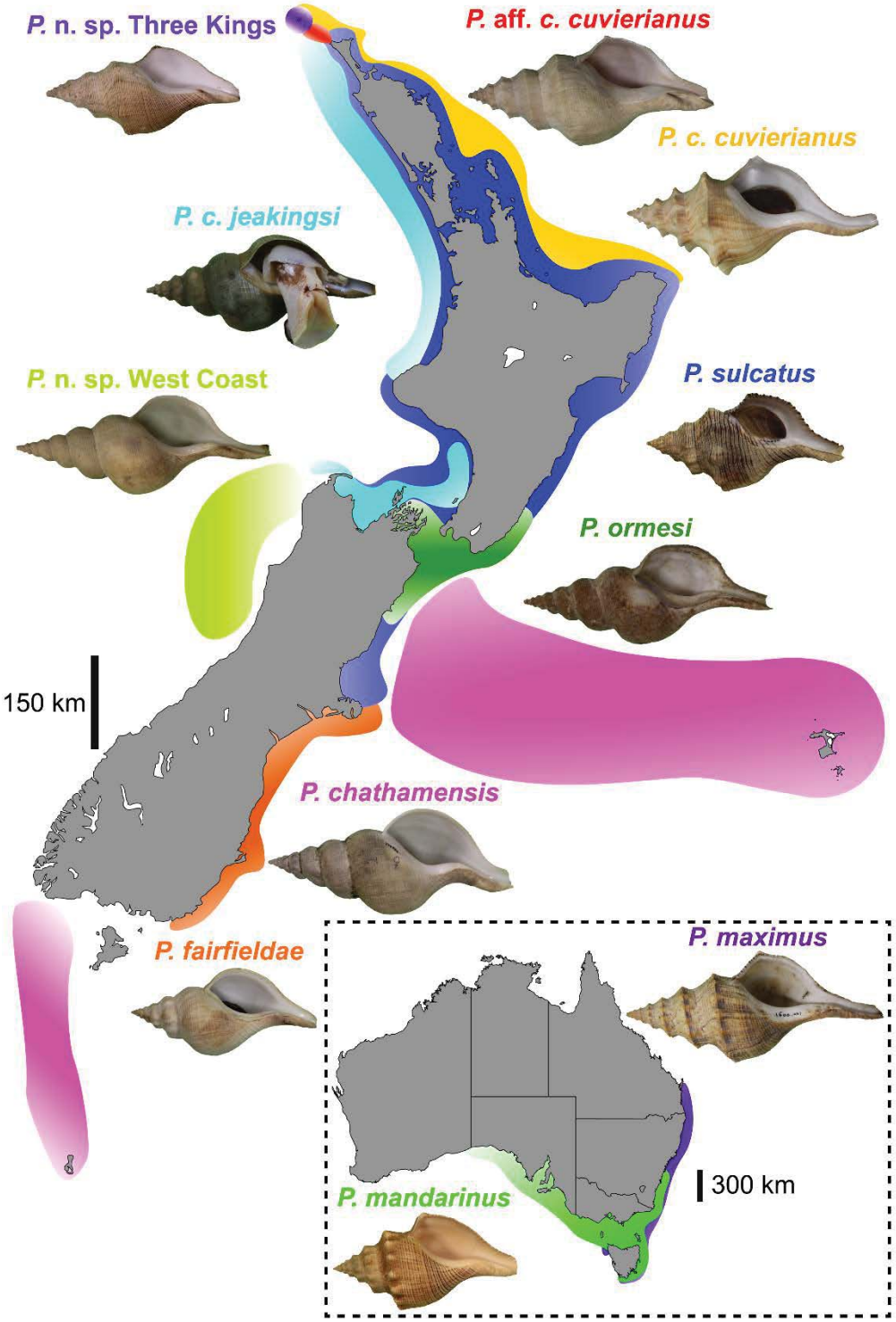
We investigate evolutionary lineages within the siphon whelk genus *Penion* Fischer, 1884 (Caenogastropoda: Neogastropoda: Buccinidae). Among benthic marine snails, morphological variation in shells and soft-body anatomy indicates that the siphon whelks are biologically and taxonomically particularly diverse (Ponder 1973, Powell 1979, Beu and Maxwell 1990, Nielsen 2003, Spencer *et al.* 2009, Spencer *et al.* 2017). Five extant species and one subspecies of *Penion* are endemic to New Zealand waters (Powell 1979, Spencer *et al.* 2017; Figure 4.1), and two further species are distributed off south-eastern Australia (Ponder 1973). The fossil record for the genus is rich and extends over 66 million years, with dozens of fossil taxa described from New Zealand (Beu and Maxwell 1990), Australia (Ponder 1973), Chile and Argentina (Frassinetti 2000, Nielsen 2003), and Antarctica (Stilwell and Zinsmeister 1992, Beu 2009). The putative high extant diversity and rich fossil record makes *Penion* an intriguing system for evolutionary study. However, a significant problem is that the taxonomic classification of siphon whelks is restricted to morphology, and the evolutionary relationships among putative taxa (species, subspecies) are uncertain.

Little is known about the ecology, reproduction or behaviour of *Penion* overall, meaning that current taxonomic classification does not consider these traits. Siphon whelks are benthic and most species occur at significant water depths (50 – 1000 m) (Dell 1956, Powell 1979, Willan *et al.* 2010). Most species occur on deep-water soft-sediment basins, although *P. sulcatus* (Lamarck, 1816), *P. c. jeakingsi* and *P. mandarinus* (Duclos, 1832) also occur in shallow-water rocky environments (Powell 1979, Willan *et al.* 2010). Siphon whelks are predator-scavengers (Spencer *et al.* 2009, Willan *et al.* 2010), and like other true whelks they are probably subject to predation by echinoderms (Brokordt *et al.* 2003). In New Zealand humans may have previously harvested *Penion* for food as shells of siphon whelks from the intertidal zone occur in the middens of historic Māori settlements (Allen 2012, Green and Pullar 1960). Likewise, the related genus *Kelletia* is commercially fished today (California Department of Fish and Game 2009, Vendetti 2009). Siphon whelks are dioecious (like most Caenogastropoda) but the mating and reproductive behaviour documented in related lineages have not been observed (Rosenthal 1970, Kenchington and Glass 1998).

Taxonomic classification is therefore based primarily on variation in shell morphology (Powell 1929, Ponder 1973, Powell 1979, Willan *et al.* 2010), especially in palaeontology as soft-body anatomy does not readily preserve (Powell 1947, Beu and

Maxwell 1990, Stilwell and Zinsmeister 1992, Frassinetti 2000, Nielsen 2003, Beu 2009). Soft-body anatomy is referred to only rarely (Dell 1956, Ponder 1973), as traits including radula morphology are considered unreliable (Dell 1956). A challenge for the evolutionary analysis of *Penion* is that siphon whelk shell morphology that is often highly variable within species. Putative species appear to vary in shell size (Powell 1927, Powell 1947, Powell 1979), in the extent and presence of many conchological features such as axial ribs (Powell 1927, Powell 1947, Ponder 1973, Ponder 1975, Dell 1956, Powell 1979, Willan *et al.* 2010), and also in patterning and colouration (Powell 1979, Willan *et al.* 2010). Shells are also thought to exhibit phenotypic plasticity resulting in significant intraspecific variation (Dell 1956, Powell 1979, Ponder 1975, Willan *et al.* 2010). Reliance on shell morphology is common in the taxonomy of true whelks (e.g. Powell 1979, Kosyan 2006, Araya 2013, Zhang and Zhang 2015), but intraspecific and within-genus molecular investigations are sparse and mostly restricted to Northern Hemisphere taxa (e.g. Iguchi *et al.* 2005, Hou *et al.* 2013, Pálsson *et al.* 2014, Azuma *et al.* 2015). In *Penion* we aim to use genetic data to identify independent evolutionary lineages, which should permit the accuracy of current taxonomy of the genus to be assessed, as a species is an arbitrary segment of an evolutionary lineage (Chapter 1).

FIGURE 4.1
Estimated geographic distributions for extant, monophyletic *Penion* from New Zealand and Australia. The range of each putative taxon is highlighted in a different colour and an example shell is shown for each putative taxon (animal included for *P. c. jeakingsi* (Powell, 1947)).



In a previous phylogenetic investigation of New Zealand true whelks, we used mitochondrial (mtDNA) genomic and nuclear ribosomal (rDNA) sequence data from a sub-set of *Penion* siphon whelk species: *P. maximus* (Tryon, 1881), *P. mandarinus, P. sulcatus, P. chathamensis, P. c. cuvierianus* (Powell, 1927) and *P. benthicolus* Dell, 1956 (Chapter 3). We inferred that *Penion* is sister to *Kelletia* Bayle, 1884, concordant with previous analyses of shell morphology and soft-body anatomy (Powell 1929, Wenz 1941, Ponder 1973, Stilwell and Zinsmeister 1992). However, DNA sequences indicated that *P. benthicolus* was misclassified as its inclusion rendered *Penion* paraphyletic with respect to *Antarctoneptunea aurora* (Hedley, 1916) (Chapter 3). A comparison of shells also indicates that the shell morphology of these two species is also similar, and we recommended reclassification as *Antarctoneptunea benthicola* (Dell, 1956) (Chapter 3).

Here we further investigate the phylogeny of *Penion*, sampling all recognised species as well as several newly identified, putative lineages. We investigate molecular variation to test if current taxonomy based on the examination of shell traits is accurate, and to determine the support for putative new species. We reconstruct phylogenetic trees using mitochondrial (mtDNA) genomic and nuclear ribosomal (rDNA) sequence data, which is complemented with short-length sequence data amplified from mtDNA and nuclear rDNA gene regions. Using next-generation sequencing of putative species, we supplement this phylogenetic analysis with an examination of single nucleotide polymorphism (SNP) variation for anonymous nuclear SNP loci. Nuclear genetic variation is investigated hierarchically, analysing variation among monophyletic *Penion* from Australia and New Zealand, and then variation among only New Zealand representatives.

Our sampling of *Penion* focusses especially upon 1) the distinction of *P. chathamensis* (Powell, 1938) and *P. fairfieldae* (Powell, 1947) and 2) a species complex containing *P. c. cuvierianus, P. c. jeakingsi* (Powell, 1947), a possible new species from the West Coast, *P. ormesi* (Powell, 1927), and a morphological variant from Cape Reinga, Northland. In previous taxonomic reviews, *P. chathamensis* was compared to *P. ormesi* and *A. benthicola* (Powell 1947, Dell 1956, Powell 1979), and *P. fairfieldae* was hypothesised to represent living descendants of a lineage containing the extinct fossil species *P. asper* and *P. imperfectus* (Powell 1947, Powell 1979). However, shells of *P. chathamensis* and *P. fairfieldae* appear to be similar in shape and they are currently

recognised as having parapatric geographic ranges off south east New Zealand (Figure 4.1).

Penion c. cuvierianus and *Penion c. jeakingsi* exhibit substantial variation in shell morphology and the taxa occupy a broad geographic range extending from Northland to the Cook Strait (Powell 1927, Powell 1979, Willan *et al.* 2010; Figure 4.1). The range of *P. c. jeakingsi* overlaps with that of *P. ormesi* in the Cook Strait (Powell 1947, Powell 1979), and these taxa can be difficult to distinguish with traditional morphology (Figure 4.1). In addition, we examine a potential new species or a previously undocumented locality for *P. c. jeakingsi* in the West Coast region, where *Penion* have not been previously recorded. West Coast specimens exhibit a similar morphology to *P. c. jeakingsi* and *P. ormesi*, but shells are often large and thin, with acutely angled teleoconch whorls, barely prominent axial ribs beyond the first three teleoconch whorls, and long siphonal canals. In the far north, seemingly restricted to Cape Reinga and possibly the Three Kings Islands, whelks with a morphological affinity to *P. c. cuvierianus* (referred to as *P.* aff. *c. cuvierianus*) exhibit a very thick shell with a short siphonal canal and an enlarged protoconch (Figure 4.1).

A single specimen of a putative new species from the Three Kings Islands (Manawatawhi) *P.* n. sp. Three Kings Islands is included (Figure 4.1). Specimens of this putative taxon have a distinctive shell morphology with a wide flattened beehive-shaped protoconch, smooth axial ribs, short siphonal canal, and striped shell colouration (Figure 4.1). The Three Kings Islands region is considered to be a ‘biodiversity hotspot’ for many organisms, including the buccinid genus *Cominella* Gray, 1850 (Willan 1978, Donald *et al.* 2015).

Methods

Taxonomy and sampling

Individuals were assigned to putative taxa primarily on the basis of traditional morphological examination of shells, with some reference to the morphology of soft-body tissues such as the radula, operculum, stomach, and gonads (Dell 1956, Ponder 1973, Powell 1979, Willan *et al.* 2010). All sampled specimens were identified by experienced molluscan taxonomists: Bruce A. Marshall (Collection Manager Sciences, Museum of New Zealand Te Papa Tongarewa) and Alan G. Beu (Palaeontologist, GNS). All extant species of *Penion* from New Zealand and Australia were sampled, including

all subspecies recognised by Powell (1979). For deeper phylogenetic comparisons all extant species of *Antarctoneptunea* Dell, 1972 and *Kelletia* were also included.

The majority of specimens examined came from museum and university collections (acknowledged below), supplemented with snails collected in the field specifically for this study (Tables 1 and 2). Specimens were obtained either by trawling (20 – 500 m depth for most sampling) or by hand within the intertidal zone (1 – 3 m). Some specimens resulted from commercial trawling fishery bycatch. Captured individuals were swiftly frozen, thawed and removed from shells, and then preserved in ample 95% ethanol.

New samples and DNA sequences collected for this investigation were supplemented by with our previous dataset produced to investigate the phylogeny of Buccinulidae (Chapter 3). This included an additional 9 individuals from 6 further species of *Penion*, and one individual each of *Kelletia kelletii* (Forbes, 1850), *K. lischkei* Kuroda, 1938, *Aeneator elegans* (Suter, 1917), *A. recens* (Dell, 1951), *Buccinulum fuscozonatum* Suter, 1908, and *B. pertinax finlayi* Powell, 1929 were used as an outgroup for phylogenetic analyses. Additional sequence data was retrieved from public databases (GenBank; Table 4.2), and sequence data amplified from the mtDNA *cox1* and 16S rRNA genes was also available from our previous investigation (Chapter 3).

All extant species of *Penion* in New Zealand and Australia recognised by Ponder (1973) and Powell (1979) were sampled, and for deeper phylogenetic comparisons all extant species of *Antarctoneptunea* and *Kelletia* were also sampled. To investigate intraspecific variation, we also sampled multiple individuals from various locations for most species. However, for six taxa: *P.* n. sp. Three Kings Islands, *P.* n. sp. West Coast, *P.* aff. *c. cuvierianus*, *A. aurora, K. kelletii,* and *K. lischkei*, only one specimen was suitable for DNA sequencing. The first four putative taxa are only known from remote regions often with narrow ranges (the Three Kings Islands; far north Northland; West Coast; and the Southern Ocean respectively; Figure 4.1), which makes sampling challenging, and issues are further complicated by difficult-to-navigate waters. Both *Kelletia* species were sampled at lower frequency because they occur outside of Australasia.

mtDNA and nuclear rDNA sequencing phylogenetics

Total genome DNA was obtained using a standardised extraction protocol described in a previous study of *Penion* to yield suitable material in these snails

(Chapter 3). DNA extracts from 7 individuals of 6 putative species were processed for high-throughput sequencing using the ThruPLEX DNA-seq Kit (Rubicon Genomics). Fragmented genomic DNA was paired-end sequenced on an Illumina HiSeq 2500 (Table 4.1). Reads for teach of the 32 individuals were de-multiplexed using standard indexes incorporated in the library-preparation kit. Resulting short-sequence reads that passed standard quality filters had adapter sequences removed using cutadapt 1.11 (Martin 2011). Geneious 9.1.3 (Kearse *et al.* 2012), was used to pair sequence reads and to edit, assemble and align sequences. We followed a standardised protocol (Chapter 3) to assemble mtDNA genome and the 45S nuclear ribosomal cassette (18S, ITS1, 5.8S, ITS2, 28S) sequences.

All sequence alignments used for phylogenetic analyses were concatenated to remove sequence gaps (Ns) and positions with ambiguous bases were removed. Gblocks 0.91b (Castresana 2000), operating under standard settings was used to eliminate poorly aligned positions and regions with low homology from DNA alignments used for phylogenetic reconstruction. Topology and signal consistency was investigated using unrooted phylogenetic networks in SplitsTree 4 (Huson and Bryant 2006). Sequence data was partitioned into protein-encoding, tRNA and rRNA genes, and the best fitting nucleotide substitution model for each gene partition was assessed using jModelTest 2.1.6 (Guindon and Gascuel 2003, Darriba *et al.* 2012). The generalised time-reversible substitution model (GTR + I + G) (Tavaré 1986), was found to be most appropriate for substitution model for the mtDNA protein-encoding, rRNA and nuclear rDNA sequences, whereas the HKY + I + G model (Hasegawa *et al.* 1985), was most suitable for the mitochondrial tRNA regions. When partitioned sequence data were used, these models were applied as unlinked substitution models. Molecular phylogenies were estimated using Bayesian MCMC inference via MrBayes 3.2 (Ronquist *et al.* 2012), and BEAST 1.8.3 (Drummond *et al.* 2012). Maximum-likelihood phylogenetic trees were also estimated using RAxML 8.2.8 (Stamatakis 2014). Posterior statistics for Bayesian MCMC parameters were evaluated using Tracer 1.6 (Rambaut *et al.* 2014). Tree outputs were viewed and edited in Figtree 1.4.2 (Figtree 2016), and node support was assessed using posterior probability. All phylogenetic reconstruction was processed using CIPRES Science Gateway (Miller *et al.* 2010).

PCR and Sanger sequencing was used to target portions of the mitochondrial genes cytochrome oxidase I (COXI) and 16S rRNA, and nuclear ribosomal 28S RNA (Table 4.2). DNA sequences for these gene regions were aligned with reference to the

whole mtDNA genome sequences. This shorter sequence data was to test species boundaries with higher replication. Variation was examined using median joining haplotype networks using the median joining network method developed for intraspecific phylogenetic inference (Bandelt *et al.* 1999), in PopART 1.7 (Leigh and Bryant 2015).

TABLE 4.1
New Zealand *Penion* DNA samples subjected to high-throughput Illumina sequencing to yield data assembled into mitochondrial genomes and nuclear ribosomal 45S sequences. All specimens were newly sequenced for this thesis.

Taxon	rDNA cassette	mtDNA genome	Voucher ID	Location
Penion c. jeakingsi	Y	Y	M.279432	Tasman Bay, Nelson
Penion c. jeakingsi	Y	Y	M.316215/1	Kahurangi Point, West Coast
Penion aff. *c. cuvierianus*	Y	Y	M.318615/1	Columbia Bank, Northland
Penion fairfieldae	Y	Y	Phoenix1	Otago Peninsula, Otago
Penion ormesi	Y	Y	M.299869/1	Cloudy Bay, Marlborough
Penion ormesi	Y	Y	M.318565/2	Pelorus Sound, Marlborough
Penion n. sp. Three Kings Islands	Y	Y	M.302876	Three Kings Islands

TABLE 4.2
New Zealand *Penion* samples used for PCR amplification of the mitochondrial *cox1*, 16S or nuclear ribosomal 28S genes. All specimens were newly sequenced for this thesis.

Taxon	mtDNA *cox1*	mtDNA 16S	Voucher ID	Location
Penion c. jeakingsi	Y	Y	M.279432/1	Tasman Bay, Nelson
Penion c. jeakingsi	Y	Y	M.279432/3	Tasman Bay, Nelson
Penion c. jeakingsi		Y	M.279432/4	Tasman Bay, Nelson
Penion c. jeakingsi	Y		M.279432/5	Tasman Bay, Nelson
Penion c. jeakingsi	Y		M.279432/7	Tasman Bay, Nelson
Penion c. jeakingsi	Y		Phoenix2	Golden Bay, Tasman
Penion c. jeakingsi	Y		Phoenix3	Golden Bay, Tasman
Penion fairfieldae		Y	M.316052/1	Otago Peninsula, Otago
Penion fairfieldae		Y	M.316052/2	Otago Peninsula, Otago
Penion ormesi	Y		M.318599/2	Pelorus Sound, Marlborough

nDNA next-generation sequencing for SNP analysis

Reduced representation high-throughput DNA sequencing was used to investigate nuclear genetic variation within species, and to test concordance between signal from anonymous nuclear loci and species identification using traditional shell traits. We focus on two instances where *Penion* taxonomy based on morphology is especially challenging. One consists of two currently recognised species with distinct geographic ranges put limited differentiation of shells (*P. chathamensis* and *P. fairfieldae* (Powell, 1947)), and the other involves a species complex with three recognised taxa (*P. c. cuvierianus, P. c. jeakingsi, P. ormesi*) and additional shell variation (*P.* aff. *c. cuvierianus* and *P.* n. sp. West Coast). 60 individuals were processed and 20 of these were suitable for downstream population genetic analyses (Table 4.3).

We used double-digest restriction site associated DNA sequencing (ddRADs) to generate a suite of SNP data across a large number of nuclear loci (Peterson 2012, Puritz *et al.* 2014). This RAD sequencing method used two restriction enzymes that cut at different sites to generate a large number of sequencing reads with broad coverage across the genome (Peterson 2012). Enzymes were chosen after consideration of genome size to yield an optimal fraction of the genome where homologous loci were represented in multiple individuals. Restriction site associated DNA (RAD) sequencing can generate abundant population genetic (e.g. Poland *et al.* 2012, Kai *et al.* 2014, Dowle *et al.* 2015), and phylogenetic data (e.g. Cariou *et al.* 2013, Wagner *et al.* 2013, Razkin *et al.* 2015), which can be used specifically for species delimitation (e.g. Leaché *et al.* 2014, Razkin *et al.* 2015, Pante *et al.* 2015, Card *et al.* 2016, Herrera and Shank 2016).

DNA extractions varied in quality and so were processed through the Agencourt AMPure XP SPRI bead purification system (Beckman Coulter, Inc.), following a similar protocol to Quail *et al.* (2009). DNA degradation and the likely persistence of mucopolysaccharides interfered with downstream enzymatic reactions – particularly during the library-preparation for ddRAD sequencing. As a result, we were not able to PCR amplify and successfully sequence all individuals that were available. This is a common experience of geneticists working with molluscs (e.g. Winnepenninckx *et al.* 1993, Skujienė and Soroka 2003, Pereira *et al.* 2011), and future investigations would benefit from improved DNA extraction and purification methods specialised for molluscs.

For next-generation sequencing, we typically treated 10 μL of DNA extract with an equal volume of beads, eluting the products to equal volumes. DNA was quantified using Qubit Fluorometric Quantitation (Life Technologies, Thermo Fisher Scientific Inc.). Following clean-up, approximately 1 μg of DNA per specimen was digested in a 30 μL reaction volume with a 1:1 DNA μg to enzyme unit ratio. We used the restriction-site enzymes NsiI-HF (low frequency 6 base pair (bp) cutter, 5'…ATGCAT… 3') and MboI (high frequency 4 bp cutter, 5'…GATC… 3') (New England BioLabs, Inc.). Examination of whole mtDNA sequences from *Penion* indicated that these restriction enzymes would yield only nuclear sequence data (Geneious; Kearse *et al.* 2012) as there was only one restriction site for the MboI enzyme in the mtDNA genome sequences a and no NSiI cut sites. NsiI-HF exhibited a low rate of activity with the siphon whelk DNA extracts, and so digestions with NsiI digest were conducted overnight (14 – 18 hours at 37°C), before addition of MboI and incubation at 37°C for an additional 2 hours. Enzymes were subsequently denatured at 80°C for 20 seconds as a precaution against DNA loss.

Adaptor sequences containing sample specific barcodes (1 – 30, in two separate library indices of 30) were ligated with digested DNA samples (Table 4.3). Ligation reaction volumes in 40 μL volumes per specimen used the T4 ligase enzyme at an incubation temperature of 65°C. Efficiency of ligation was tested using PCR primers targeting ligation barcodes and gel electrophoresis. Individuals were pooled into the two libraries for indexing, each with a volume of 600 μL (20 μL from 30 individuals). Pooled samples were then cleaned of the previous PCR primers and degraded DNA using the QIAquick PCR Purfication Kit (Qiagen N.V.), resulting in a final volume per pool of 30 μL. Pooled samples were then size-selected by gel electrophoresis separation using 2% agarose. Fragments of 250 – 350 bp band were excised via observation under blue light with reference to a 1 kb+ DNA ladder. Gel cuts were then extracted using the Qiaquick gel extraction kit (Qiagen N.V.). Illumina index sequences were added to each set of pooled samples using a short-cycle, high-fidelity PCR amplification reaction. The annealing reaction volume was 20 μL per index, using the Phusion high-fidelity *Taq* DNA polymerase enzyme (New England BioLabs, Inc.). The annealing and extension temperature was 72°C, with 15 cycles of denaturation, annealing and extension.

Pooled, barcoded and indexed DNA libraries were sequenced via massive parallel, high-throughput sequencing (Illumina HiSeq 2500 via New Zealand Genomics Limited). High-throughput sequencing yielded 5,946,742 reads for Index 1, and

12,115,776 reads for Index 2. Reads with poor sequence quality and ambiguous barcodes or RAD-tag were removed, leaving 97.95% and 98.99% of reads for each Index respectively. DNA reads of approximately 125 bp were de-multiplexed into stacks of reads per individuals using the STACKS 1.01 pipeline (Catchen *et al.* 2011, Catchen *et al.* 2013). As no nuclear genome has been assembled for a neogastropod mollusc, we were not able to align our RADseq reads to a reference genome before compiling stacks. Only individuals that yielded 10 mb or more of sequence data were used, as files with fewer reads did not contain sufficient loci for analysis, meaning that only 20 out of 60 process individuals were used for analysis (Table 4.3).

TABLE 4.3
Individuals used in next generation sequencing. Row shading reflects the two Illumina libraries of 30 individuals used for ddRAD sequencing. Individuals are listed in the order that they were by annealed barcodes within library pools. Only specimens marked in the 'used for analysis' column were used for SNP analysis. A handful of specimens were original collection by the authors, however the majority of specimens were collected by a combination of National Museum of New Zealand Te Papa Tongarewa and National Institute of Water and Atmospheric Research (NIWA), and the two sampled Australian taxa (marked with an asterisk) were loaned by the Australian Museum. All specimens were newly sequenced for this thesis.

#	Taxon	Voucher ID	Origin	Used for analysis?
1	*P. chathamensis*	M.190091/1	N of Mernoo Bank, Chatham Rise	
2	*P. chathamensis*	M.190091/4	N of Mernoo Bank, Chatham Rise	
3	*P. chathamensis*	M.190091/5	N of Mernoo Bank, Chatham Rise	
4	*P. chathamensis*	M.190087/1	N of Mernoo Bank, Chatham Rise	
5	*P. chathamensis*	M.190091/7	N of Mernoo Bank, Chatham Rise	
6	*P. chathamensis*	M.190095/2	N of Mernoo Bank, Chatham Rise	
7	*P. chathamensis*	M.190085/x	N of Mernoo Bank, Chatham Rise	
8	*P. chathamensis*	M.190077/2	E of Mernoo Bank, Chatham Rise	
9	*P. chathamensis*	M.190070/2	E of Mernoo Bank, Chatham Rise	
10	*P. chathamensis*	M.190085/1	E of Mernoo Bank, Chatham Rise	
11	*P. chathamensis*	M.190077/1	E of Mernoo Bank, Chatham Rise	
12	*P. chathamensis*	M.274986/1	Mernoo Bank, Chatham Rise	
13	*P. chathamensis*	M.274986/2	Mernoo Bank, Chatham Rise	
14	*P. chathamensis*	M.274985/2	Mernoo Bank, Chatham Rise	
15	*P. chathamensis*	M.274992/2	Mernoo Bank, Chatham Rise	
16	*P. chathamensis*	M.274985/1	Mernoo Bank, Chatham Rise	
17	*P. chathamensis*	M.190108/3	N of Chatham Islands, Chatham Rise	
18	*P. chathamensis*	M.190123/1	N of Chatham Islands, Chatham Rise	
19	*P. chathamensis*	M.190108/2	N of Chatham Islands, Chatham Rise	
20	*P. chathamensis*	M.190123/2	N of Chatham Islands, Chatham Rise	
21	*P. chathamensis*	M.190356	Auckland Islands, Southland	
22	*P. chathamensis*	M.274013/1	Auckland Islands, Southland	
23	*P. chathamensis*	M.274013/2	Auckland Islands, Southland	
24	*P. chathamensis*	M.274013/4	Auckland Islands, Southland	
25	*P. chathamensis*	M.274013/5	Auckland Islands, Southland	
26	*P. fairfieldae*	M.316052/2	Karitane Canyon, Otago Peninsula, Otago	Y
27	*P. fairfieldae*	M.316051	Karitane Canyon, Otago Peninsula, Otago	
28	*P. fairfieldae*	Phoenix1	Otago Peninsula, Otago	
29	*P. chathamensis*	M.190355/1	Auckland Islands, Southland	Y
30	*P. chathamensis*	M.190355/2	Auckland Islands, Southland	Y
31	*P. chathamensis*	M.190065/2	N of Mernoo Bank, Chatham Rise	Y
32	*P. chathamensis*	M.190075/2	N of Mernoo Bank, Chatham Rise	
33	*P. chathamensis*	M.190082/1	N of Mernoo Bank, Chatham Rise	
34	*P. chathamensis*	M.190070/3	E of Mernoo Bank, Chatham Rise	Y
35	*P. chathamensis*	M.190100/3	E of Mernoo Bank, Chatham Rise	
36	*P. chathamensis*	M.274013/3	Auckland Islands, Southland	Y
37	*A. benthicola*	M.190102/1	E of Mernoo Bank, Chatham Rise	
38	*A. benthicola*	M.190102/3	E of Mernoo Bank, Chatham Rise	
39	*A. benthicola*	M.190130	E of Mernoo Bank, Chatham Rise	
40	*A. benthicola*	M.190068/1	N of Mernoo Bank, Chatham Rise	
41	*A. benthicola*	Phoenix-Z1	Chatham Rise	
42	*A. benthicola*	M.190068/2	N of Mernoo Bank, Chatham Rise	
43	*A. benthicola*	M.190073	N of Mernoo Bank, Chatham Rise	
44	*A. benthicola*	M.274268	Cape Kidnappers, Hawke's Bay	

45	*A. benthicola*	Phoenix-W1	Wairarapa, Wellington	
46	*P. c. cuvierianus*	M.183792/1	Red Mercury Island, Coromandel, Waikato	Y
47	*P. c. cuvierianus*	M.183927	Mayor Island, Bay of Plenty	Y
48	*P. c. cuvierianus*	Phoenix1	Auckland	Y
49	*P. c. jeakingsi*	M.279432/1	Tasman Bay, Nelson	Y
50	*P. c. jeakingsi*	M.279432/2	Tasman Bay, Nelson	Y
51	*P. c. jeakingsi*	M.279432/3	Tasman Bay, Nelson	Y
52	*P. c. jeakingsi*	M.279432/4	Tasman Bay, Nelson	Y
53	*P. c. jeakingsi*	M.279432/5	Tasman Bay, Nelson	Y
54	*P. c. jeakingsi*	M.279432/10	Tasman Bay, Nelson	
55	*P. mandarinus*	C.487643*	Gabo Island, Victoria, Australia	Y
56	*P. maximus*	C.450316*	Terrigal, New South Wales, Australia	Y
57	*P. ormesi*	M.299869/1	White Bluffs, Cloudy Bay, Marlborough	Y
58	*P. sulcatus*	Phoenix9	Auckland	Y
59	*P. sulcatus*	Phoenix11	Mahia Peninsula, Hawkes Bay	Y
60	*P. sulcatus*	Phoenix10	Karaka Bay, Wellington, Wellington	Y

For the results presented, settings for the de novo assembly using STACKS were -m3 -N7 -M5 –n15 to focus on variation between species. We used an optimum coverage of 3 reads per individual (excluding stacks with lower coverage; -m3), allowing for a maximum of five mismatches between alleles from a single individual (-M5), and seven mismatches between primary and secondary reads within the de novo assembly pipeline (-N7). Fifteen mismatches were allowed between loci when building the catalogue among multiple individuals (-n15). We assume that the combination of mismatches for loci (e.g. –n15) does mask some intraspecific variation. Previous studies investigating multiple congeneric species have used similar settings (e.g. Wagner *et al.* 2013, Dowle *et al.* 2014, Dowle *et al.* 2015, Mastretta-Yanes *et al.* 2015, Rodríguez-Expeleta *et al.* 2016). We aimed to use settings that balanced the number of loci and individuals available for analysis, which is an important trade-off to consider for species delimitation (Leaché *et al.* 2014, Takahashi and Moreno 2015, Pante *et al.* 2015, Herrera and Shank 2016). It is common for the number of available nuclear loci to be low between highly divergent species (Pante *et al.* 2015), however a small number of loci can be adequate to distinguish genetic lineages (Leaché *et al.* 2014). Likewise, large numbers of individuals are not always necessary to identify genetic differentiation among species, and a small number of individuals (n = 4) can suitable (Willing *et al.* 2012). Outputs of the STACKS pipeline were then used to populate a MySQL database (Oracle Corporation) for subsequent interrogation of the data.

We analysed single-nucleotide polymorphic (SNP) variation among groups of interest (i.e. putative species) for our anonymous nuclear loci using the populations program within STACKS. For each dataset, we assumed only one ‘population’ among all sampled individuals (-p1), so that any identified SNP variation that supported groups was acquired naïvely in the absence of any *a priori* hypothesis. This setting also made it more likely that loci analysed were represented in more than one species (especially in our dataset with few individuals per species), which is desirable for investigation of interspecific genetic variation.

We analysed variation among individuals hierarchically using several investigative groups. The first group included all sampling from monophyletic *Penion* from Australian and New Zealand. The second group contained only taxa from the monophyletic New Zealand clade. These were used to investigate species delimitation between closely related species (based on phylogenetic results), with one group containing specimens of *P. chathamensis, P. fairfieldae* (Powell, 1947) and *P. c.*

cuvierianus, and another including *P. c. cuvierianus*, *P. ormesi* and *P. c. jeakingsi*. For each investigative group (dataset), we adjusted the stringency for the representation of loci among individuals within a putative population (-r 0.33 – 0.9), as setting higher representation yielded fewer loci but high assignment probabilities (see below). This means that multiple SNP datasets with variable numbers of loci were produced for each group of specimens (Supplementary Table 4.1). To avoid confounding variation due to genetic linkage, all analyses used only the first SNP of each locus (--write_single_snp; e.g. Dowle *et al.* 2014, Dowle *et al.* 2015, Mastretta-Yanes *et al.* 2015, Rodríguez-Expeleta *et al.* 2016).

Using individual snail genotypes, we inferred population structure and identified the optimal number of genetic clusters using the Bayesian assignment approach of STRUCTURE 2.3.4 (Falush *et al.* 2007), which is considered to be a suitable program for analysing RAD sequencing results with many loci or numerous species (Choi 2016). We used an admixture model with correlated frequencies to investigate genetic structure among individuals. For each dataset we followed the recommended settings for STRUCTURE (Falush *et al.* 2003, Pritchard *et al.* 2010), with 10 iterations of each number of groups (K, ranging from 1 – 9) using an MCMC length of 100,000 generations with a burn-in of 10,000. Similar MCMC settings have been used for this type of study (e.g. Evanno *et al.* 2005, Coulon *et al.* 2006, Porras-Hurtado *et al.* 2013, Dowle *et al.* 2014), although sometimes longer chain lengths are used (e.g. Coulon *et al.* 2006, Cornille *et al.* 2012, Dowle *et al.* 2015, Fuchs *et al.* 2015, Rodríguez-Expeleta *et al.* 2016), particularly for values of K that are of especial interest (e.g. Coulon *et al.* 2006). Authors frequently test 10 iterations of each value of K (e.g. Cornille *et al.* 2013, Rodríguez-Expeleta *et al.* 2016). Convergence of Bayesian parameters was investigated using trace plots produced within STRUCTURE. Results from STRUCTURE were examined using STRUCTURE HARVESTER 0.6.94 (Earl and von Holdt 2012), before being averaged across the 10 replications using CLUMPP 1.1.2 (Jakobsson and Rosenberg 2007). Output genotype structure graphs were generated in DISTRUCT 1.1 (Rosenberg 2004).

Results

Phylogenetics

We assembled new mitochondrial genomes and nuclear 45S rDNA sequences (18S, 5.8S, 28S rRNA genes) from 7 individuals representing 6 putative taxa (Table

4.1). We also PCR amplified and Sanger sequenced 19 further individuals, targeting the mtDNA 16S rRNA and *cox1* genes (Table 4.2). All sequenced mtDNA genomes contained the standard gene complement and order described for previously sequenced neogastropod species (Simison *et al.* 2006, Cunha *et al.* 2009, Hills *et al.* 2011; Chapter 3). Assembled mtDNA genome sequences varied between 15,227 and 15,238 bp in length, and nuclear rDNA sequences were all 5339 bp in length (prior to any trimming). Sequences appeared to carry similar levels of phylogenetic information as estimated in the previous investigation of Buccinulidae (Chapter 3; composition details in Supplementary Tables 4.2 and 4.3).

For mtDNA sequences, gblocks retained 99% of the original mtDNA protein-encoding nucleotide positions, and 80% and 83% of the mitochondrial tRNA and rRNA positions respectively. This analysis resulted in sequence lengths of 9399 bp, 945 bp and 1019 bp respectively for mtDNA protein-encoding, tRNA and rRNA sequence regions. Most (99%) of the nuclear rDNA (excluding ITS1 and 2) nucleotide positions were also retained, leaving an alignment sequence length of 4673 bp available for phylogenetic reconstruction.

Phylogenetic relationships inferred separately from mtDNA and nuclear rDNA data were broadly similar (Figures 4.2 and 4.3). Bayesian and maximum-likelihood trees were also mostly consistent with one another (Figures 4.2 and 4.3, Supplementary Figures 4.3 and 4.4). However, the phylogenetic placement of some individuals (e.g. the single specimen of *P.* n. sp. Three Kings Islands) varied between the mtDNA and nuclear rDNA trees (Figures 4.2 and 4.3). This difference is most likely is due to the shorter sequence length, smaller number of variable sites, and low level of phylogenetic signal provided by the rDNA sequence alignment (see Chapter 3). When investigated as a splits network it is apparent that the phylogenetic signal within mtDNA is much more constrained than in the rDNA data (Supplementary Figures 4.1 and 4.2). Multiple alternative evolutionary relationships are evident within the rDNA data (Supplementary Figure 4.2).

Phylogenetic analyses using mtDNA and nuclear rDNA sequence data showed concordance with the previous study of Buccinulidae (Chapter 3), that most *Penion* form a monophyletic clade sister to *Kelletia* and *Antarctoneptunea* (Figures 4.2 and 4.3). However, *P. benthicolus* is not part of the *Penion* clade, but instead groups with *Antarctonetpunea aurora* (Figure 4.4; in agreement with Chapter 3). Australian (*P. mandarinus, P. maximus*) and New Zealand taxa (all other species) form two separate

clades within *Penion* (Figures 4.2 and 4.3). Both mtDNA and rDNA sequence data supported a clade including *P. sulcatus, P. chathamensis* and *P. fairfieldae*, although posterior support was low for mtDNA data (Figures 4.2 and 4.3). Phylogenies with higher sampling of individuals, based on sequence alignments of mtDNA *cox1*, 16S rRNA and nuclear rDNA 28S rRNA genes, presented relationships similar to the overall mtDNA and nuclear rDNA trees (Figures 4.4 and 4.5, Supplementary Figure 4.5). Where multiple individuals from the same species were sampled they were found to be genetically clustered in most cases (Figures 4.4 and 4.5, Supplementary Figure 4.5).

FIGURE 4.2
Evolutionary relationships among marine snail species illustrated with a Bayesian phylogeny based on an alignment of 22 concatenated mitochondrial genome (incorporating protein-encoding, tRNA and rRNA genes) sequences. Two sequence partitions were used: 1) protein-encoding genes (9339 bp), and 2) tRNA and rRNA genes (1964 bp) using the GTR + I + G and HKY + I + G substitution models respectively. No outgroup or monophyly was enforced for this tree. BEAST 1.8.3 using and MCMC length of 100 million, 1000 sample frequency and a 10% burn-in was used to generate this phylogeny. Posterior support values are also shown at nodes, but only if support was less than 1.0. The full list of parameters used provided in the text.

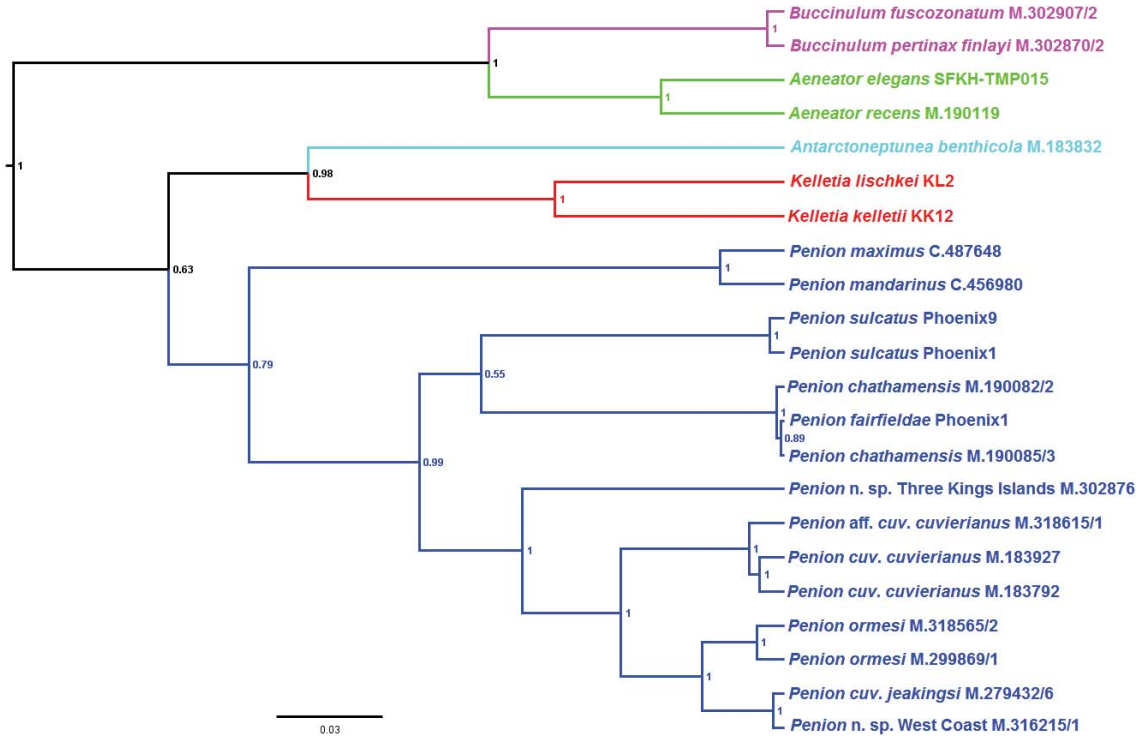


FIGURE 4.3
Evolutionary relationships among marine snail species illustrated with a Bayesian phylogeny based on a 4673 bp alignment of 22 concatenated nuclear rDNA sequences (18S, 5.8S, 28S rRNA genes). Sequence data was not partitioned and the GTR + I + G substitution model was used. No outgroup or monophyly was enforced for this tree. BEAST 1.8.3 using and MCMC length of 100 million, 1000 sample frequency and a 10% burn-in was used to generate this phylogeny. Posterior support values are also shown at nodes, but only if support was less than 1.0. The full list of parameters used provided in the text.

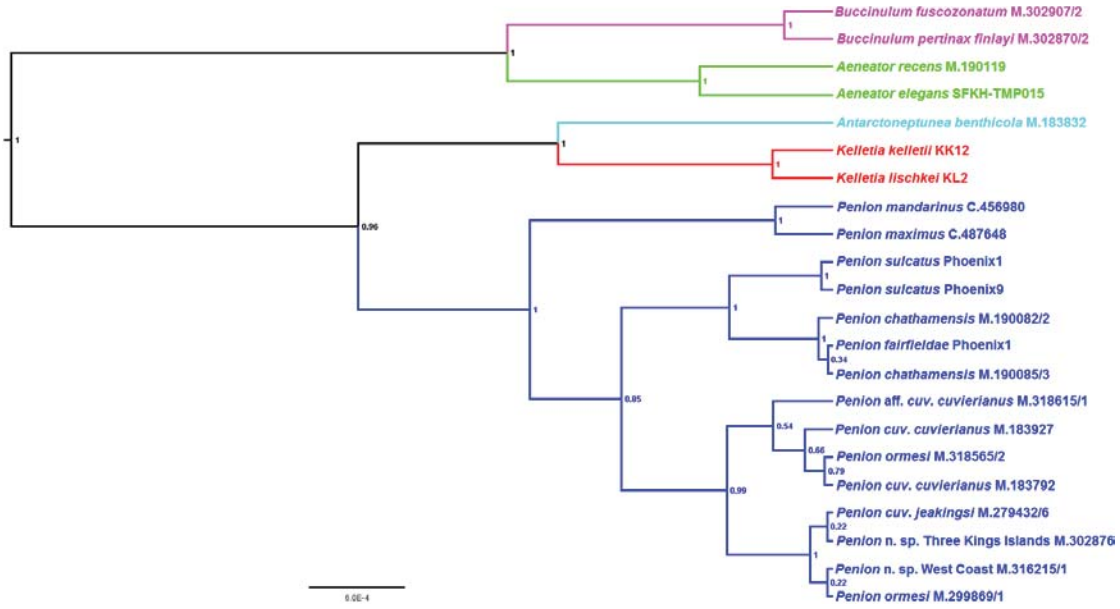


FIGURE 4.4
A Bayesian phylogeny based on a 492 bp alignment of 37 mitochondrial *cox1* gene sequences. The GTR + I + G substitution model was used, and no outgroup or monophyly was enforced for this tree. The phylogeny was produced using a Bayesian method (100 million MCMC, 10% burn-in, 1000 sample frequency, node labels are posterior support values), via BEAST 1.8.3.

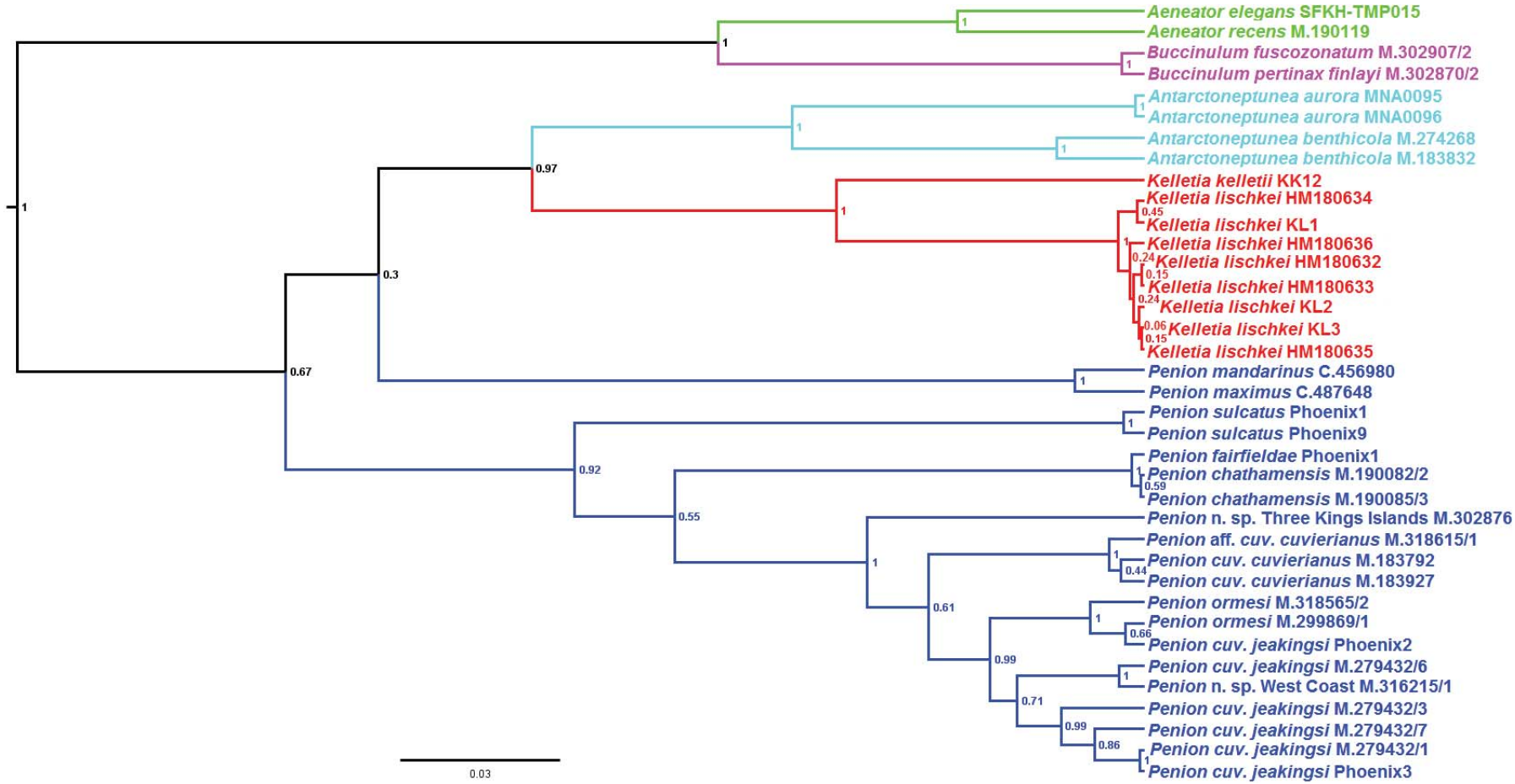


FIGURE 4.5
A Bayesian phylogeny based on a 243 bp alignment of 44 mitochondrial 16S RNA gene sequences. The GTR + I + G substitution model was used, and monophyly was enforced for *Aeneator* Finlay, 1926 and *Buccinulum* Deshayes, 1830, and *Antarctoneptunea*, *Penion* and *Kelletia* as two separate clades. The phylogeny was produced using a Bayesian method (100 million MCMC, 10% burn-in, 1000 sample frequency, node labels are posterior support values), via BEAST 1.8.3.

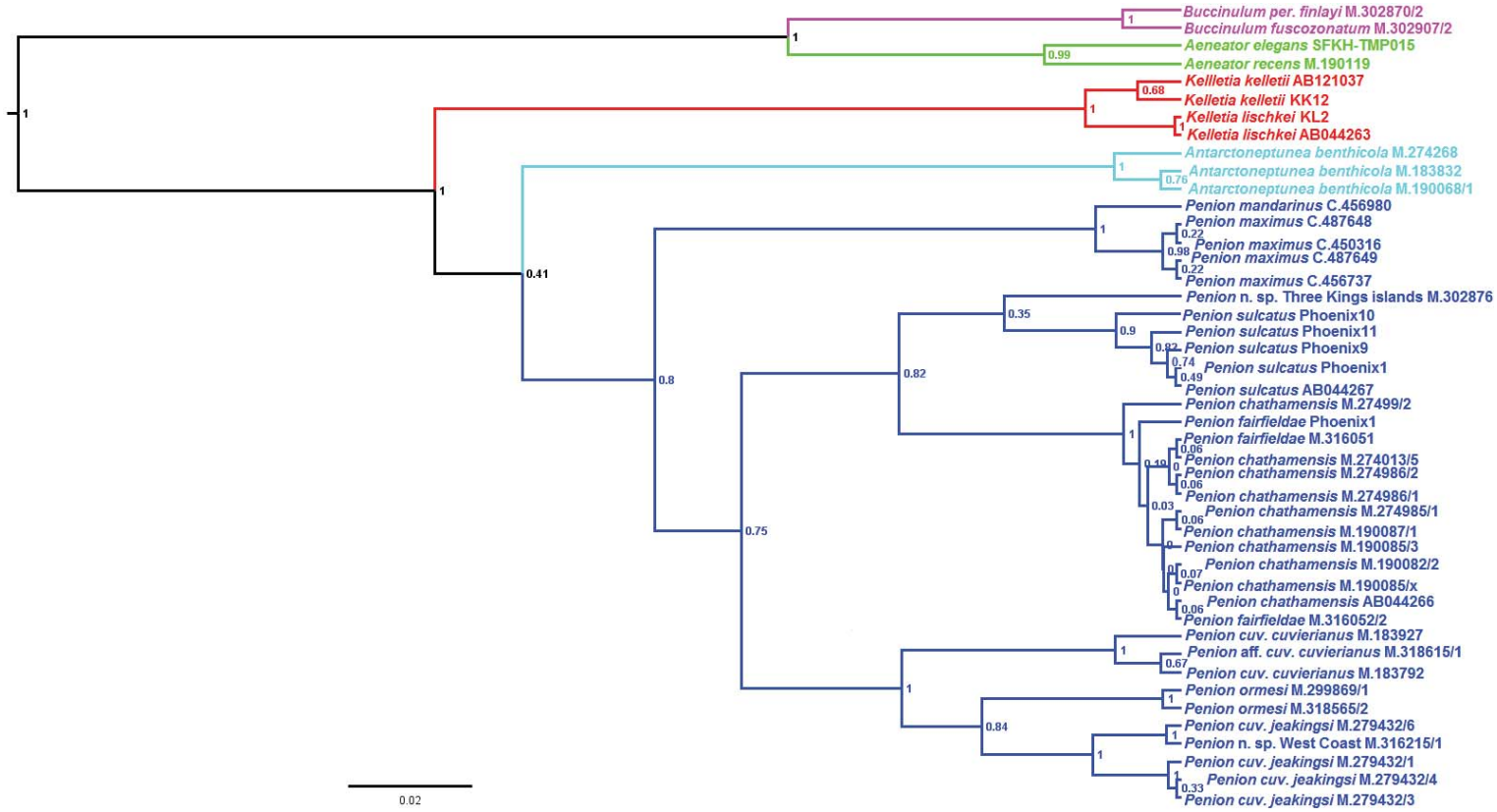
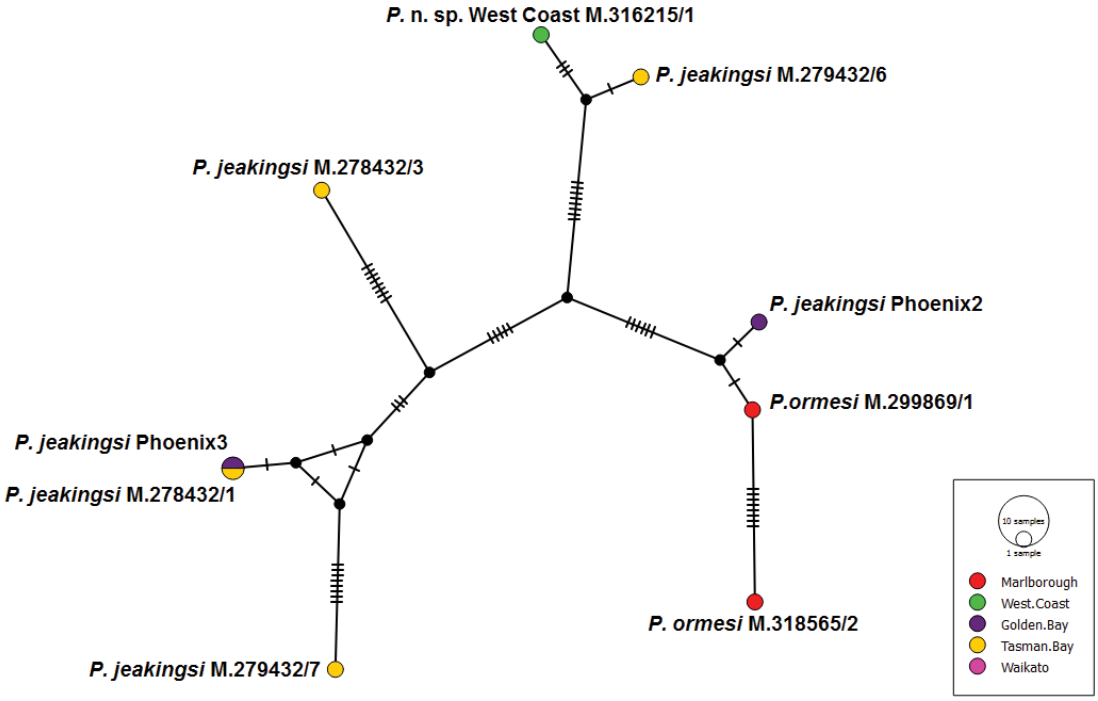


FIGURE 4.6
Genetic variation among 9 individuals classified as *Penion ormesi*, *P. cuvierianus jeakingsi* and *P.* n. sp. West Coast. The median joining haplotype network (Bandelt *et al.* 1999), is derived a fragment of the mitochondrial *cox1* gene (490 bp). The diagram was generated using PopArt 1.7 (Leigh and Bryant 2015), to investigate Short bisecting lines between individuals indicate estimated substitution events between sequences. The size of circles indicates the number of individuals that share the same haplotype, and the colouration of individual sequences reflects geographic location (key shown in figure).



SNP analysis of RAD sequencing reads

ddRAD sequencing yielded nuclear DNA sequence data from 20 individuals of 8 putative taxa (Table 4.3). High-throughput sequencing returned 5,946,742 reads for Index 1, and 12,115,776 reads for Index 2. Datasets with different numbers of loci (dependent on stringency settings described in methods) were available for each investigative group of interest (Supplementary Table 4.3).

For all 20 sampled individuals of *Penion* under stringent settings (-r 0.9 with 17 loci; meaning that each locus had to be present in at least 18 of the individuals), two clusters were identified as being optimal according to the delta K statistic (Figure 4.7, Supplementary Figure 4.4). These two clusters corresponded to the division of Australian (*P. mandarinus, P. maximus*) and the monophyletic New Zealand *Penion* species (Figure 4.7). Under the K = 4 model, four groups could be identified corresponding to: 1) *P. mandarinus* and *P. maximus*, 2) *P. sulcatus*, 3) *P. chathamensis* and *P. fairfieldae*, and 4) *P. c. cuvierianus, P. c. jeakingsi* and *P. ormesi* (Figure 4). Lower stringency datasets had reduced structure among specimens but the optimal number of clusters presented concordant patterns (Figure 4.4). For sampling of just the monophyletic New Zealand *Penion* under stringent settings (-r 0.9 with 36 loci), five clusters were identified as being optimal according to the delta K statistic (Figure 4.8, Supplementary Figure 4.5). These five clusters corresponded closely to each of the species sampled, with the exception of *P. fairfieldae* that was not distinguished from *P. chathamensis* (Figure 4.5). Lower stringency datasets had reduced structure among specimens but the optimal number of clusters presented concordant patterns (Figure 4.5).

To investigate *P. chathamensis, P. fairfieldae* and *P. c. cuvierianus* data from stringent STACKS settings (-r 0.9 with 89 loci) supported two clusters (Figure 4.9). These clusters distinguished *P. c. cuvierianus* from *P. chathamensis* and *P. fairfieldae*, which were not separated. Results at lower stringency settings were mostly consistent, although *P. c. cuvierianus* was distinguished less clearly as more loci were included (Figure 4.9). *Penion c. cuvierianus* was chosen instead of *P. sulcatus* to test the signal for the relationship of *P. chathamensis* and *P. fairfieldae* because this provided datasets with more loci (at –r 0.9 89 loci with *P. c. cuvierianus* versus 74 loci with *P. sulcatus*).

For the second investigative group including specimens of *P. c. cuvierianus, P. c. jeakingsi* and *P. ormesi* under moderately stringent settings (-r 0.75 with 249 loci), four clusters were identified as optimal (Figure 4.10). These clusters correspond to the distinction of each taxon, although one specimen of *P. c. cuvierianus* (M.183927 from

the Bay of Plenty) was separated from the other individuals of that species (Figure 4.10). Output from the three cluster models distinguished the three taxa (Figure 4.10), and results were mostly similar at lower STACKS stringency settings (Figure 4.10). Results at the highest stringency setting (-r 0.9 with 39 loci) were inconsistent, seemingly because few alleles were shared among individuals, which was difficult to resolve using STACKS settings when only 9 individuals were sampled (Figure 4.10).

The number of loci available for analysis at high STACKS stringency settings tended to be low, but the ready distinction of most taxa indicates sufficient data to investigate species delimitation. Similar findings have been reported by other RADseq studies with low numbers and few individuals per putative species (e.g. Leaché *et al.* 2014, Pante *et al.* 2015), and simulated investigations have predicted such sample sizes to be adequate (e.g. Willing *et al.* 2012).

FIGURE 4.7
Optimal clustering of individuals based on SNP variation for sequenced anonymous loci among the 20 sampled individuals of monophyletic *Penion* from Australia and New Zealand (bar diagrams on left). Individuals are separated as columns along the X-axis of each plot, and the colour and proportion of each column represents the assignment probability of SNP variation to clusters that have been estimated by STRUCTURE. The optimal number of clusters identified (K) for each dataset where the representation of a locus among specimens was varied (-r 0.9 with 17 loci; 0.5 with 1884 loci, 0.33 with 3712 loci). Clustering was estimated via Bayesian assignment using STRUCTURE 2.3.4. Plots on the left show the optimal number of clusters (K; marked with an asterisk on the left-hand cluster plots) that was identified using the delta K statistic by STRUCTURE HARVESTER 0.6.94 for each presented dataset. Higher delta K values indicate higher support for numbers of clusters (K) estimated from the SNP data.

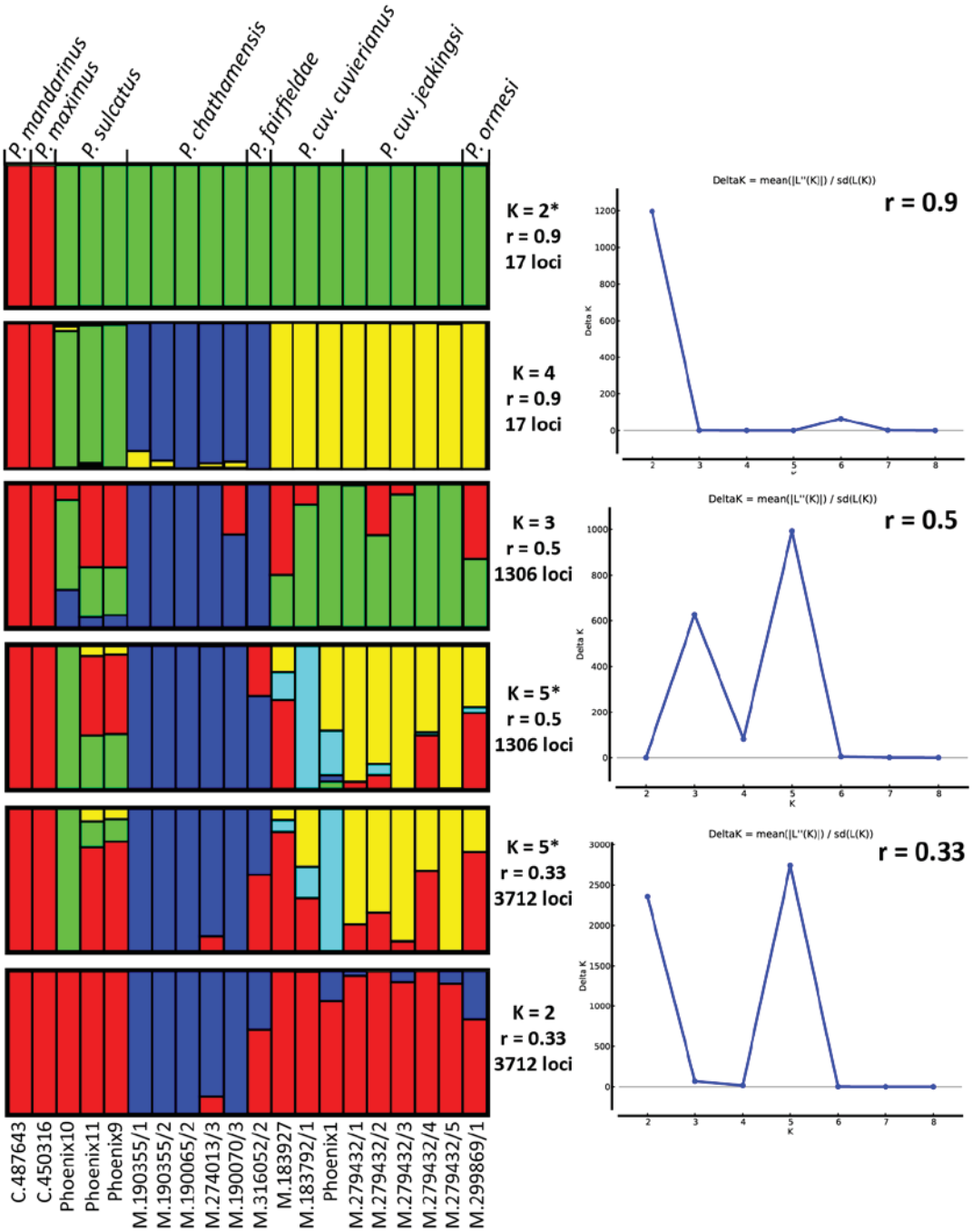


FIGURE 4.8
Optimal clustering of individuals based on SNP variation for sequenced anonymous loci among the 18 sampled individuals of monophyletic *Penion* from New Zealand (bar diagrams on left). Individuals are separated as columns along the X-axis of each plot, and the colour and proportion of each column represents the assignment probability of SNP variation to clusters that have been estimated by STRUCTURE. Different plots show the number of clusters identified (K) for each dataset where the representation of a locus among specimens was varied (-r 0.9 with 36 loci; 0.5 with 1885 loci, 0.33 with 5191 loci). Clustering was estimated via Bayesian assignment using STRUCTURE 2.3.4. Plots on the left show the optimal number of clusters (K; marked with an asterisk on the left-hand cluster plots) that was identified using the delta K statistic by STRUCTURE HARVESTER 0.6.94 for each presented dataset. Higher delta K values indicate higher support for numbers of clusters (K) estimated from the SNP data.

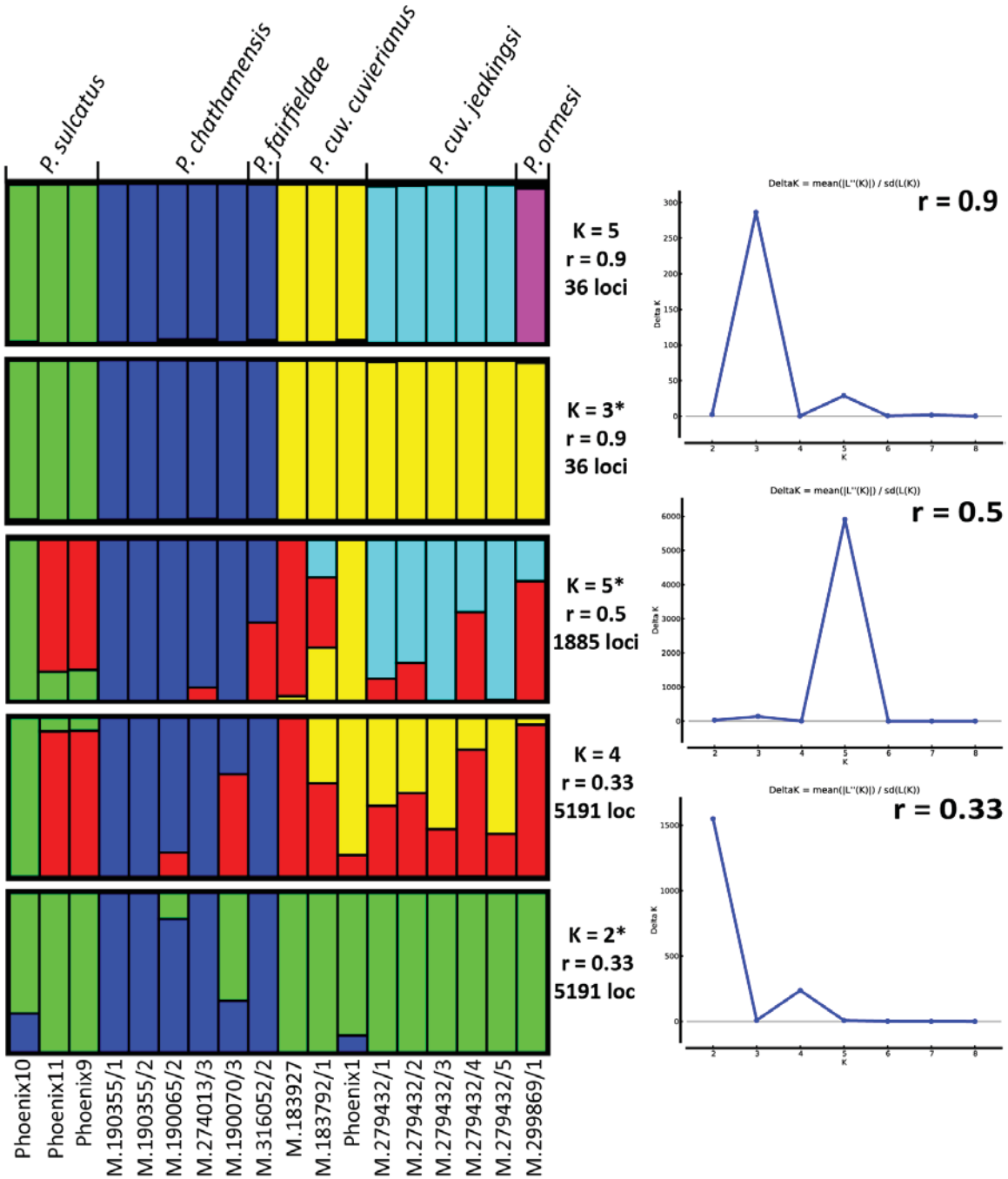


FIGURE 4.9
Optimal clustering of individuals based on SNP variation for sequenced anonymous loci among the 9 sampled individuals of *P. chathamensis*, *P. fairfieldae* and *P. c. cuvierianus* (bar diagrams on left). Individuals are separated as columns along the X-axis of each plot, and the colour and proportion of each column represents the assignment probability of SNP variation to clusters that have been estimated by STRUCTURE. Different plots show the number of clusters identified (K) for each dataset where the representation of a locus among specimens was varied (-r 0.9 with 89 loci; 0.5 with 3732 loci, 0.33 with 9013 loci). Clustering was estimated via Bayesian assignment using STRUCTURE 2.3.4. Plots on the left show the optimal number of clusters (K; marked with an asterisk on the left-hand cluster plots) that was identified using the delta K statistic by STRUCTURE HARVESTER 0.6.94 for each presented dataset. Higher delta K values indicate higher support for numbers of clusters (K) estimated from the SNP data.

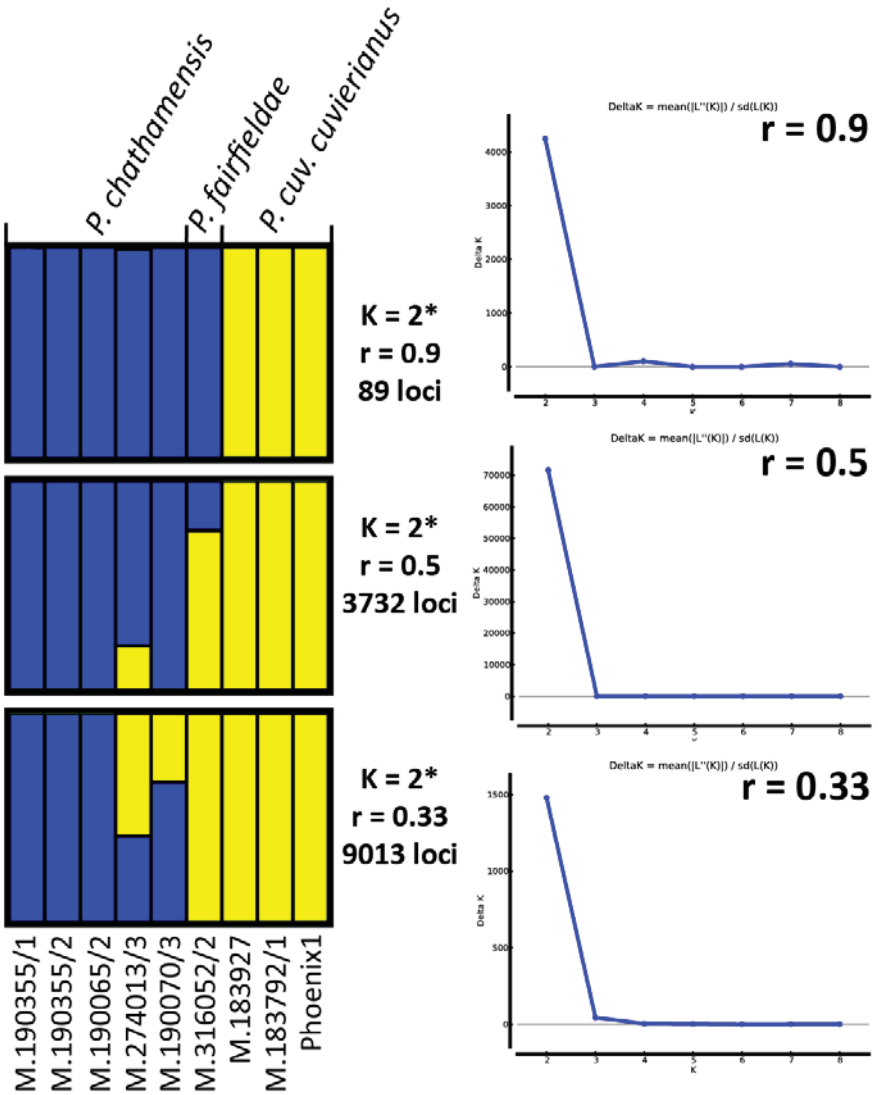
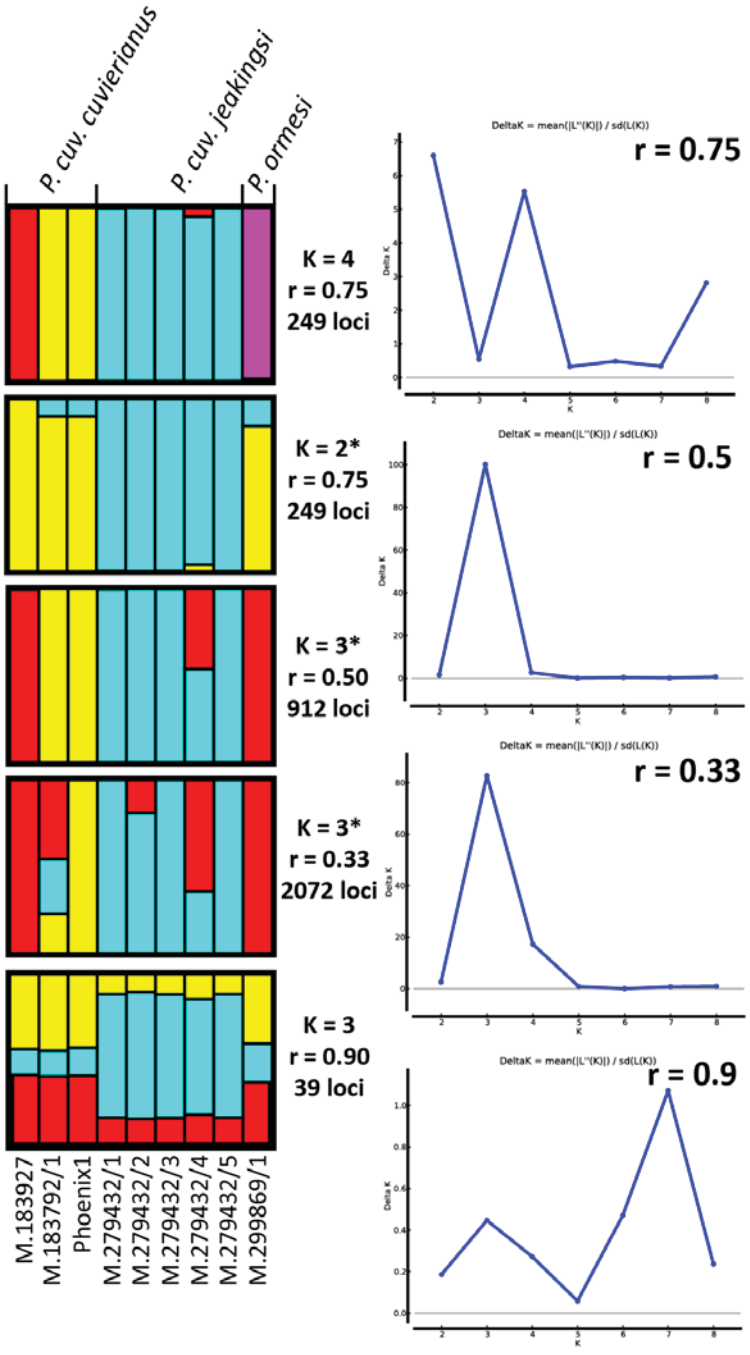


FIGURE 4.10
Optimal clustering of individuals based on SNP variation for sequenced anonymous loci among the 10 sampled individuals of *P. c. cuvierianus*, *P. c. jeakingsi*, and *P. ormesi* (bar diagrams on left). Individuals are separated as columns along the X-axis of each plot, and the colour and proportion of each column represents the assignment probability of SNP variation to clusters that have been estimated by STRUCTURE. Different plots show the number of clusters identified (K) for each dataset where the representation of a locus among specimens was varied (-r 0.9 with 39 loci; 0.75 with 249 loci; 0.5 with 912 loci, 0.33 with 2072 loci). Clustering was estimated via Bayesian assignment using STRUCTURE 2.3.4. Plots on the left show the optimal number of clusters (K; marked with an asterisk on the left-hand cluster plots) that was identified using the delta K statistic by STRUCTURE HARVESTER 0.6.94 for each presented dataset. Higher delta K values indicate higher support for numbers of clusters (K) estimated from the SNP data.



Discussion

We aimed to investigate the evolution of *Penion* using molecular data. Phylogenetic results from mtDNA and nuclear rDNA sequence data demonstrate that (with the exception of *P. benthicolus*) the majority of New Zealand *Penion* form a reciprocally monophyletic group with Australian taxa (Figures 4.2 and 4.3). This study indicates that at least six living *Penion* species are present in New Zealand waters. At least two of these genetically supported taxa (*P. sulcatus* and *P. ormesi*) have recognised fossil records in New Zealand (Beu and Maxwell 1990). However, phylogenetic analysis questions the validity of some currently recognised taxa (e.g. *P. fairfieldae*; Figures 4.2 – 4.5), which are discussed below.

Analysis of SNP variation indicated that there were substantial genotypic differences between *Penion* from Australia and New Zealand (Figure 4.7). These differences are not surprising, given that the last common ancestor of the two regions was estimated to have a median age of 68 Ma (Chapter 3). Regardless of the stringency of STACKS settings used to filter data, *P. mandarinus* and *P. maximus* together were always distinguished from New Zealand taxa (Figure 4.7). For the highest stringency dataset (-r 0.9) using just 16 loci, three were identified within New Zealand: *P. sulcatus*; *P. chathamensis* and *P. fairfieldae*; *P. c. cuvierianus, P. c. jeakingsi* and *P. ormesi* (Figure 4.7). Results from phylogenetic analysis of mtDNA and nuclear rDNA sequence data and ddRAD SNP data were consistent with one another (Figures 4.2 and 4.3).

Penion sulcatus

Previous studies (Hayashi 2005, Chapter 3), placed *P. sulcatus* sister to other New Zealand *Penion*, but denser sampling of the clade reveals *P. sulcatus* as sister to *P. chathamensis* (Figures 4.2 and 4.3). However, although nuclear rDNA sequence data had high support for this relationship (Figure 4.3), the posterior probability for this relationship was low for the Bayesian mtDNA tree (Figure 4.2), and the same relationship was not shown by the mtDNA maximum-likelihood tree (Supplementary Figure 4.3). The lack of resolution for this relationship in the mtDNA data is likely due to conflicting phylogenetic signal (see Supplementary Figure 4.1). For anonymous nuclear loci, individuals of *P. sulcatus* were readily distinguished from other New Zealand taxa (Figure 4.8). Analysis at low stringency (-r 0.5 with 1306 loci) also indicated that *P. sulcatus* shared alleles (SNPs) with *P. mandarinus* and *P. maximus* (Figure 4.7).

Analysis of SNP variation with reduced stringency settings (-r 0.5), with 1306 and 1885 loci for the overall and New Zealand monophyletic *Penion* datasets respectively, indicated that considerable genotypic variation among the three *P. sulcatus* individuals (Figures 4.7 and 4.8). This variation was not observed for high stringency settings with and without Australian taxa. The level of intraspecific genetic variation within *P. sulcatus* is consistent with the broad geographic range of the species and former identification of multiple subspecies and morphotypes (Powell 1927, Powell 1947, Ponder 1973, Powell 1979, Willan *et al.* 2010).

Penion chathamensis and *Penion fairfieldae*

Genetic data cannot distinguish between our sample of *P. fairfieldae* and individuals of *P. chathamensis*, with very low sequence variation evident (Figures 4.2 – 4.5, Supplementary Figure 4.1, Supplementary Tables 4.2 and 4.3). Whole mtDNA sequence data from two *P. chathamensis* and one *P. fairfieldae* individuals had a mean pair-wise site variability of 3.6%. Analysis of SNP variation at 89 anonymous nuclear loci from five *P. chathamensis* and one *P. fairfieldae* also failed to identify variation consistent with the two taxa (Figures 4.7 – 4.9). Given the geographic range of our sampling (sites >480k m apart; Figure 4.1; Tables 4.1 – 4.3), the low level of genetic variation detected is notable. Analysis of shell morphology is needed to assess whether the differences used to distinguish the shells of these two species are reliable (Chapter 6).

Penion n. sp. Three Kings Islands

The mitochondrial genome and nuclear ribosomal 45S cassette was sequenced for a single individual of a putative new species identified from waters off the Three Kings Islands. mtDNA sequence data indicate a distinct lineage (Figure 4.2), whereas nuclear rDNA derived trees placed *P.* n. sp. Three Kings Islands in a polytomy with taxa closely related to *P. c. cuvierianus* (Figure 4.3). Analysis of mtDNA *cox1* with higher sampling from some other species matched the overall mtDNA tree (Figure 4.4), whereas trees based on the mtDNA 16S rRNA and nuclear rDNA 28S genes presented scenarios similar to the overall nuclear rDNA tree (Figure 4.5, Supplementary Figure 4.1). The relationships presented by 16S rRNA and nuclear ribosomal data may reflect a lack of phylogenetic information, as these genes evolve more slowly and stochastically than protein-encoding mtDNA genes (Simon *et al.* 1996, e.g. Donald *et al.* 2015).

However, it also possible that genetic introgression has occurred between *P.* n. sp. Three Kings and *P. c. cuvierianus*. Previous work on Buccinulidae demonstrated that the phylogenetic information carried by the 16S rRNA gene is low for comparisons at the species-level (see Supplementary Figure 3.1). We did not obtain ddRAD data from the Three Kings specimen.

Due to the remoteness of the region and difficult-to-navigate waters, it is unlikely that further individuals of *P.* n. sp. Three Kings Islands suitable for molecular sequencing will be collected in the near future. On the basis of mtDNA results (Figures 4.2 and 4.3), it seems appropriate to consider *P.* n. sp. Three Kings Islands as a new, genetically distinct species of siphon whelk subject to future research with other nuclear markers to test alternative phylogenetic signal (Figure 4.3), and morphometric analysis (Chapter 6). In particular, given the results from the more conserved gene regions it needs to be tested whether the shells of this putative species can be differentiated from *P. c. cuvierianus* (Figures 4.3, 4.5, Supplementary Figure 4.1).

Penion c. cuvierianus, Penion c. jeakingsi, P. aff. *c. cuvierianus*

We investigated the species *P. cuvierianus* that is currently divided in two subspecies (*P. c. cuvierianus, P. c. jeakingsi*) and an additional morphological variant identified in the course of this work (*P.* aff. *c. cuvierianus*). Two individuals of *P. c. cuvierianus,* and one individual each of *P. c. jeakingsi* and *P.* aff. *c. cuvierianus* were included for our phylogenetic analysis of mtDNA and nuclear rDNA sequences. The *P. c. jeakingsi* specimen had a genetically distinct mtDNA lineage but *P.* aff. *c. cuvierianus* and *P. c. cuvierianus* could not be distinguished (Figures 4.2 and 4.3). Based on mtDNA sequence data (from 1 – 6 specimens) *P. c. jeakingsi* is more closely related to *P. ormesi* than *P. c. cuvierianus* (Figures 4.2 and 4.3), although some nuclear rDNA data struggle to provide little signal differentiating *P. c. cuvierianus, P. c. jeakingsi, P. ormesi,* and *P.* n. sp. West Coast (e.g. Supplementary Figure 4.1). This is likely due to recent evolutionary divergence within the clade (and/or hybridisation) and low phylogenetic resolution in the nuclear rDNA data for the *Penion* lineage. Relationships are also likely affected by the low phylogenetic information carried by nuclear rRNA genes for species-level comparisons (see Chapter 3).

Analysis of SNP variation for anonymous nuclear loci in three individuals classified as *P. c. cuvierianus* and six *P. c. jeakingsi* readily distinguished the two taxa (-r 0.9 with 36 loci; Figure 4.8), although shared alleles could be observed at lower

tested stringencies (-r 0.33 – 0.5 with 5191 – 1885 loci; Figure 4.8). Notably, in a dataset including these taxa and one individual of *P. ormesi*, all three taxa were readily distinguished (-r 0.75 with 249 loci; Figure 4.10). Thus mtDNA and nuclear data are concordant: *P. c. cuvierianus* and *P. c. jeakingsi*, can be distinguished genetically from one another.

In previous taxonomic reviews, differences in shell morphology linked with geographic restriction have been assumed to provide accurate estimation of evolutionary relationships (e.g. Ponder 1973, Powell 1979). However, results here indicate that *P.* aff. *c. cuvierianus* despite being morphologically conspicuous and occurring at the western-most extreme of the range of *P. c. cuvierianus*, is closely related to typical specimens of *P. c. cuvierianus* (Figures 4.2 and 4.3). Perhaps *P.* aff. *c. cuvierianus* represents a recent speciation, and the genetic markers sequenced in this study have not yet diverged or achieved reciprocal monophyly (Shaffer and Thomson 2007, e.g. Sturge *et al.* 2016). Alternatively, *P.* aff. *c. cuvierianus* may represent morphological plasticity within the species that is responsive to local environmental conditions. Nuclear SNP variation did indicate substantial intraspecific variation among three individuals of *P. c. cuvierianus*, but *P.* aff. *cuvieranus* was not sampled (Figures 4.7 and 4.8, 4.10). Morphological analysis of *P.* aff. *c. cuvierianus* and *P. c. cuvierianus* would be informative (Chapter 6). More individuals of both taxa – especially within the Northland region, also need to be sequenced for mtDNA and nuclear DNA to see if any consistent genetic differences can be identified, even if they are small.

In contrast, individuals of *P. c. jeakingsi* could be distinguished from *P. c. cuvierianus* under certain stringency settings (Figures 4.7 and 4.8, 4.10) despite morphological variation among northern and southern populations appearing to overlap (Powell 1947, Powell 1979), and the two taxa being challenging to differentiate in absence of geographic data. The shell morphology of these taxa is said to differ in terms of maximum shell size, the prominence of axial ribs and the angle of teleoconch spire whorls (Powell 1947, Powell 1979). Research is now needed to test if these differences are sufficient to distinguish *P. c. cuvierianus* from *P. c. jeakingsi* (Chapter 6). For soft-body anatomy, *P. c. jeakingsi* has been noted to have a distinctive central radula tooth compared to *P. c. cuvierianus* (Powell 1979), but this trait is not suitable for the swift identification of live specimens or fossils. The investigation of these three taxa demonstrates the potential of molecular data to aid our interpretation of morphological variation.

Penion ormesi, *Penion c. jeakingsi*, *P.* n. sp. West Coast

Phylogenetic analysis of mtDNA and nuclear rDNA sequence data indicated that one individual each of *P. c. jeakingsi* and *P.* n. sp. West Coast in the available sample were closely related to two individuals of *P. ormesi* (Figures 4.2 and 4.3). This pattern was also apparent in analysis of multiple individuals of *P. c. jeakingsi* and *P. ormesi* for mtDNA *cox1* and 16S rRNA (Figures 4.4 and 4.5), and no clear taxonomic or geographic patterns were apparent in a *cox1* haplotype network (Figure 4.6). We included three specimens identified as *P. c. cuvierianus,* six *P. c. jeakingsi* and one individual of *P. ormesi* in the nulclear SNP dataset, but these taxa could not be distinguished (Figure 4.7). Low stringency settings (-r 0.33 – 0.5 with 5191 – 1885 loci) suggested a mixture of shared SNP alleles among these snails (Figure 4.10). When SNP variation was analysed with three indidivuals of *P. c. cuvierianus,* results indicated that the three taxa of interest could be readily distinguished with few shared alleles (Figure 4.10).

The exact evolutionary relationship among *P. ormesi, P. c. jeakingsi* and *P.* n. sp. West Coast is unclear. DNA sequencing of mtDNA and rDNA certainly indicate that all of our samples are closely related (Figures 4.2 and 4.3, 4.7 and 4.8). mtDNA haplotypes from six specimens of *P. c. jeakingsi* and the single specimens of *P.* n. sp. West Coast were not shared (Figures 4.4 – 4.6), and there was no obvious geographic distribution or morphological pattern concordant with this mtDNA variation (Figure 4.6).

Morphological variation needs to be investigated among these taxa to determine if there is discrete phenotypic variation or perhaps clines that reflect genetic variation (Figures 4.2 – 4.5, 4.7 and 4.8, 4.10; see Chapter 6). In particular, are there morphological traits that can readily separate individuals assigned to the putative species *P.* n. sp. West Coast? With the removal of *P. c. jeakingsi* from *P. c. cuvierianus,* the range of the latter species is now restricted to the north coast of the North Island (east coast of Northland to Hawke's Bay). The species complex of *P. ormesi, P. c. jeakingsi* and *P.* n. sp. West Coast occupy adjacent geographic ranges (centred on Cook Strait; Powell 1947), suggesting the possibility of continued gene flow.

Conclusion

Although the New Zealand members of Penion are monophyletic (excluding *P. benthicolus*), the genetic distinctiveness of some species provides reasons to question

their validity as independent evolutionary lineages. Molecular results confirm a recognised species (e.g. *P. sulcatus*), support the existence of an undescribed taxon (*P.* n. sp. Three Kings Islands), and indicate the species-level separation of two subspecies (*P. c. cuvierianus* and *P. c. jeakingsi*). However, genetic data was unable to distinguish two putative species with adjacent geographic ranges (*P. chathamensis* and *P. fairfieldae*), and mixed results from phylogenetic and SNP data indicate the existence of a species complex (*P. ormesi, P. c. jeakingsi, P.* n. sp. West Coast). It now needs to be determined if the exposed differences between current taxonomy and our molecular results can be resolved with detailed analysis of shell morphology (Chapter 6), the primary phenotypic trait used for siphon whelk species classification.

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Supplementary Data for Chapter Four

Supplementary Tables

SUPPLEMENTARY TABLE 4.1
The variation in the number of anonymous nuclear loci available for each investigative group of interest where the stringency for the representation of loci was varied (-r 0.3 – 0.9 in STACKS population settings). Specifically, -r refers to the minimum percentage of individuals in a population required to possess a locus for that population (i.e. for a locus to be used, at least e.g. one third of individuals within a population must exhibit that locus). Since we assumed in all groups that there was only one population, this means that the stringency reflects the representation of a locus within the entire dataset. Investigative groups were used to investigate SNP variation among individuals of *Penion* hierarchically. The first group included all sampling from monophyletic *Penion* from Australian and New Zealand, the second contained only monophyletic taxa from New Zealand. The last two groups were used to investigate species delimitation between closely related species (based on phylogenetic results), with one group containing specimens of *P. chathamensis, P. fairfieldae* and *P. c. cuvierianus*, and another including *P. c. cuvierianus, P. ormesi* and *P. c. jeakingsi*.

		Number of loci under different stringencies			
Investigative Group	**Number of Individuals**	**0.9**	**0.75**	**0.5**	**0.33**
All *Penion*	20	17	-	1306	3712
New Zealand *Penion*	18	36	-	1885	5191
P. chathamensis group	9	89	-	3732	9013
P. cuvierianus group	10	39	249	912	2072

SUPPLEMENTARY TABLE 4.2

A summary of statistics for the length and nucleotide composition for the concatenated DNA sequences for the nuclear rDNA 18S, 5.8S and 28S rRNA genes (the internal transcribed spacer regions are not included). All listed specimens were newly sequenced for this study.

		Concatenated 18S, 5.8S, 28S (no gap removal, just ITS removal)					
Species	**Museum ID**	**Length (bp)**	**% A**	**% C**	**% G**	**% T**	**GC bias**
Penion fairfieldae	2006177	5339	23.3	24.4	29.9	22.3	54.3
Penion n. sp. Three Kings Islands	M.302876	5339	23.3	24.4	29.9	22.3	54.3
Penion aff. *c. cuvierianus*	M.318615/1	5339	23.3	24.3	30.0	22.4	54.3
Penion ormesi	M.299869/1	5339	23.3	24.4	29.9	22.3	54.3
Penion ormesi	M.318565/2	5339	23.3	24.3	30.0	22.3	54.3
Penion c. jeakingsi	M.279432	5339	23.3	24.4	29.9	22.3	54.3
Penion n. sp. West Coast	M.316215/1	5339	23.3	24.4	29.9	22.3	54.3

SUPPLEMENTARY TABLE 4.3

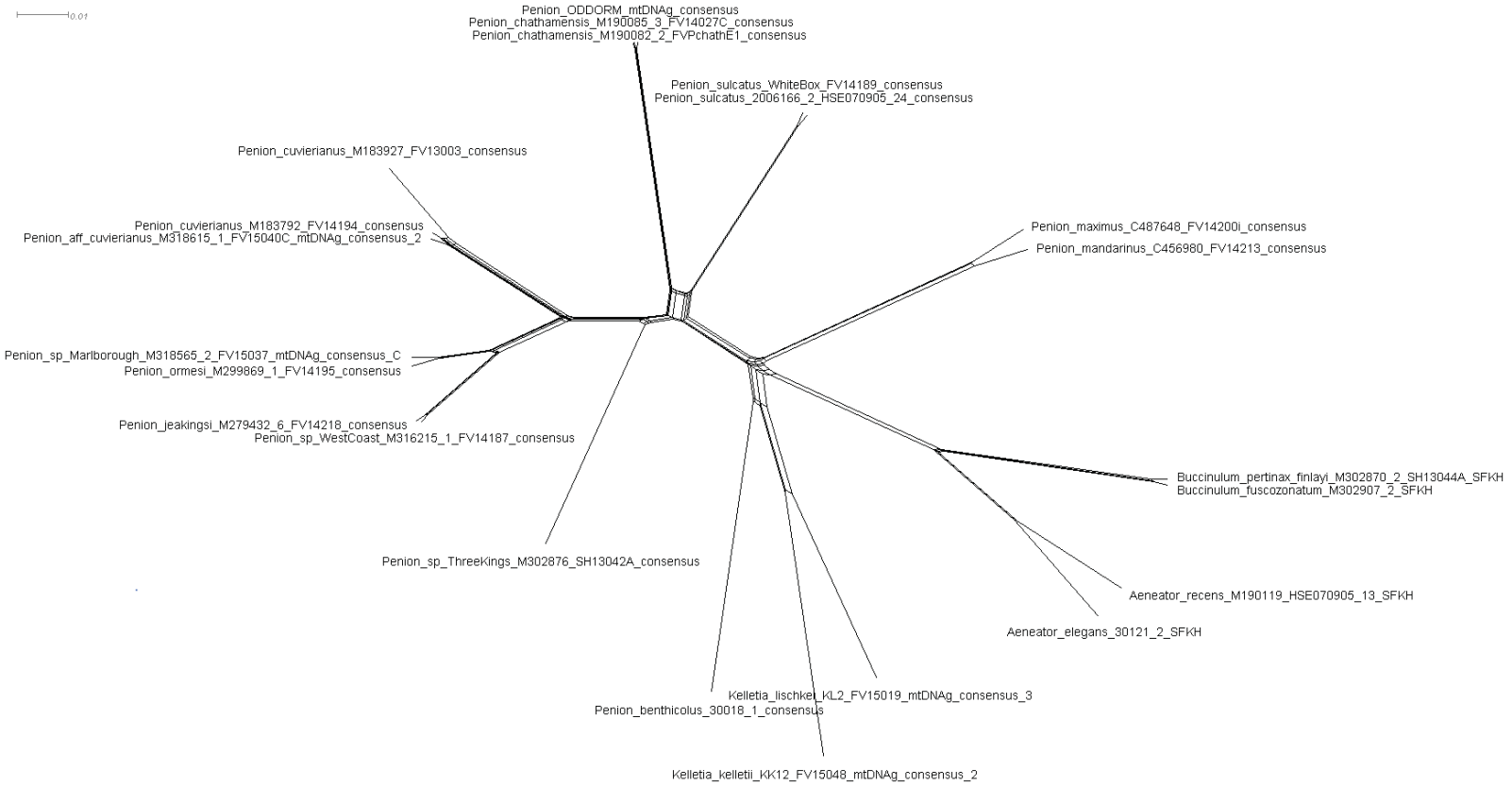
A summary of the statistics for the length and nucleotide composition for the mitochondrial genomes newly sequenced as part of this study. Specimens marked with one asterisk (*) exhibit drops in read coverage for some small regions, for example *P.* n. sp. Three Kings Islands has 45 bp missing from ND5. A specimen of *P. ormesi* is marked with two asterisks (**) as the genome has large gaps in genome coverage for some regions, including 133 bp, all bases, and 270 bp missing from the COX2, tRNA-Asp and 16S rRNA genes respectively.

		mtDNA genome						
Species	**Museum ID**	**Length (bp)**	**% A**	**% C**	**% G**	**% T**	**GC bias**	
Penion fairfieldae	2006177	15227	28.0	17.2	18.3	36.5	35.5	*
Penion n. sp. TKI	M.302876	15234	29.0	16.7	17.6	36.7	34.3	*
Penion aff. *c. cuvierianus*	M.318615/1	15236	28.7	16.9	17.7	36.7	34.6	
Penion ormesi	M.299869/1	15235	28.8	16.7	17.7	36.8	34.4	
Penion ormesi	M.318565/2	15235	28.8	16.8	17.6	36.7	34.4	**
Penion c. jeakingsi	M.279432	15237	28.8	16.8	17.7	36.7	34.5	
Penion n. sp. West Coast	M.316215/1	15238	28.8	16.7	17.7	36.7	34.4	

Supplementary Figures

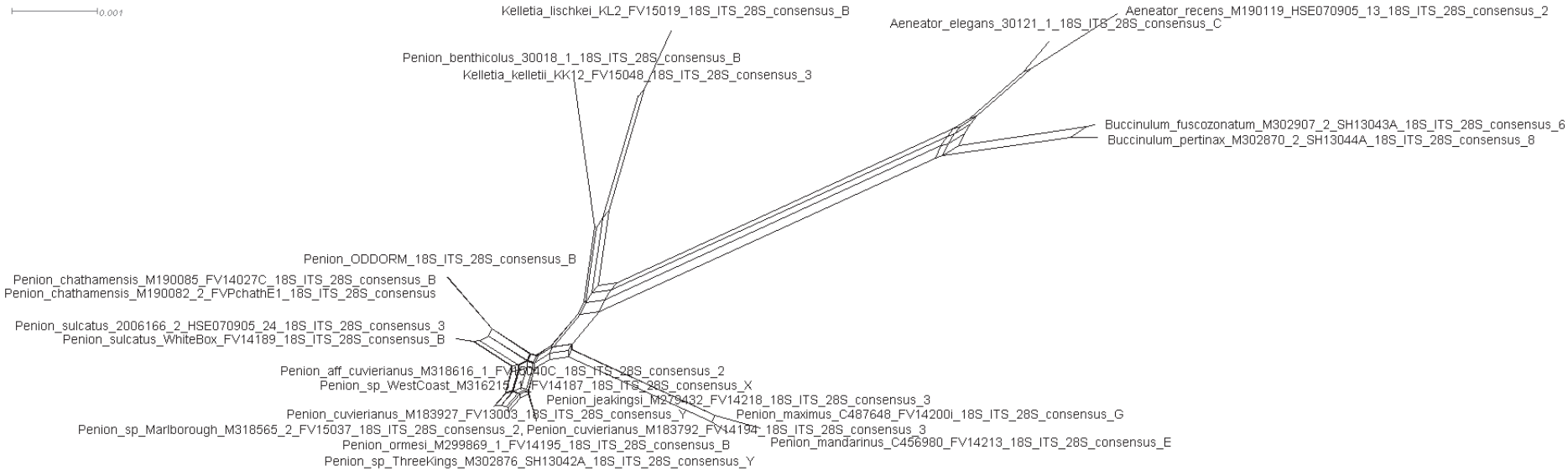
SUPPLEMENTARY FIGURE 4.1

A splits network of based on an alignment an 11,303 bp alignment of 22 concatenated mitochondrial genome sequences (incorporating protein-encoding, tRNA and rRNA genes). Splits were generated using the Neighbor-Net algorithm in SplitsTree 4. The splits network presents all possible relationships among sequences, but it does not consider conflict between alternative phylogenetic relationships or likelihood. Increased split length indicates increased genetic distance, and box structures within the network indicate that alternative relationships are possible (rather than relationships converging on one trajectory).

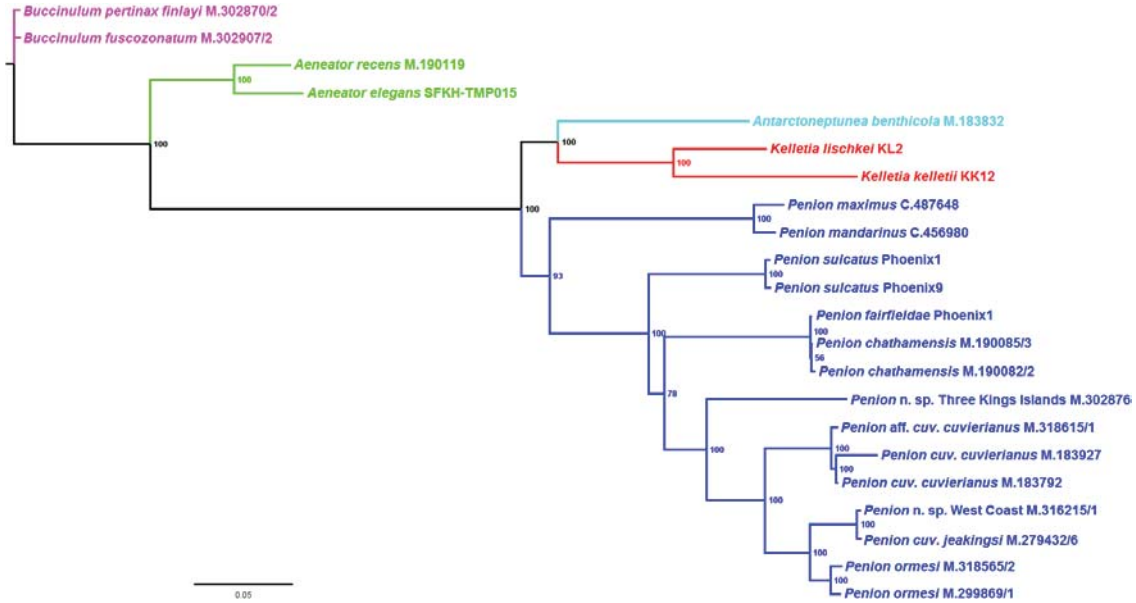


SUPPLEMENTARY FIGURE 4.2

A splits network of based on a 4673 bp alignment of 22 concatenated nuclear rDNA sequences (18S, 5.8S, 28S rRNA genes). Splits were generated using the Neighbor-Net algorithm in SplitsTree 4. The splits network presents all possible relationships among sequences, but it does not consider conflict between alternative phylogenetic relationships or likelihood. Increased split length indicates increased genetic distance, and box structures within the network indicate that alternative relationships are possible (rather than relationships converging on one trajectory).

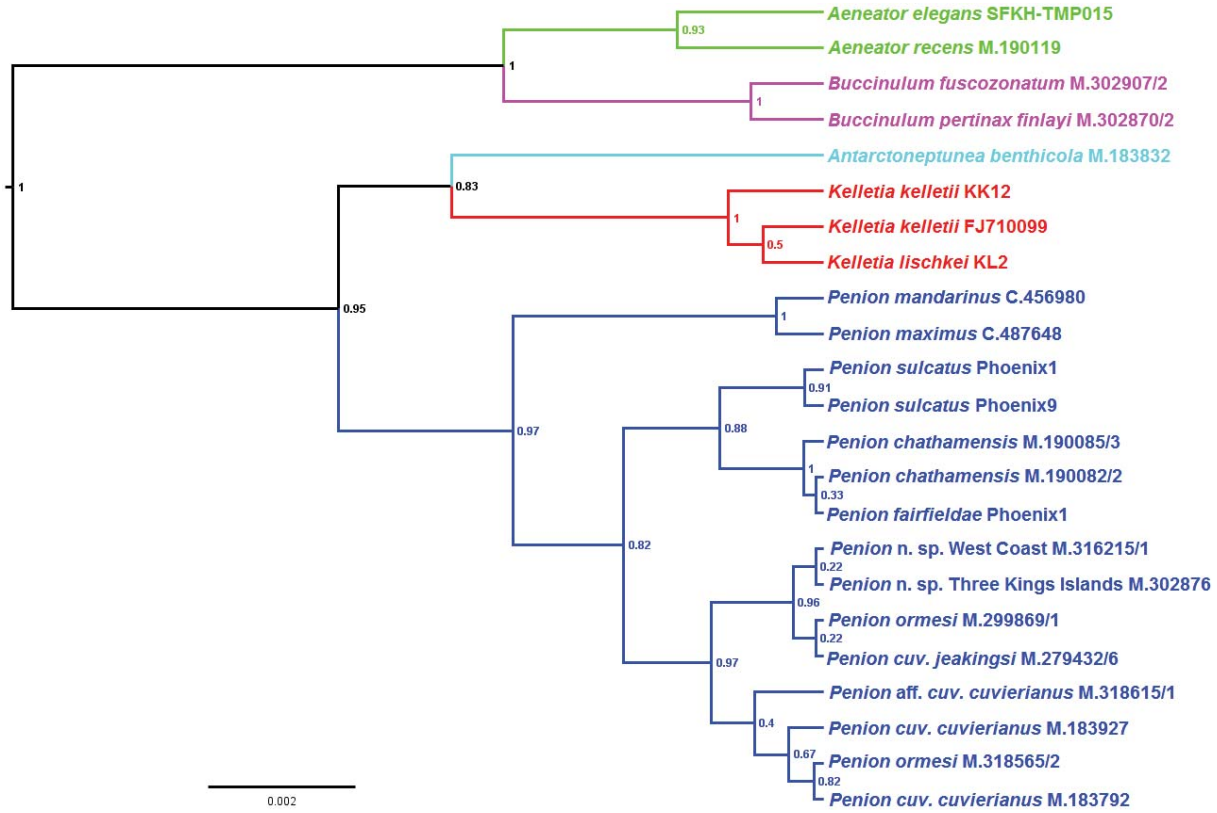


SUPPLEMENTARY FIGURE 4.3
A maximum-likelihood derived phylogeny (generated using RAxML 8.2.8) based on a 11,303 bp alignment of 22 concatenated mitochondrial genome sequences (incorporating protein-encoding, tRNA and rRNA genes). No partitions were used. No outgroup or monophyly was enforced for this tree.



SUPPLEMENTARY FIGURE 4.4
A maximum-likelihood derived phylogeny (generated using RAxML 8.2.8) based on a 4673 bp alignment of 22 concatenated nuclear rDNA sequences (18S, 5.8S, 28S rRNA genes). No partitions were used. No outgroup or monophyly was enforced for this tree.

SUPPLEMENTARY FIGURE 4.5
A Bayesian phylogeny based on an alignment of nuclear ribosomal 28S RNA gene sequences obtained from 23 individuals (1367 concatenated bp with ambiguous bases removed). The GTR + I + G substitution model was used. The phylogeny was produced using a Bayesian method (100 million MCMC, 10% burn-in, 1000 sample frequency, node labels are posterior support values), via BEAST 1.8.3.



Chapter Five

Geometric morphometric analysis reveals that the shells of male and female siphon whelks *Penion chathamensis* are the same size and shape

Bodies of male (above) and female (below) *P. cuvierianus jeakingsi*.

Introduction

Secondary sexual dimorphism can make the distinction of intra- and interspecific variation difficult. If males and females of the same species differ significantly in shape or size, the identification of separate evolutionary lineages can be challenging, and taxonomic over-splitting can occur. Sexual dimorphism has been a source of confounding variation in the analysis of extant organisms (e.g. Reskind 1965, Campos 2013, Khorozyan 2014, Underhill and Illiev 2014), and it is especially problematic in palaeontology where genetic and behavioural data are not usually available (e.g. Dodson 1975, Kimbel and White 1988, Huynen *et al.* 2003). Investigations of morphological stasis and change in the fossil record can identify and focus on single evolutionary lineages if sexual dimorphism is not exhibited.

Although caenogastropod snails are dioecious, sexual dimorphism is understudied within the group. Variation in shell morphology frequently informs gastropod taxonomy (e.g. Reid *et al.* 1996, Harasewych and Kantor 1999, Kantor 2003, Araya 2013), particularly in palaeontology (e.g. Beu and Maxwell 1990, Frasinetti 2000, Nielsen 2000), and morphometric analyses of shells are increasingly applied at the population and species-level (e.g. Tokeshi *et al.* 2000, Iguchi *et al.* 2005, Hills *et al.* 2012, Smith and Hendricks 2013). Historically it was assumed that secondary sexual dimorphism was rare in marine snails (Son and Hughes 2000), but investigations of soft-body anatomy and shell morphology have since indicated otherwise. Females are typically the larger sex (e.g. Simone 1996, Kenchington and Glass 1998, Kurata and Kikuchi 2000, Son and Hughes 2000, Minton and Wang 2011), although exceptions occur (e.g. Kurata and Kikuchi 2000), and sexual differences in shell shape have been identified in some taxa (e.g. ten Hallers-Tjabbes *et al.* 1994, Pastorino 2007, Minton and Wang 2011, Avaca *et al.* 2013, Mahilum and Demayo 2014).

We investigate whether there is evidence of sexual dimorphism in the shell morphology of the siphon whelk *Penion chathamensis* (Powell, 1938). Siphon whelks are large, benthic true whelks endemic to Australia and New Zealand (Ponder 1973, Powell 1979). The current taxonomy of *Penion* Fischer, 1884 is based upon variation in traditional analysis of shell morphology and soft-body anatomy (Dell 1956, Ponder 1973, Powell 1979), and there is a rich, intensively collected fossil record for the genus across the Southern Hemisphere (Ponder 1973, Beu and Maxwell 1990, Frasinetti 2000, Nielsen 2003, Beu 2009). *Penion chathamensis* is a large species (shell height 120 – 215 mm), found in deep-water (112 – 410 m) on the Chatham Rise and Campbell

Plateau southeast of New Zealand. Variation in shell morphology of *P. chathamensis* includes adult shell size, length of the siphonal canal, and prominence and persistence of axial ribs on the teleoconch whorls. Such variation is common in true whelks (e.g. Powell 1979, Beu and Maxwell 1990, Nielsen 2003, Araya 2013), and is thought to partially reflect environmental plasticity in response to depth, turbidity and substrate (Powell 1927, Ponder 1971). In a close relative of *Penion* (Hayashi 2005), *Kelletia kelletii* (Forbes, 1850), females in mating pairs were found to be on average 13 mm larger than their male partners (Rosenthal 1970), but no explicit investigation of sexual dimorphism has been conducted within the clade.

To gauge the potential effect of sexual dimorphism on shell variation, we also investigate interspecific variation by comparing shells of *P. chathamensis* to *P. sulcatus* (Lamarck, 1816). *Penion sulcatus* is endemic to New Zealand waters but occurs at shallower depths than *P. chathamensis* (1 – 165 m), near the North Island and northern South Island coasts. Siphon whelk species appear to exhibit significant intra- and interspecific variation in shell morphology, and the differentiation of species is often challenging (Powell 1979). However, although *P. chathamensis* and *P. sulcatus* are closely related according to mitochondrial 16S RNA gene DNA sequences (Hayashi 2005), they differ significantly in body size, shell colouration, protoconch morphology and the presence of other shell features such as axial ribs (Powell 1979).

Using a geometric morphometric approach, we investigate whether male and female *P. chathamensis* differ in shell shape or size, and generate preliminary information about the utility of geometric morphometric analysis applied to *Penion.*

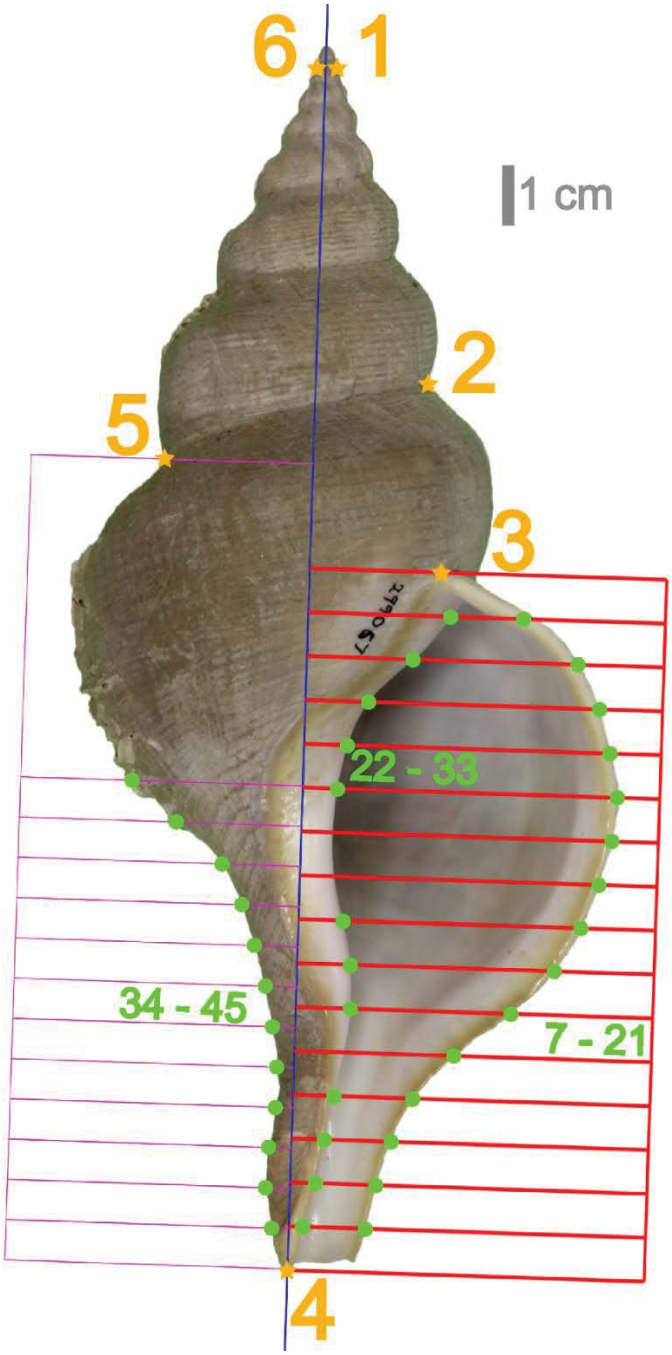
Materials and Methods

Specimens used for this study were collected by trawling (20 – 620 m) or by hand within intertidal depths (1 – 3 m). Most specimens are held at Museum of New Zealand Te Papa Tongarewa and the National Institute of Water and Atmospheric Research, but additional museum collections were also used (see Supplementary Tables 1 – 2 and Acknowledgements). A total of 124 *P. chathamensis* shells were sampled across the entire geographic range of the species, including 21 females and 11 males from western Chatham Rise (east of Mernoo Bank), 4 females from north of the Chatham Islands, and 2 females from the Auckland Islands. The remaining 86 shells came from unsexed individuals. Sexed snails were identified based on the genital anatomy (presence/absence of penis) of live-caught individuals (see Ponder 1973 for

description of reproductive anatomy). Since only the Chatham Rise sampling included identified males, and in order to exclude potential inter-population variation, most analyses were restricted to this group of 32 snails. We also sampled 190 shells of *P. sulcatus* (Lamarck, 1816). Only adult shells that were complete or near-complete with intact edges were included. Maturity was estimated by the presence of at least six teleoconch whorls, thickening of the outer aperture lip, and ascent of the end of the last whorl. Although sexual maturity can occur earlier (Jones 1938), shell maturity is usually treated as a proxy for adulthood in snails as it indicates when a snail is no longer growing in size (Goodfriend 1986).

We analysed shell morphology using two-dimensional landmark-based geometric morphometrics, following recommendations listed by Webster and Sheets (2010). Shells were mounted in fine-grade silica sand and photographed with the aperture facing upward using a Canon EF-S 600D camera with an 18 – 55 mm IS II lens (Figure 5.1). A 50 mm scale bar was included in each digital image. The roll, pitch and yaw were adjusted so that the shells were balanced along the vertical axis (spire to siphonal canal) and the inner lip of the aperture faced directly upward, towards the camera (Figure 5.1). All positioning, photography and subsequent digitisation was conducted by one person to minimise experimental error (Schilthuizen and Haase 2010), which was found to be negligible when investigated (see Supplementary Data). Liveshoot options allowed us to target the camera focus on the aperture and protoconch. For the majority of photographs the camera was mounted on a Kaiser copy stand (RS1, RA-1 arm), but for very large shells it was necessary to use a Compact Action Manfrotto tripod (MKCOMPACTACN) to accommodate large shells within the central field of view using the same camera lens.

FIGURE 5.1
Shell orientation and the configuration of all 45 landmarks (6 landmarks (orange stars), 39 semi-landmarks (green circles)) digitised and used for the morphometric analysis of shell morphology in *Penion*.



Virtual digital combs were aligned to the central axis of the shell and biologically homologous positions such as the end of the siphonal canal in Adobe Photoshop CS6 to provide consistent points for digitisation of semi-landmarks (Figure 5.1). Digital images were organised into thin plate spline (TPS) files using tpsUtil (Rohlf 2013), with the order of specimens randomised to reduce potential experimental bias. Landmarks and semi-landmarks were then identified on each image photographed using a Wacom Cintiq 22HD Pen Display tablet, and then scale-calibrated and slid using tpsUtil, tpsDig (Rohlf 2013), and IMP (Sheets 2014). This yielded X – Y coordinates for points digitised on shells. We used a total of 45 landmarks to digitally summarise shell shape (Figure 5.1). Six fixed landmarks captured biologically homologous points such as the top of the teleoconch, and 39 semi-landmarks described the inner and outer curves of the aperture and siphonal canal. This number of landmarks was selected after optimisation based on principal component loadings (Supplementary Data). Following the interpretation of Gunz *et al.* (2005), all of our landmarks (*sensu stricto*) are Type I as defined by Bookstein (1991). Semi-landmarks were 'slid' to minimise the effect of the arbitrary placement of points on the curves of interest. Sliding was achieved by minimising Procrustes distances (Bookstein 1996, Zelditch *et al.* 2004, Perez *et al.* 2006), using the IMP program Semiland7 (Sheets 2014).

Partial Procrustes superimposition was achieved using MorphoJ 1.06c (Klingenberg 2011), which aligns and superimposes landmarks for all specimens to remove confounding variation due to differences in the size, translation (position) and orientation of objects (Webster and Sheets 2010, Mitteroecker *et al.* 2013, Monteiro 2013, Polly *et al.* 2013). Procrustes superimposition is the preferred method when morphological variation is relatively small (Perez *et al.* 2006). A covariance matrix was generated from the X – Y coordinates of the superimposed landmarks, providing input for principal components analysis (PCA) in MorphoJ 1.06c (Klingenberg 2011). The principal components reflect variation in the shape of objects, and centroid size acts as a proxy for size variation (independent of shape). Statistically significant principal components (PCs) were identified using the broken-stick test on eigenvalues, implemented in the R package vegan 2.2-1 (Jackson 1993, Oksanen *et al.* 2015). We used PCA ordination to estimate the separation of *a priori* groups (e.g. males and females), using 90% mean confidence ellipses of group means to determine whether groups were likely to overlap. Canonical variates analysis (CVA) was used to test statistically the ability to differentiate these groups, with discrimination success

determined using cross-validation scores; the number of individuals correctly assigned to each *a priori* group based on Mahalanobis distance of each individual from group means. CVA was conducted using either MorphoJ 1.06c (Klingenberg 2011), analysing the original X – Y landmark coordinates, or the R package MASS 7.3-26 (R Core Team 2014; Venables and Ripley 2002) using PCs generated from PCA in MorphoJ. For groups with fewer specimens than the number of landmarks used, we used PCA as a dimensionality-reducing method to allow *a priori* groups to be tested with CVA.

We also wanted to investigate what groupings could be naïvely identified using the shell morphological data alone, without relying on *a priori* hypotheses based on data such as genetics, taxonomy, or geography. To investigate naïve groupings, we conducted model-based Bayesian assignment analysis using the R package mclust 5.2 (Fraley and Raftery 2002). Mclust can analyse both PCs (shell shape) and centroid size (shell size), and it identifies the clustering model that most efficiently explains variation in a dataset without any prior classification of specimens. The fit of a model is tested with an iterative expectation-maximisation (EM) method using Gaussian mixture modelling (Fraley and Raftery 2012). The models tested by mclust differ in the expected distribution of data, as well as the volume, shape and orientation of the covariance matrices generated from observed data (Fraley and Raftery 2012; model parameters listed in Supplementary Table 3). Bayesian information criterion (BIC) scores were used to determine the relative support for competing clustering models. In mclust, BIC scores are multiplied by -1 and therefore higher BIC values indicate stronger support for a given model. Where centroid size was included with PCs for mclust analyses, variables were scaled (using the base function in R) because centroid size is expressed on a much larger numerical scale than PCs. Different numerical scales are problematic for mclust analysis as multiple models tested assume the same variance across all variables or estimated clusters (Fraley and Raftery 2012).

Results and Discussion

According to CVA, the shape of *P. chathamensis* shells could not be used to successfully differentiate males from females based on jack-knifed cross-validation scores (Figure 5.2). More than half of the specimens from Chatham Rise were misassigned to the opposite sex (61.9 % of females and 45.5% of males misassigned), and discrimination was similarly poor across the entire species (29.6% females and 54.5% males misassigned). Using PCA with any combination of the significant PCs

(variation at PC1 = 39.4%, PC2 = 19.5%, PC3 = 11.4%; PC4 = 7.2%), the similarity in shell shape between males and females was readily apparent (Figure 5.3, Supplementary Figure 5.1). Male and female shape overlapped in morphospace based on 90% mean confidence ellipses. The mean distribution of male and female shape lay close to the mean for the species (*P. chathamensis* as a whole; Figure 5.3). The mean centroid sizes of males and females were almost identical (male mean = 34.34, SD = 4.57; all female mean = 34.43, SD = 3.99; Chatham Rise only female mean = 35.13, SD = 3.86), indicating that the sexes also do not differ significantly in shell size.

FIGURE 5.2
Canonical variates analysis produced using the R package MASS 7.3-26 (Venables and Ripley 2002). Results indicate that the shells of male and female *P. chathamensis* cannot be distinguished, based on the mutual misassignment of individuals (overlapping columns; jack-knifed cross-validation) and the short distance between individuals belonging to each group. The distribution of specimens is shown across all locations (left plot) and for sexed individuals from western Chatham Rise only (right). Individuals are coloured according to identified sex: females (dark grey), males (light grey).

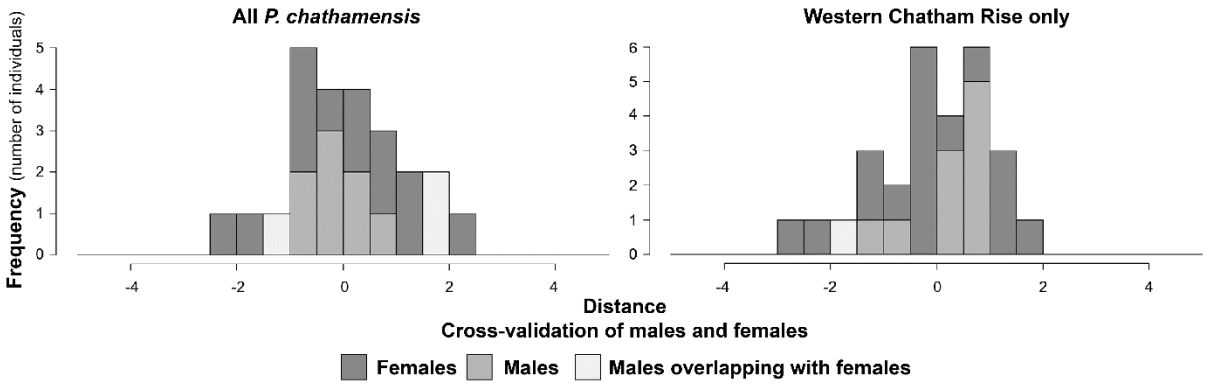
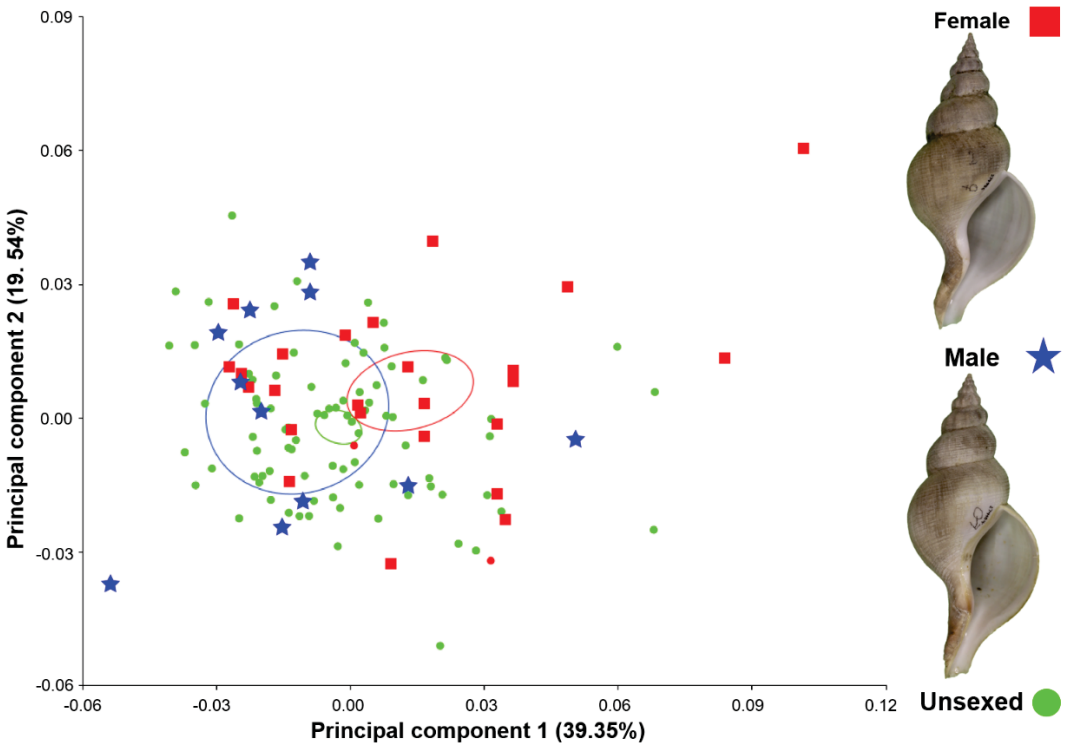


FIGURE 5.3
A principal components analysis plot produced using MorphoJ 1.06c (Klingenberg 2011), showing variation among individuals of *P. chathamensis* for principal components generated from geometric morphometric measurements of shells. Principal components 1 (39.35% of variation) and 2 (19.54%) are shown. Females are illustrated as red square symbols, males as blue stars, and shells from unsexed individuals are shown as green circles. 90% mean confidence ellipses are illustrated for each group (in matching colouration), and indicate that the means of all three groups are likely to overlaps. The sexes were no more distinguishable when the other statistically significant principal components (3 and 4) were included (Supplementary Figure 5.1).



Canonical variates analysis and the ellipses estimated in PCA indicate that males and females are similar, but both analyses rely on the *a priori* classification of individuals (i.e. sex). Neither analysis explicitly attempts to identify the most suitable groupings within the data. We therefore conducted naïve, model-based cluster analysis using mclust. For the sampled of sexed individuals from Chatham Rise (n = 32), mclust supported only one cluster, whether using the significant principal components alone (PC1 – PC4) or with centroid size included (Supplementary Figure 5.2). This led us to accept the null hypothesis that there are no identifiable groupings within the data; males and females cannot be naïvely distinguished based on shell morphology.

Failure to detect sexual dimorphism in the shells of *P. chathamensis* might indicate that our chosen morphometric landmarks were unsuitable for the detection of relevant morphological variation (a Type II error). To test this, we analysed a dataset containing our sampling of *P. chathamensis* (n = 124) and *P. sulcatus* (n = 190). Based on CVA using cross-validation scores, *P. chathamensis* and *P. sulcatus* were readily distinguished from one another, with only 0.8% and 1.1% of individuals respectively being misassigned to the wrong species. Using any combination of the significant PCs (PC1 = 45.6%, PC2 = 19.6%, PC3 = 8.7%), PCA demonstrates that the two species means are widely separated in morphospace (Figure 5.4). Some individuals of the two species in overlapped morphospace, but this is to be expected given the highly variable morphology of siphon whelks (Ponder 1973, Powell 1979). Mclust analysis using the significant principal components (PC1 – PC3) with or without centroid size, found best support for two clusters of shells (Supplementary Figure 5.3). These two clusters corresponded closely with the identification of specimens despite the taxonomic classification being derived from traits not captured by our landmarks (e.g. protoconch and axial rib morphology, shell colouration), and accuracy generally improved when centroid size was included (Figure 5.5). Since mclust was able to naïvely distinguish the species with such high accuracy (Figure 5.5), despite some overlap in shape space among individuals (Figure 5.4), we infer that our landmarks are adequate for capturing morphological variation in the shells of siphon whelks. This means that the lack of evidence for secondary sexual dimorphism in *P. chathamensis* likely reflects biological reality.

FIGURE 5.4
A principal components analysis plot produced using MorphoJ 1.06c (Klingenberg 2011), showing variation among individuals classified as *P. chathamensis* and *P. sulcatus* for principal components generated from geometric morphometric measurements of shells. Principal components 1 (45.65% of variation) and 2 (19.62%) are shown. Specimens classified as *P. chathamensis* and *P. sulcatus* are illustrated as green and blue circles respectively. 90% mean confidence ellipses are illustrated for each group (in matching colouration), and indicate that the means of the two species are widely separated morphospace. The taxa were also readily distinguished using the remaining statistically significant principal component 3.

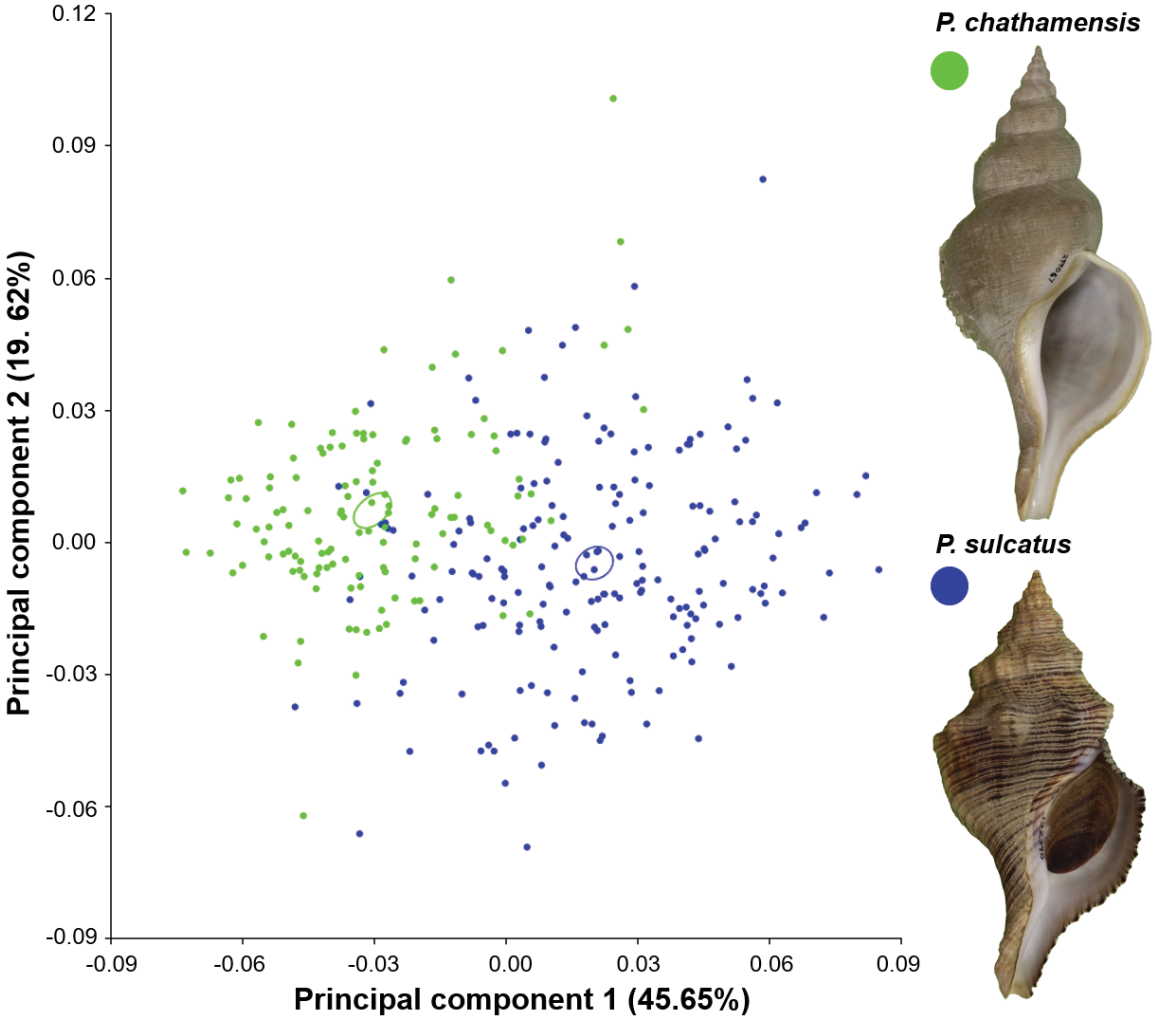
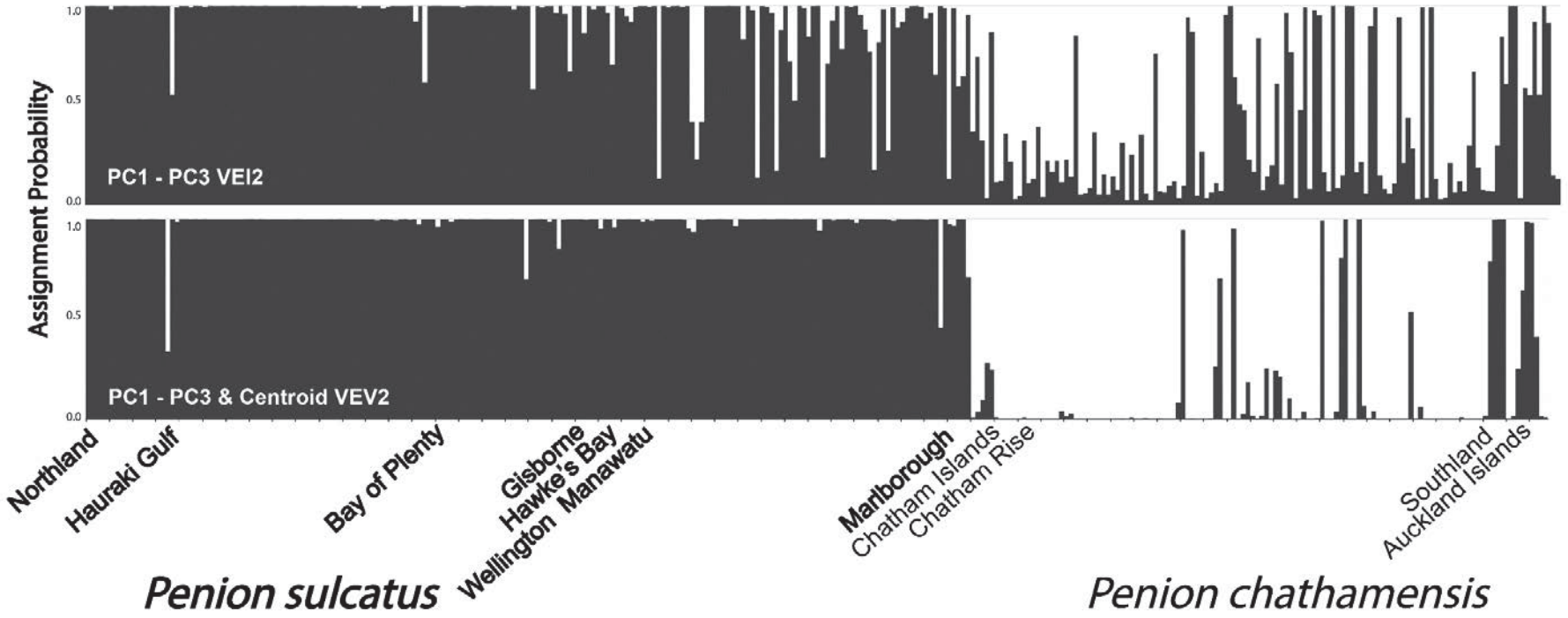


FIGURE 5.5
Bayesian assignment probability for each siphon whelk shell belonging to one of the two clusters estimated by modelling shell shape variation of *Penion chathamensis* and *P. sulcatus* in the R clustering package mclust 5.2 (Fraley and Raftery 2002). The best supported models are shown for analyses using the statistically significant principal components (1 – 3) and centroid size with support estimated using the Bayesian Information Criterion. When centroid size was included, variables were scaled because centroid size is expressed on a much larger scale than principal components. The top plot shows the VEI2 model using PC1 – PC3 only, the bottom plot shows the VEV2 model using PC1 – PC3 and centroid size. See Supplementary Table 3 for a list of the different parameters used for each clustering model. Species and geographic locations are labelled along the X-axis, with tick marks that indicate every fifth specimens. Clusters identified using the mclust modelling (grey, white) correspond closely with prior species-level identification, despite the analysis not using any *a priori* classification. The inclusion of centroid size mostly increases confidence.



We conclude that sexual dimorphism is not exhibited in shell morphology of *P. chathamensis*, in contrast to results from other Caenogastropoda that suggest sexes differ in shell shape, and that females are usually larger than males (see references in introduction). It should be noted, however, that some of the previous studies found only weak evidence for sexual dimorphism (e.g. Kenchington and Glass 1998, Son and Hughes 2000). It is also possible that sexual dimorphism is exhibited in a region of the shell not captured by our 2D landmarks (such as the protoconch or the interior of the shell), but this seems unlikely as our landmarks focus on the aperture, which is the end of the generating curve of the shell. Siphon whelks may still exhibit secondary sexual dimorphism in soft-body anatomy. If we assume that other siphon whelk species are similar to *P. chathamensis*, future studies of *Penion* shell variation are likely to be free of the confounding effects of significant secondary sexual dimorphism. This is especially beneficial for palaeontological research where many fossil taxa are known only from single localities or few individuals (Beu and Maxwell 1990, Nielsen 2003, Beu 2009).

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Supplementary Data for Chapter Five

In this supplementary material we provide further details of our data and method of geometric morphometric analysis, with details and results of our digitisation error study.

Landmark data

A comma separate file (.csv), which containing the X – Y coordinates for the 45 landmark dataset (output from IMP (Sheets 2014)) is available to download (from http://evolves.massey.ac.nz/DNA_Toolkit.htm). The file provides the museum specimen identification and a binned geographic locality for every individual, and an additional 'Figure' column is included so the geographic localities labelled in Figure 5.5 can be interpreted. This file is ready to be imported into Procrustes analysis software such as MorphoJ once centroid size and the additional label column are removed.

Individuals are labelled in the following fashion:
Genus_species_Museum collection lot ID_Individual ID within collection_Classifier string

The classifier string is used in MorphoJ to extract groups such as species (-7, -6), sex and error study (-11, -9), sex (-9, -9), and location (-3, -1). PS = *P. sulcatus*, PF = *P. chathamensis*; F = female, M = male; HAU = Hauraki Gulf, CHA = Chatham Rise etc. (all can be deciphered using the 'Figure' column).

Any analysis involving a subset of the data must be re-analysed independently as principal components and other variables are not comparable between datasets.

Optimisation of number of landmarks

Originally, 49 landmarks were used to digitise shell shape. However, we investigated the principal component loadings for each landmark (exported from MorphoJ 1.06c; Klingenberg 2011), using the R package vegan (Oksanen *et al.* 2015). We decided to remove the original semi-landmarks 28, 29 and 33 (position can be inferred by gaps on the inner aperture curve in Figure 5.1), as these points contributed little to the shape variation estimated in the dataset. We also removed landmark 1 that

was placed on top of the protoconch, as this feature was not present in all shells (position again can be seen in Figure 5.1). The reduction of redundant landmarks improves statistical power because it raises the degrees of freedom relative to the dimensionality of the data.

Estimation of experimental error

The positioning of specimens, the camera-specimen distance during photography, and variation in the placement of landmarks and combs on images were expected to be main sources of experimental error. Following previous methods (e.g. Dowle *et al.* 2015), we investigated error by re-positioning and photographing a single shell of *P. chathamensis* five times at three different heights (103, 125, 135 cm) and digitising all photos once independently. Camera height was considered as a potential source of variation because *P. chathamensis* and other siphon whelks vary in size and cannot easily be photographed from a single height. A single photo from the 103 cm height was also digitised a further four times to disentangle photographic and digitisation error. Experimental error was visualised using principal components analysis, with neither repeated photography or digitisation appearing to have a significant effect (PCA; Supplementary Figure 5.4).

Supplementary References

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Supplementary Tables

SUPPLEMENTARY TABLE 5.1
Shells of *P. chathamensis* used for this study, organised by museum collection lot ID with museum and broad geographic sampling region (used for mclust plot) also listed.

Museum	Collection ID	Region	Number of shells used
Te Papa	M.117114	Auckland Islands	3
Te Papa	M.118744	Auckland Islands	2
Te Papa	M.147001	Auckland Islands	1
Te Papa	M.190356	Auckland Islands	1
Te Papa	M.274006	Auckland Islands	1
Te Papa	M.274011	Auckland Islands	1
Te Papa	M.274012	Auckland Islands	1
Te Papa	M.274013	Auckland Islands	1
Te Papa	M.086741	Chatham Islands	1
Te Papa	M.090040	Chatham Islands	1
Te Papa	M.190108	Chatham Islands	2
Te Papa	M.190123	Chatham Islands	2
Te Papa	M.111884	Chatham Rise	2
Te Papa	M.116995	Chatham Rise	10
Te Papa	M.117002	Chatham Rise	1
Te Papa	M.117016	Chatham Rise	2
Te Papa	M.117027	Chatham Rise	1
Te Papa	M.118863	Chatham Rise	5
Te Papa	M.118993	Chatham Rise	24
Te Papa	M.127025	Chatham Rise	22
Te Papa	M.190065	Chatham Rise	1
Te Papa	M.190070	Chatham Rise	2
Te Papa	M.190077	Chatham Rise	1
Te Papa	M.190082	Chatham Rise	1
Te Papa	M.190091	Chatham Rise	2
Te Papa	M.190095	Chatham Rise	2
Te Papa	M.190100	Chatham Rise	3
Te Papa	M.274095	Chatham Rise	4
Te Papa	M.274099	Chatham Rise	2
Te Papa	M.274104	Chatham Rise	3
Te Papa	M.274985	Chatham Rise	3
Te Papa	M.274986	Chatham Rise	2
Te Papa	M.274989	Chatham Rise	1
Te Papa	M.274992	Chatham Rise	2
Te Papa	M.275003	Chatham Rise	1
Te Papa	M.275004	Chatham Rise	2

Te Papa	M.275005	Chatham Rise	2
Te Papa	M.275006	Chatham Rise	1
Te Papa	M.275011	Chatham Rise	1
Te Papa	M.299066	Chatham Rise	2
Auckland Museum	MA70077	Chatham Rise	1
Te Papa	M.090055	Southland	1

SUPPLEMENTARY TABLE 5.2
Shells of *P. sulcatus* used for this study, organised by museum collection lot ID with museum and broad geographic sampling region (used for mclust plot) also listed.

Museum	Collection ID	Region	Number of shells used
Australian Museum	C103917	Bay of Plenty	2
Museum Victoria	F17896	Bay of Plenty	1
Te Papa	M.002541	Bay of Plenty	1
Te Papa	M.005437	Bay of Plenty	2
Te Papa	M.005467	Bay of Plenty	1
Te Papa	M.005491	Bay of Plenty	2
Te Papa	M.005521	Bay of Plenty	1
Te Papa	M.005522	Bay of Plenty	2
Te Papa	M.005527	Bay of Plenty	1
Te Papa	M.005529	Bay of Plenty	1
Te Papa	M.005599	Bay of Plenty	1
Te Papa	M.005600	Bay of Plenty	1
Te Papa	M.032516	Bay of Plenty	9
Te Papa	M.036248	Bay of Plenty	1
Te Papa	M.065237	Bay of Plenty	1
Te Papa	M.070988	Bay of Plenty	1
Te Papa	M.117630	Bay of Plenty	1
Te Papa	M.126318	Bay of Plenty	2
Te Papa	M.130182	Bay of Plenty	1
Te Papa	M.132382	Bay of Plenty	1
Te Papa	M.144015	Bay of Plenty	1
Te Papa	M.278793	Bay of Plenty	1
Te Papa	M.278820	Bay of Plenty	1
Te Papa	M.279127	Bay of Plenty	1
Te Papa	M.306291	Bay of Plenty	1
Auckland Museum	MA71414	Bay of Plenty	1
Auckland University	RX025	Bay of Plenty	1
Auckland University	RX026	Bay of Plenty	1
GNS Science	267	Gisborne	1
GNS Science	269	Hauraki Gulf	1
GNS Science	270	Hauraki Gulf	1

GNS Science	271	Hauraki Gulf	1
GNS Science	4803	Hauraki Gulf	1
GNS Science	2678a	Hauraki Gulf	2
Australian Museum	C1258	Hauraki Gulf	2
Australian Museum	C244132	Hauraki Gulf	1
Australian Museum	C44588	Hauraki Gulf	1
Australian Museum	C53246	Hauraki Gulf	3
Australian Museum	C75789	Hauraki Gulf	1
Museum Victoria	F221249	Hauraki Gulf	1
Museum Victoria	F221250	Hauraki Gulf	1
Museum Victoria	F221251	Hauraki Gulf	1
Museum Victoria	F221252	Hauraki Gulf	1
Auckland University	G4661	Hauraki Gulf	1
Te Papa	M.001411	Hauraki Gulf	3
Te Papa	M.011611	Hauraki Gulf	1
Te Papa	M.021089	Hauraki Gulf	1
Te Papa	M.038836	Hauraki Gulf	2
Te Papa	M.083830	Hauraki Gulf	15
Te Papa	M.083831	Hauraki Gulf	1
Te Papa	M.083840	Hauraki Gulf	1
Te Papa	M.083845	Hauraki Gulf	2
Te Papa	M.083846	Hauraki Gulf	1
Te Papa	M.083847	Hauraki Gulf	3
Te Papa	M.083849	Hauraki Gulf	1
Te Papa	M.132376	Hauraki Gulf	2
Te Papa	M.132377	Hauraki Gulf	3
Te Papa	M.132381	Hauraki Gulf	1
Te Papa	M.132383	Hauraki Gulf	1
Te Papa	M.132390	Hauraki Gulf	1
Te Papa	M.132393	Hauraki Gulf	1
Te Papa	M.136021	Hauraki Gulf	2
Te Papa	M.136025	Hauraki Gulf	1
Te Papa	M.136026	Hauraki Gulf	1
Te Papa	M.137294	Hauraki Gulf	1
Te Papa	M.145036	Hauraki Gulf	1
Te Papa	M.153330	Hauraki Gulf	1
Te Papa	M.153331	Hauraki Gulf	1
Te Papa	M.278785	Hauraki Gulf	1
Te Papa	M.278786	Hauraki Gulf	1
Te Papa	M.278791	Hauraki Gulf	1
Te Papa	M.278792	Hauraki Gulf	1

Te Papa	M.278794	Hauraki Gulf	1
Te Papa	M.278795	Hauraki Gulf	1
Te Papa	M.278796	Hauraki Gulf	1
Te Papa	M.278801	Hauraki Gulf	1
Te Papa	M.278826	Hauraki Gulf	2
Auckland Museum	MA72164	Hauraki Gulf	1
Massey University	Phoenix9	Hauraki Gulf	1
Te Papa	M.086723	Hawke's Bay	1
Te Papa	M.278806	Hawke's Bay	1
Te Papa	M.005865	Marlborough	1
Te Papa	M.011199	Marlborough	1
Te Papa	M.011202	Marlborough	1
Te Papa	M.045155	Marlborough	1
Te Papa	M.050799	Marlborough	1
Te Papa	M.318565	Marlborough	1
GNS Science	3591	Northland	1
Te Papa	M.002543	Northland	1
Te Papa	M.132379	Northland	2
Te Papa	M.132380	Northland	2
Te Papa	M.137085	Northland	1
Te Papa	M.137101	Northland	1
Te Papa	M.137138	Northland	1
Te Papa	M.137267	Northland	1
Te Papa	M.137271	Northland	1
Te Papa	M.137348	Northland	1
Te Papa	M.143999	Northland	1
Te Papa	M.151069	Northland	1
Te Papa	M.278788	Northland	1
Te Papa	M.278804	Northland	1
GNS Science	173	Wellington Manawatu	1
GNS Science	260	Wellington Manawatu	1
GNS Science	266	Wellington Manawatu	1
GNS Science	268	Wellington Manawatu	3
GNS Science	3260	Wellington Manawatu	1
GNS Science	3573	Wellington Manawatu	7
Auckland University	G6630	Wellington Manawatu	1
GNS Science	GNS01	Wellington Manawatu	1
Te Papa	M.005871	Wellington Manawatu	5
Te Papa	M.009017	Wellington Manawatu	5
Te Papa	M.010144	Wellington Manawatu	1
Te Papa	M.011026	Wellington Manawatu	3

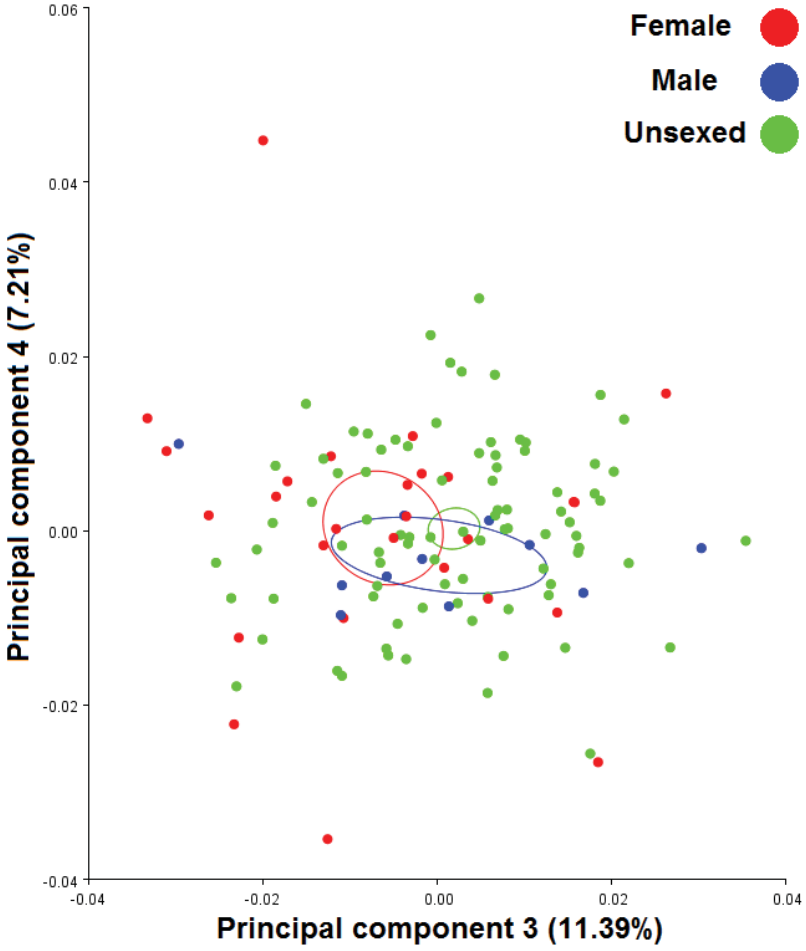
Te Papa	M.011602	Wellington Manawatu	1
Te Papa	M.013328	Wellington Manawatu	1
Te Papa	M.019626	Wellington Manawatu	1
Te Papa	M.132386	Wellington Manawatu	1
Te Papa	M.153317	Wellington Manawatu	1
Te Papa	M.303376	Wellington Manawatu	2
Massey University	Phoenix7	Wellington Manawatu	1
GNS Science	RM3368	Wellington Manawatu	4
GNS Science	RM5866	Wellington Manawatu	3

SUPPLEMENTARY TABLE 5.3
The parameters used for each mclust model, adapted from Fraley and Raftery 2012. The HC column lists models that are available for hierarchical clustering, and the EM column lists those available for iterative expectation-maximisation Gaussian mixture-modelling cluster analysis (the standard mclust analysis). The explicit mathematical formulae used for models are listed in Fraley & Raftery 2012.

Model name	HC?	EM?	Distribution	Volume	Shape	Orientation
E	Y	Y	(univariate)	equal		
V	Y	Y	(univariate)	variable		
EII	Y	Y	Spherical	equal	equal	N/A
VII	Y	Y	Spherical	variable	equal	N/A
EEI		Y	Diagonal	equal	equal	coordinate axes
VEI		Y	Diagonal	variable	equal	coordinate axes
EVI		Y	Diagonal	equal	variable	coordinate axes
VVI		Y	Diagonal	variable	variable	coordinate axes
EEE	Y	Y	Ellipsoidal	equal	equal	equal
EEV		Y	Ellipsoidal	equal	equal	variable
VEV		Y	Ellipsoidal	variable	equal	variable
VVV	Y	Y	Ellipsoidal	variable	variable	variable

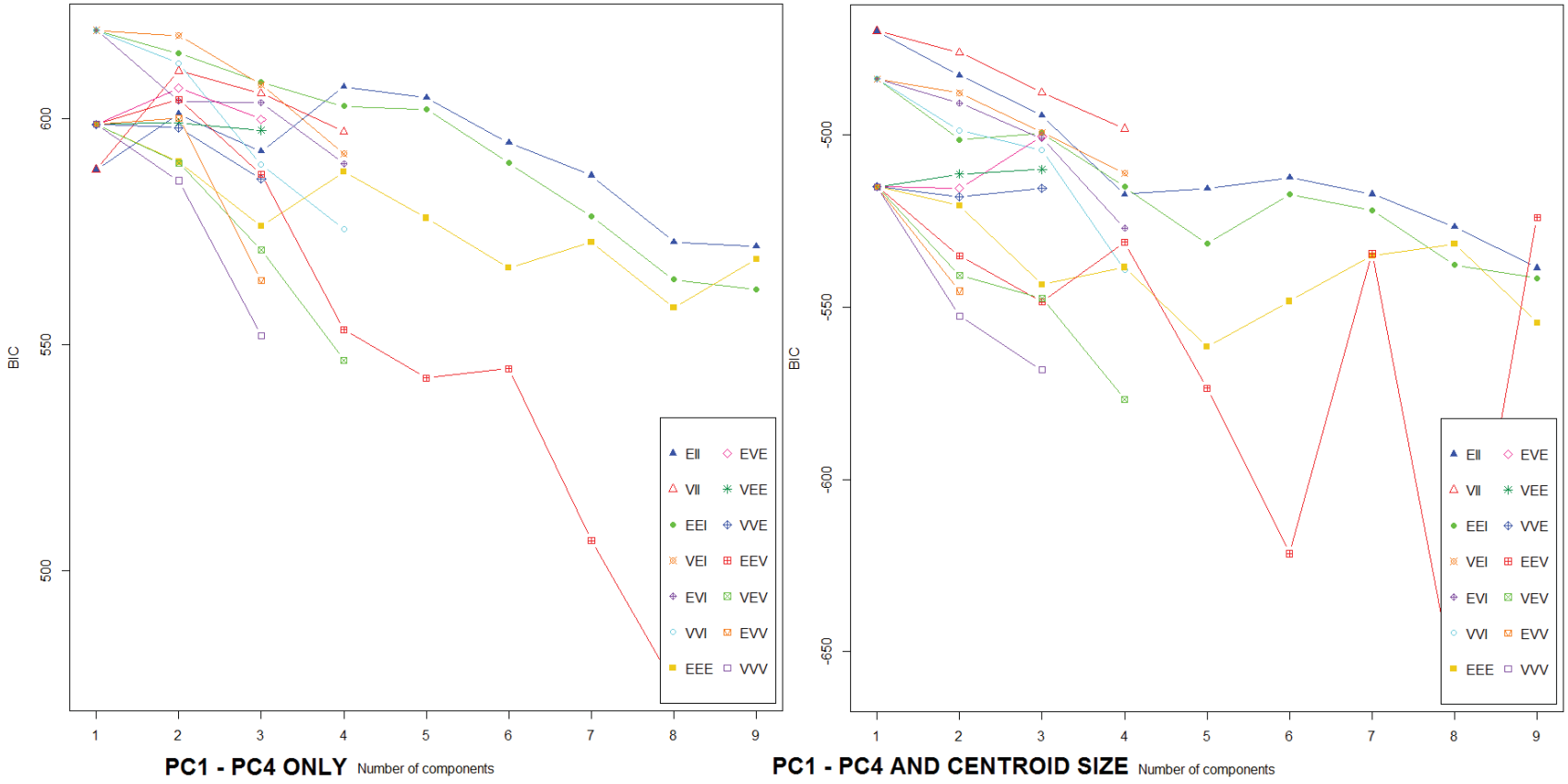
Supplementary Figures

SUPPLEMENTARY FIGURE 5.1
A principal components analysis plot produced using MorphoJ 1.06c (Klingenberg 2011), showing variation among individuals of *P. chathamensis* for principal components generated from geometric morphometric measurements of shells. Principal components 3 (11.39% of variation) and 4 (7.21%) are shown. Females are coloured in red, males in blue, and shells from unsexed individuals in green. 90% mean confidence ellipses are illustrated for each group (in matching colouration), and indicate that the means of all three groups are likely to overlaps. The sexes were no more distinguishable when the other statistically significant principal components (1 and 2) were included (Figure 3).



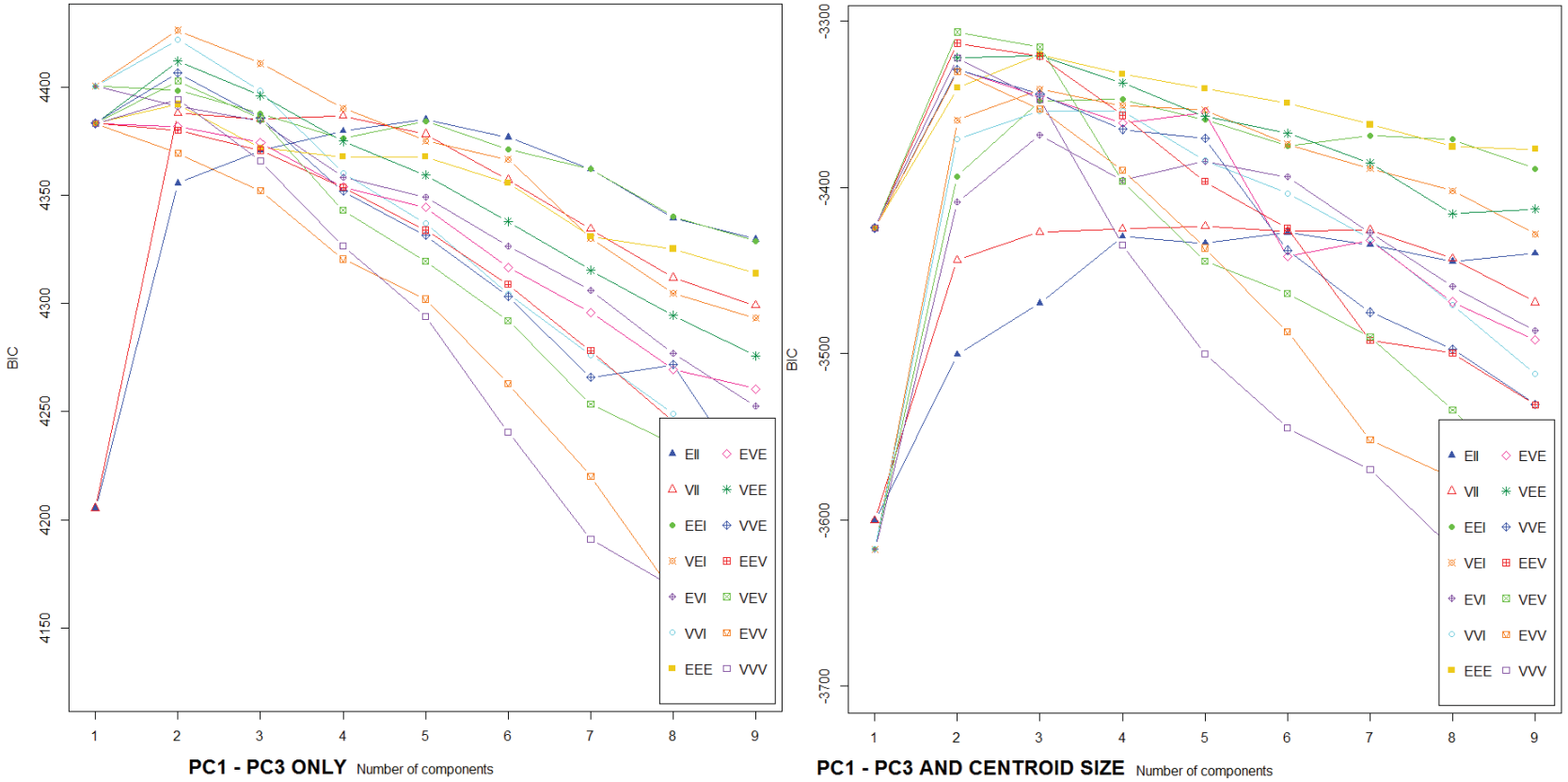
SUPPLEMENTARY FIGURE 5.2

A plot of Bayesian information criterion (BIC) scores for fitting various clustering models (in the R package mclust 5.2; Fraley and Raftery 2002) to variation among only male and female *P. chathamensis* from western Chatham Rise. The left diagram shows BIC scores when only PC1 – PC4 were analysed, and the right diagram shows when PC1 – PC4 and centroid size were analysed. When centroid size was included, variables were scaled because centroid size is expressed on a much larger scale than principal components. In mclust, BIC scores are multiplied by -1 and therefore higher BIC values indicate stronger support. See Fraley and Raftery (2012), for an explanation of the models listed.

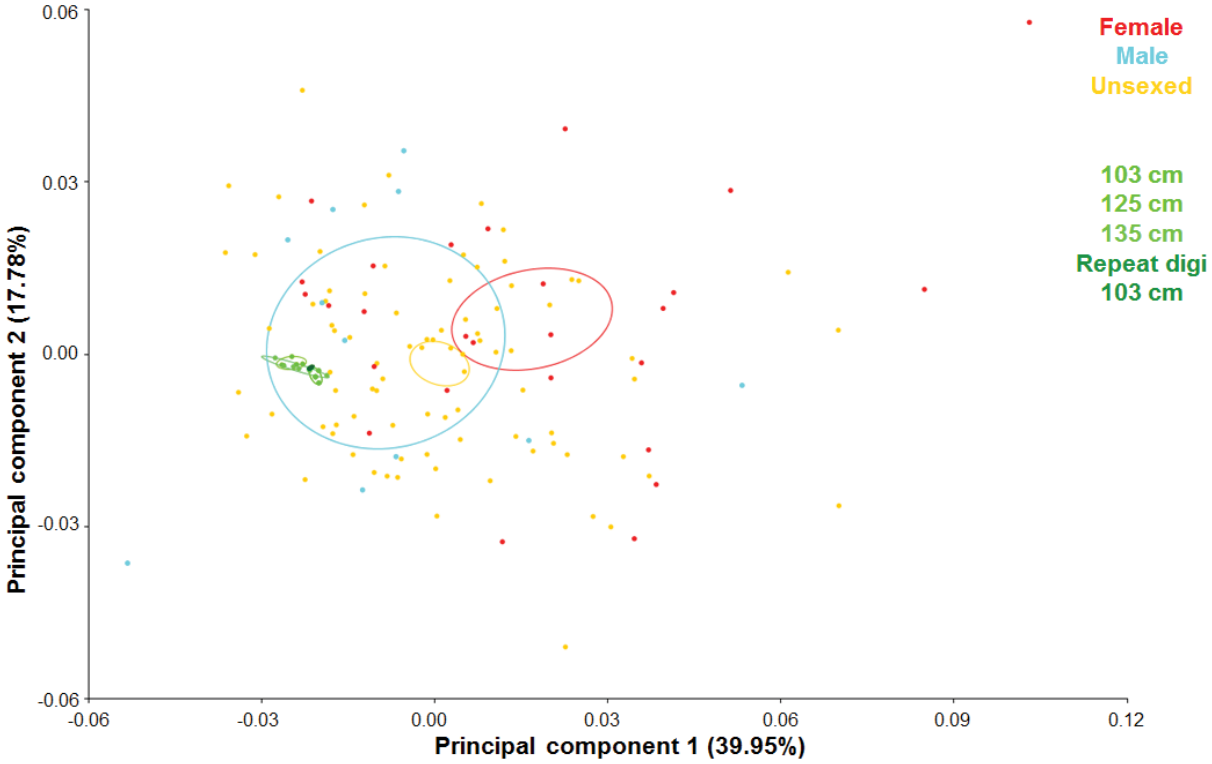


SUPPLEMENTARY FIGURE 5.3

A plot of Bayesian information criterion (BIC) scores for fitting various clustering models (in the R package mclust 5.2; Fraley and Raftery 2002) to variation among samples of *P. chathamensis* and *P. sulcatus*. The left diagram shows BIC scores when only PC1 – PC3 were analysed, and the right diagram shows when PC1 – PC3 and centroid size were analysed. When centroid size was included, variables were scaled because centroid size is expressed on a much larger scale than principal components. In mclust, BIC scores are multiplied by -1 and therefore higher BIC values indicate stronger support. See Fraley and Raftery (2012), for an explanation of the models listed.



SUPPLEMENTARY FIGURE 5.4
A principal components analysis plot produced using MorphoJ 1.06c (Klingenberg 2011), showing variation among all sampled *P. chathamensis* shells, including repeats of a single shell used for an error investigation. Principal components 1 (39.95% of variation) and 2 (17.78%) are shown. Shells from females are coloured in red, males in cyan, and shells with unknown sex are coloured in yellow. For the error study, repeated digitisations of a single shell are coloured in shades of green, with groups corresponding to the three camera heights (103, 125, 135 cm), and the repeated digitisation of a single 103 cm photograph. 90% mean confidence ellipses are illustrated for each group (in matching colouration), and indicate that the repeated error study groups all closely overlap in morphospace. Using the other statistically significant principal components (PC3 – PC5), did not alter results.



DRC 16

MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: **Felix Vaux**

Name/Title of Principal Supervisor: **Mary Morgan-Richards**

Name of Published Research Output and full reference:

Vaux, F., Crampton, J.S., Marshall, B.A., Trewick, S.A., Morgan-Richards, M. (2016). Geometric morphometric analysis reveals that the shells of male and female siphon whelks Penion chathamensis are the same size and shape. Molluscan Research (accepted).

In which Chapter is the Published Work: **Chapter Five**

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate:

  and / or

- Describe the contribution that the candidate has made to the Published Work:

  I proposed the research topic, surveyed the literature, and conducted all sampling and analysis. I wrote the original manuscript, and processed iterative feedback from co-authors and reviewers. Co-authors assisted with specimen identification, choice of statistical methods and figures, and provided feedback on manuscript drafts.

Felix Vaux — Digitally signed by Felix Vaux

Candidate's Signature

26/10/2016

Date

Morgan-Richards, Mary

Principal Supervisor's signature

28th October 2016

Date

GRS Version 3– 16 September 2011

Chapter Six

Shell morphology can estimate evolutionary lineages of siphon whelks (*Penion*)

Shells of *P. c. cuvierianus* (left) and *P.* aff. *c. cuvierianus* (right).

Introduction

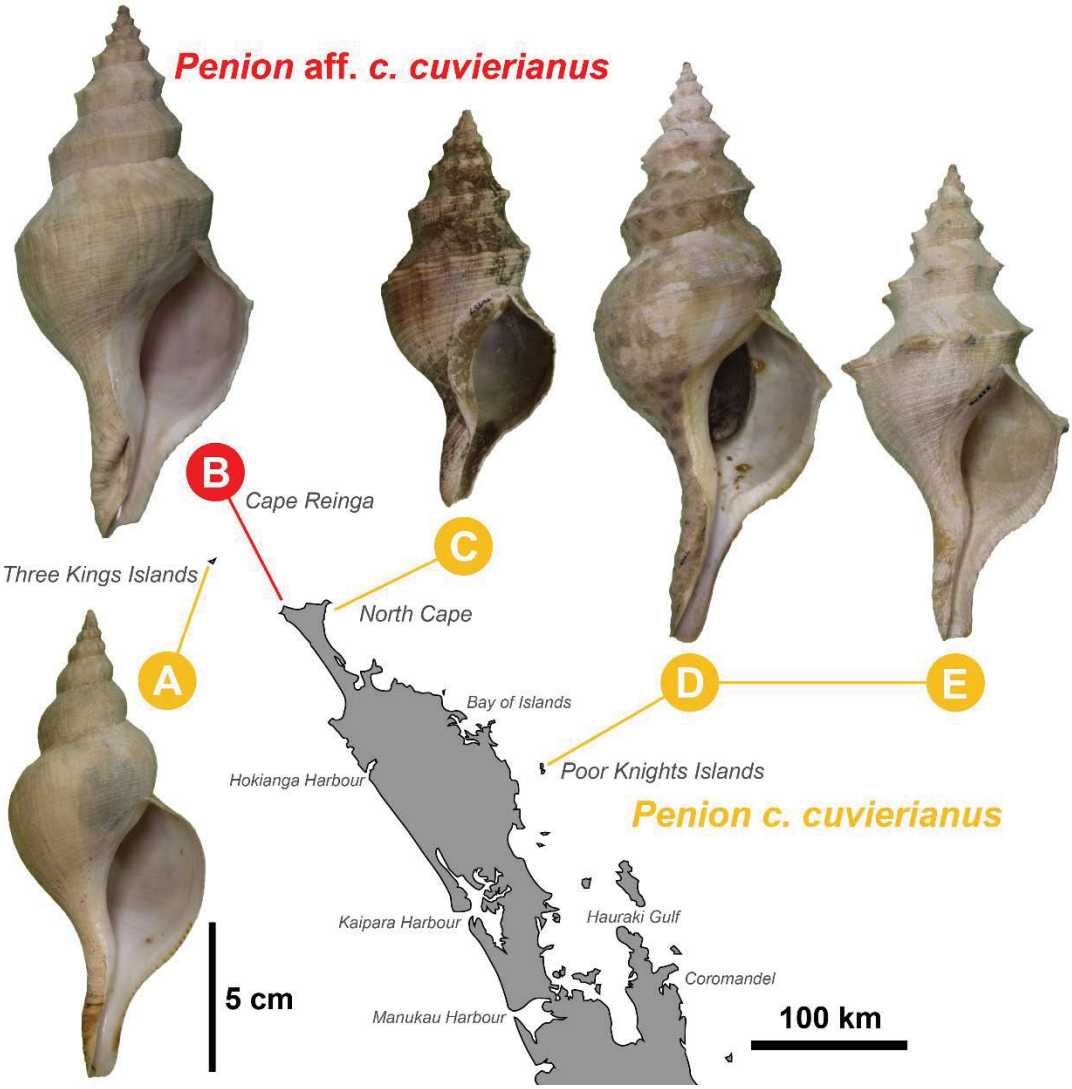
A persistent problem for evolutionary biology is whether morphology accurately reflects phylogeny and speciation in a given set of organisms. Morphological traits are desirable to study because they can be considered across the entire range of living systems from subcellular pathogens (e.g. Roberts and Compans 1998, Diaz-Avalos *et al.* 2005), to unicellular (e.g. Siefert and Fox 1998), and multicellular organisms (e.g. Niklas 2000, Valentin *et al.* 2002, Hills *et al.* 2012, Dowle *et al.* 2015). Morphology can be considered at multiple levels, including nucleic acid and protein structure (e.g. Ender and Schierwater 2003, Sakamaki *et al.* 2015), gametes (e.g. Landry *et al.* 2003), and body plans (e.g. Niklas 2000, Valentin *et al.* 2002). It includes obvious traits, often likely to be under selection, that are intuitive to observe and easy to measure. Morphology is the predominant evidence preserved in the fossil record, which is our only source of primary data for the majority of evolutionary time. However, a significant problem for evolutionary analysis is that morphological variation does not necessarily concord with the splitting and divergence of evolutionary lineages (Bapst 2013, Chapter 1: Vaux *et al.* 2016). Consequently, there is immense interest when morphological changes concords with phylogeny, as such instances provide the best opportunity to estimate rates of evolution over long periods of time (Hunt 2013).

Since marine molluscs have some of the best preserved fossil records of all animals (Wagner 2001, Crampton *et al.* 2006), many lineages are popular systems to investigate speciation and models of evolution (e.g. Michaux 1989, Wagner 2001, Monnet *et al.* 2011, Hills *et al.* 2012, Collins *et al.* 2016, Combosch and Giribet 2016). The marine molluscan fossil record is rich because calcareous shells preserve readily, and fossils are often in suitable condition to be compared to living specimens. Shell morphology is a useful phenotypic trait to study because it can reflect habitat and niche adaptation (Seilacher and Gunji 1993, Vermeij 1995), as it captures features of development and growth (e.g. Seilacher and Gunji 1993), and because it can indicate the morphology of non-preserved soft-body anatomy (e.g. Runnegar and Bentley 1983). However instead of genetic difference, variation in shell morphology can also represent phenotypic plasticity in response to environmental variation (e.g. Palmer 1990, Trussell 2000, Hollander *et al.* 2006), or sexual dimorphism (e.g. Kurata and Kikuchi 2000, Avaca *et al.* 2013), and likewise similarity among shells can be the result of convergent evolution (e.g. Serb *et al.* 2011).

Most recent systematic studies of Gastropoda focus on molecular data and variation in soft-body anatomy (e.g. Bieler 1992, Wagner 2001, Bouchet *et al.* 2005). However, the traditional examination and measurement of shell morphology often appears to be more informative than soft-body anatomy for species-level classification (e.g. Dell 1956, Willan 1978, Kantor 2003, Walker *et al.* 2008), and many genus-level taxonomic studies only consider shell traits (e.g. Chiba 1999, Gittenberger *et al.* 2012, Araya 2013). Since most paleontological studies are also constrained to the analysis of shell morphology, it is valuable to know if variation in shell morphology reflects evolutionary relationships.

FIGURE 6.1
A – E shows putative intraspecific morphological variation among individuals classified as *P. c. cuvierianus* (Powell, 1927) (with J classified as *P.* aff. *c. cuvierianus*). All classification is based on traditional morphological examination of shell traits, rather than geometric morphometric analysis or molecular data.

A: M.071981 [NMNZ] from north of the Three Kings Islands (481 – 503 m depth); B: M.279151 [NMNZ] from North Cape, classified as *P.* aff. *c. cuvierianus*; C: M.074957 [NMNZ] north of North Cape (95 – 105 m depth); D: M.279121 [NMNZ] from near Poor Knights Islands; E: M.033574 [NMNZ] from off Poor Knights Islands.



We investigated whether shell morphology accurately reflects evolutionary lineages of *Penion* Fischer, 1884 siphon whelks (Neogastropoda: Buccinoidea: Buccinidae). The current taxonomy of *Penion* is based primarily on the traditional examination of shell morphology (Ponder 1975, Powell 1979, Willan *et al.* 2010), with limited consideration of soft-body anatomy (Ponder 1975), as traits such as radulae morphology appear unreliable (Dell 1956). Siphon whelks are problematic for evolutionary analysis and taxonomic interpretation as there appears to be significant inter- and intraspecific variation in shell morphology (Ponder 1975, Powell 1979). Putative species can vary wildly in shell size, shape and colouration within small geographic distances (Figure 6.1). Siphon whelks also exhibit a rich fossil record (Ponder 1975, Beu and Maxwell 1990, Frasinetti 2000, Nielsen 2003, Beu 2009), but interpretation of this is also plagued by gross variation in shell morphology.

The phylogeny of Buccinulidae true whelks was previously inferred using mitochondrial (mtDNA) and nuclear ribosomal DNA (rDNA) sequence data (Chapter 3). Results indicated that *Penion* is sister to the genera *Kelletia* Bayle, 1884 and *Antarctoneptunea* Dell, 1972 (Chapter 3). However, *P. benthicolus* Dell, 1956 rendered *Penion* paraphyletic with *Antarctoneptunea*, suggesting reclassification as *A. benthicola* (Dell, 1956) (Chapter 3). The evolutionary relationships of all extant *Penion* were investigated further, again using mtDNA and nuclear rDNA, as well as single nucleotide polymorphic (SNP) variation for anonymous nuclear loci (Chapter 4). Results indicated that several recognised species were conspecific and suggested the existence of at least one new species (Chapter 4). Here, the concordance of variation in shell morphology among individuals of *Penion* with these phylogenetic results is investigated (Supplementary Figure 6.1), and whether results can be reconciled with current taxonomy.

Instead of using traditional morphological inference, we analysis shell morphology using landmark-based two dimensional geometric morphometrics. The morphometric landmarks used in this study capture key shell measurements traditionally used in gastropod taxonomy (e.g. shell height, aperture height) along with more information regarding shape. Previous studies have compared molecular data with traditional morphological measurements (e.g. Reid *et al.* 1996, Iguchi *et al.* 2005, Kantor *et al.* 2013), but few studies have instead used geometric morphometric data with extant (e.g. Pfenninger *et al.* 2006, Dowle *et al.* 2015), and fossil taxa (e.g. Hills *et al.* 2012, Smith and Hendricks 2013).

Geometric morphometric methods are widely considered to be superior to traditional morphological measurements as they can compare mathematical shape while controlling for variation in the size, translation (position) and orientation of objects (Webster and Sheets 2010, Mitteroecker *et al.* 2013, Monteiro 2013, Polly *et al.* 2013). Geometric morphometric methods are multivariate analyses, which are statistically more powerful and robust than the uni- or bivariate approaches conducted using linear measurements (Webster and Sheets 2010, Polly *et al.* 2013). With the integration of Kendall's 'shape space' (Kendall 1984), the methods have a strong theoretical underpinning in mathematics (Bookstein 1995). Lastly, geometric morphometric analyses can also reveal unexpected variation that is not obvious to human observers (Webster and Sheets 2010).

We investigate variation in shell morphology hierarchically, taking a bottom-up approach along the estimated phylogenetic tree of the *Antarctonetpunea*, *Kelletia* and *Penion* clade (Supplementary Figure 6.1). First we looked for evidence of shape and size differences between shells of different buccinid genera and deep phylogenetic splits (i.e. *Antarctoneptunea, Kelletia,* Australian *Penion*, New Zealand *Penion*; Supplementary Figure 6.1). We then investigated variation within the monophyletic New Zealand *Penion* clade, and finally explored variation between closely related putative species (Supplementary Figure 6.1).

Three sets of closely related lineages were investigated: 1) *P. chathamensis* (Powell, 1938) and *P. fairfieldae* (Powell, 1947), which were previously found to be genetically indistinguishable (Supplementary Figure 6.1; Chapter 4) and have adjacent geographic ranges. Previous taxonomic reviews considering shell traits in these taxa such as the prominence of teleoconch spire ridges and siphonal canal lengths did not consider the two species to be closely related (Powell 1947, Dell 1956, Powell 1979). 2) *P. c. cuvierianus* (Powell, 1927) and *P.* aff. *c. cuvierianus*, where the latter taxon is suspected to represent a distinct lineage based on traditional examination of shells, focussing on shell thickness and protoconch morphology. It is geographically restricted to the westernmost range limit of *P. c. cuvierianus*. However, DNA sequence data from a single individual resolved no genetic differentiation (Supplementary Figure 6.1; Chapter 4). 3) 'The *P. ormesi* complex' consists of *P. ormesi* (Powell, 1927), *P. c. jeakingsi* (Powell, 1947) and *P.* n. sp. West Coast (Supplementary Figure 6.1). These taxa are difficult to distinguish using traditional morphological traits. They may have fairly distinct geographic ranges with limited overlap at some locations (see Figure 4.1).

Molecular results were mixed for the distinction of the taxa, with multiple mtDNA haplotypes being shared among all three taxa, but anonymous nuclear loci readily separating *P. ormesi* and *P. c. jeakingsi* (Chapter 4). For each of these sets of taxa, we assessed whether variation in shell morphology estimated via geometric morphometrics supported the phylogenetic or traditional taxonomic hypothesis.

Methods

Previous molecular evidence

DNA sequence data provided the primary phylogenetic hypothesis of *Penion* (Chapter 4), against which to test variation in siphon whelk shell morphology. A DNA phylogeny of Buccinulidae (Chapter 3), provided a basis for consideration between earlier evolutionary splits. We consult single nucleotide polymorphic (SNP) variation for anonymous nuclear loci within and among some species of *Penion*, which was generated via double-digest restriction site associated DNA sequencing (Chapter 4).

Taxonomy

Individuals were assigned to putative taxa based on traditional morphological examination of shells and soft-body anatomy (Dell 1956, Ponder 1973, Powell 1979, Willan *et al.* 2010). The traditional classification of specimens specifically took into account traits such as body size, shell colouration, protoconch morphology and the presence of shell features such as axial ribs (Powell 1979). All sampled genetic individuals and shells were classified by experienced malacological taxonomists (Bruce A. Marshall [Te Papa Tongarewa Museum of New Zealand], Alan G. Beu [GNS Science]). We treated the taxonomic classification of specimens as a working hypothesis to describe arbitrary segments of evolutionary lineages (see Chapter 1), which can be tested using genetic and phenotypic data. Current taxonomy and the operational taxonomic units (OTUs) used for this thesis are reviewed in Chapter 8.

We interpret the phylogeny of *Penion* in two ways: 1) 'maximal OTUs' where all putative taxa are included, 2) 'revised OTUs' where the taxonomy is revised based on the molecular phylogenetic results (Chapters 3 and 4). The second approach specifically combines *P. ormesi* with *P. cuvierianus jeakingsi* and *P.* n. sp. West Coast; *P. chathamensis* with *P. fairfieldae*; and *P. c. cuvierianus* with *P.* aff. *cuvierianus cuvierianus*. In both approaches, *P. c. cuvierianus* and *P. c. jeakingsi* are treated separately, given the clear phylogenetic evidence for separation (Chapter 4). Taking this dual approach permits us to separately assess the concordance of morphometric data with the phylogenetic hypothesis and current taxonomy.

Shell sampling

All extant species of *Penion* from New Zealand and Australia, including all subspecies recognised by Powell (1979), and all extant species of *Antarctoneptunea* and

Kelletia were sampled (see Figures 3.7 and 4.1 for species distributions). We sampled a substantial number of shells per putative species (see Table 6.1), with at least 46 shells per species where possible, as this amount exceeds the final number of landmarks used (45). For downstream analyses adequate sampling ensures that the degrees of freedom exceed the shape dimensionality of the data; meaning that there are an adequate number of principal components for later analyses. We attempted to sample individuals from more than one location per species in order to capture population-level variation (Figure 6.1). However, morphological sampling was limited for six taxa of interest: *P.* n. sp. Three Kings Islands, *P.* n. sp. West Coast, *P.* aff. *c. cuvierianus*, *A. aurora*, *K. kelletii* (Forbes, 1850), and *K. lischkei* (Table 6.1). The first four taxa are known only from small or remote regions (the Three Kings Islands; West Coast; far north Northland; and the Southern Ocean respectively), which makes sampling challenging, a problem compounded by waters difficult-to-navigate. Both *Kelletia* species were sampled at low frequency because they occur outside of Australasia with collection dependent on overseas colleagues. Molecular sampling for these taxa was also limited for the same reasons (Chapter 4).

The majority of shells were sourced from museum and university collections (listed in Acknowledgements). Most specimens were obtained by trawling at depths of 20 – 500 m, or as fishery by-catch. Some specimens were collected by hand intertidally and snorkelling (1 – 5 m), or gathered from live or recently deceased snails wash ashore on beaches. Complete or near-complete shells (specimens with intact edges and points encompassed by landmarks) with reliable provenance data were used. Among our shell sampling of *Antarctoneptunea, Kelletia* and *Penion*, 31 specimens had provided genetic data (Chapters 3 and 4). Only conchologically mature shells were photographed, with maturity being estimated by the presence of at least six teleoconch whorls, thickening of the outer aperture lip and ascent of the end of the last whorl. Although sexual maturity can occur earlier (Jones 1938), shell maturity is usually treated as a proxy for adulthood in snails as it indicates when a snail is no longer growing in size (Goodfriend 1986).

TABLE 6.1
Sampling of extant, mature *Penion, Kelletia* and *Antarctoneptunea* shells for morphometric analysis.

Genus	species	Broad geographic location	Number of shells included in dataset
Antarctoneptunea	*aurora*	Antarctica	1
Antarctoneptunea	*benthicola*	New Zealand	60
Kelletia	*kelletii*	USA, Mexico	24
Kelletia	*lischkei*	Japan, South Korea	8
Penion	*chathamensis*	New Zealand	125
Penion	*c. cuvierianus*	New Zealand	200
Penion	aff. *c. cuvierianus*	New Zealand	21
Penion	*c. jeakingsi*	New Zealand	78
Penion	n. sp. West Coast	New Zealand	4
Penion	*fairfieldae*	New Zealand	48
Penion	*mandarinus*	Australia	89
Penion	*maximus*	Australia	114
Penion	*ormesi*	New Zealand	50
Penion	*sulcatus*	New Zealand	187
Penion	n. sp. Three Kings Islands	New Zealand	25
TOTAL			**1034**

Photography and geometric morphometric analysis of shells

Variation in shell morphology was analysed using the same two-dimensional landmark-based geometric morphometric method used to investigate sexual dimorphism in *P. chathamensis* (for detailed method see Chapter 5). In summary, shells were photographed with the aperture facing upward using a Canon EF-S 600D camera with an 18 – 55 mm IS II lens (see Figure 5.1), and the positioning and orientation of shells was controlled carefully (see discussion by Webster and Sheets 2010; Supplementary Data). Combs, aligned to the central axis of the shell, were added to photographs in Adobe Photoshop CS6 so that semi-landmarks could be placed consistently. We used a total of 45 landmarks to summarise shell shape (Figure 5.1). Six fixed landmarks captured biologically homologous points such as the top of the teleoconch, and 39 semi-landmarks described the inner and outer curves of the aperture and siphonal canal. Following the interpretation of Gunz *et al.* (2005), all of our landmarks are Type I as defined by Bookstein (1991). Landmarks and semi-landmarks were digitised and scale-calibrated using tpsUtil, tpsDig (Rohlf 2013), and the IMP program CoordGen7 (Sheets 2014), yielding X – Y Procrustes coordinates. Semi-landmarks were 'slid' to minimise the effect of the arbitrary placement of points on the curves of interest. Sliding was achieved by minimising Procrustes distances (Bookstein 1996, Zelditch *et al.* 2004, Perez *et al.* 2006), using the IMP program Semiland7 (Sheets 2014).

Experimental error was investigated and found to be negligible (see Supplementary Data). Individuals sequenced for molecular data (Chapters 3 and 4), were determined to represent an adequate range of morphological variation among monophyletic New Zealand *Penion* (Supplementary Data). Based on results from *P. chathamensis* (Chapter 5), we assume that secondary sexual dimorphism is not a source of significant, confounding variation in the shell morphology of all sampled species of *Penion* and perhaps also *Antarctoneptunea*. However, there is possibly a size difference between males and females of *Kelletia* (Rosenthal 1970).

Partial Procrustes superimposition was conducted using MorphoJ 1.06c (Klingenberg 2011), which aligns and superimposes landmarks for all specimens to remove confounding variation due to differences in the size, translation (position) and orientation of objects (Webster and Sheets 2010, Mitteroecker *et al.* 2013, Monteiro 2013, Polly *et al.* 2013). Procrustes superimposition is the preferred method when morphological variation is relatively small (Perez *et al.* 2006). A covariance matrix was generated from the X – Y coordinates of the superimposed landmarks, providing input

for principal components analysis (PCA) in MorphoJ 1.06c (Klingenberg 2011). The principal components reflect (mathematically independent) variation in the shape of objects, and centroid size acts as a proxy for size variation (independent of shape). Statistically significant principal components (PCs) were identified using the broken-stick test on eigenvalues, implemented in the R (R Core Team 2016) package vegan 2.2-1 (Jackson 1993, Oksanen *et al.* 2015). We used PCA ordinations to estimate the separation of *a priori* groups (e.g. monophyletic clades, taxonomic species, populations). We used 90% mean confidence ellipses of group means to determine if groups were likely to overlap. Canonical variates analysis (CVA) was used to statistically test the ability to differentiate these groups, with the success of discrimination determined using cross-validation scores (the number of individuals correctly assigned to each *a priori* group). CVA was conducted using either MorphoJ 1.06c (Klingenberg 2011), analysing the original X – Y landmark coordinates, or the R package MASS 7.3-26 (Venables and Ripley 2002) using PCs generated from PCA in MorphoJ. For taxa with fewer specimens than the number of landmarks used, we used PCA as a dimensionality-reducing method to allow *a priori* groups to be tested with CVA.

We also wanted to investigate what groupings could be naïvely identified using the shell morphological data (shape and size) alone, without relying on *a priori* hypotheses based on other information such as genetics, taxonomy, or geography. To investigate naïve groupings, we conducted model-based cluster analysis using the R package mclust 5.2 (Fraley and Raftery 2002). Mclust can analyse both PCs (shell shape) and centroid size (shell size), and it attempts to identify the clustering model that most efficiently explains variation in a dataset without prior classification of specimens. The fit of a model is tested with an iterative expectation-maximisation (EM) method using Gaussian mixture modelling (Fraley and Raftery 2012). The models used by mclust differ in the expected distribution of data, as well as the volume, shape and orientation of the covariance matrices generated from observed data (parameters for mclust models listed in Supplementary Table 5.3; Fraley and Raftery 2012). Bayesian information criterion (BIC) scores were used to determine the relative support for competing clustering models. In mclust, BIC scores are multiplied by -1 and therefore higher BIC values indicate higher support. Where centroid size was included with PCs for mclust analyses, variables were scaled (using the base function in R) because centroid size is expressed on a much larger numerical scale than the PCs. Different

numerical scales are problematic for mclust analysis as multiple models tested assume the same variance across all variables or estimated clusters (Fraley and Raftery 2012).

Results and Discussion

Morphometric variation across deep evolutionary splits

Two statistically significant PCs were identified (broken-stick test): PC1 (60.6% of variation), and PC2 (14.5%) for the first dataset including all sampling of extant *Penion, Kelletia* and *Antarctoneptunea*. When analysed without *a priori* classification, 3 clusters were best supported when only the significant PCs were analysed (based on BIC score using mclust; Supplementary Figure 6.5), and 4 had highest support when centroid size was also included (Supplementary Figure 6.5). Clusters identified by mclust appeared to be nested hierarchically after >3 clusters, and were consistent across models (Figures 6.2a – 6.2d), although BIC scores became homogenous for models after >4 clusters (Supplementary Figure 6.1). The hierarchical nature of the data can best be observed by comparing the assignment probability of individuals across models and varying number of clusters (Figure 6.2a). The assignment of specimens across clustering models can also be compared directly (Figure 6.2b). As can be seen, the accuracy of cluster assignment generally improves as more clusters are included, and groupings remain hierarchically quite consistent. We attempted to see if this hierarchical pattern could be corroborated by specific hierarchical clustering methods, such as the mclust 5.2 hc function (Fraley and Raftery 2012), however our dataset does not readily conform to the models available for this analysis and therefore results were deemed to be unreliable. Specifically, few models for hierarchical clustering include both equal and variable covariance matrix components (see Supplementary Table 5.3), which are often supported for our datasets using the standard EM clustering method.

The clusters identified by mclust correspond quite closely to the lineages identified via molecular phylogenetics (Figures 6.2a, also 6.2d and 6.2e). For example, for the VVE3 model using PC1 – PC2, cluster 1 appears to represent *Kelletia* (90.6% of genus, 74.4% of cluster 1; see Figure 2). For the EVE4 model using PC1 – PC2 and centroid size, cluster 2 contains almost all specimens of *P. sulcatus* (Lamarck, 1816) (95.0% of species, 53.6% of cluster 2; Figure 6.2a) and many *P. fairfieldae* (79.2% of species, 11.9% of cluster 2; Figure 6.2a), which are potentially phylogenetically sister according to mtDNA and rDNA (Chapter 4). See Supplementary Table 5.3 for an explanation of the different parameters used by mclust models. The results are mostly

concordant with current taxonomy, and where disagreements occur (e.g. many *A. benthicola* being clustered with *Kelletia* and *A. aurora*), this is consistent with the molecular phylogeny. The inclusion of centroid size also helped to identified *A. benthicola* as a separate cluster.

Using PCA, shell shape was able to separate some species recognised under maximal OTUs (Figure 6.2e), and many included within revised OTUs (Figure 6.2f). Overlap of PC distributions was more frequent between the closely related species in the monophyletic New Zealand *Penion* clade, indicating that the significant PCs were dominated by generic-level variation in shell shape. Compared to maximal OTUs (Figure 6.2e), revised OTUs (based on mtDNA) resulted in groups that were generally overlapped less (Figure 6.2f). Cross-validation via CVA, showed clear separation of scores from the three putative genera (with the re-classification of *P. benthicolus* to *Antarctoneptunea* as *A. benthicola*), as were most revised and maximal taxonomic units (Supplementary Table 6.1). This was apparent via CVA ordination plots (Figure 6.2g). Misassignment of individuals was reduced when phylogenetic groups were followed, and taxa that remained difficult to differentiate exhibited low sampling. Although CVA ordination plots showed separation of deeper phylogenetic splits, the monophyletic New Zealand *Penion* samples exhibited considerable overlap (Supplementary Figure 6.6).

Shape variation among individuals represented by PC1 appeared to reflect variation in the width of the shell, being most obvious in the aperture, body whorl, and siphonal canal, whereas PC2 appeared to reflect variation in the height of the spire and aperture (Figure 6.2h). This is consistent with the traditional taxonomic classification, as *Penion* and *Kelletia* are said to differ primarily in those traits (e.g. Ponder 1975), and suggests that our landmarks were capturing biologically (or at least taxonomically) meaningful variation.

FIGURE 6.2a
Bayesian assignment probability of all sampled *Penion*, *Kelletia* and *Antarctoneptunea* shells to clusters estimated by the R model-based clustering package mclust 5.2. Specimens (each individual is one vertical line) are coloured by assignment probability to clusters, organised following the molecular phylogeny (labelled by species) and by geographic distribution within species (not labelled due to space constraints). Colours used for each cluster are identified within a key. The VVE3 model (top) was the best supported model using only the statistically significant PCs 1 – 2, whereas the EVE4 model (middle) received the highest BIC support when centroid size was also included. When 8 clusters were considered, the VEE8 model (bottom) using PCs 1 – 2 and centroid size received the highest support among alternative models. See Supplementary Figure 6.5 for a comparison for BIC values among clustering models.

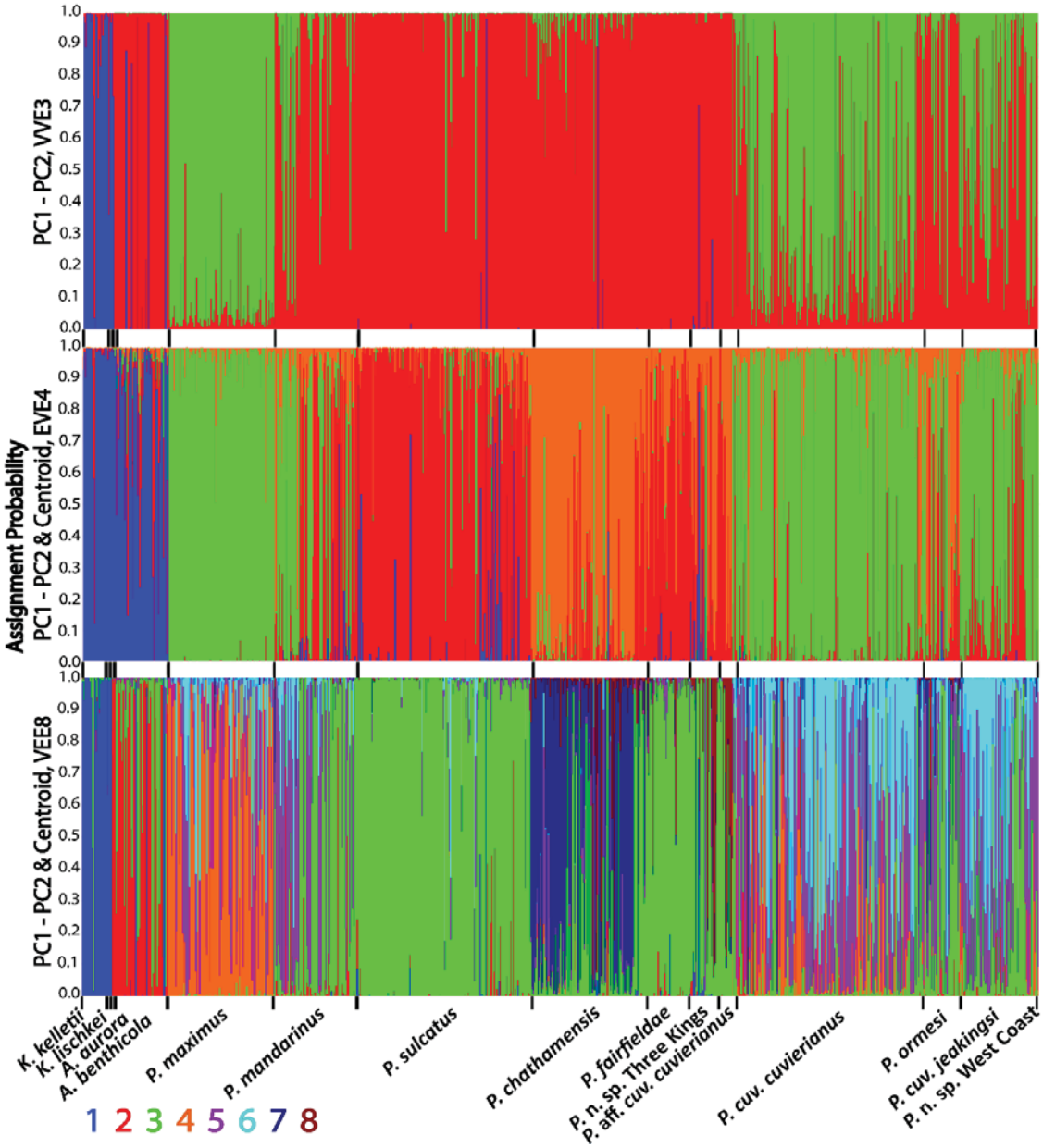


FIGURE 6.2b
A comparison of how all sampled *Penion*, *Kelletia* and *Antarctoneptunea* shells are assigned to clusters estimated by the R model-based clustering package mclust 5.2. On the top diagram, the X axis shows the assignment of shells to clusters estimated by the EVE4 model using PCs 1 – 2 and centroid size, whereas the Y-axis shows the assignment of specimens under the VEE8 model using the same set of variables. On the bottom diagram, the X axis shows the assignment of specimens under the VVE3 model using only PCs 1 – 2, whereas the Y axis shows assignment under the EVE4 model using PCs 1 – 2 and centroid size. On both diagrams, each specimen is marked as a separate increment on the X axis to allow the re-classification of specimens between clusters to be observed.

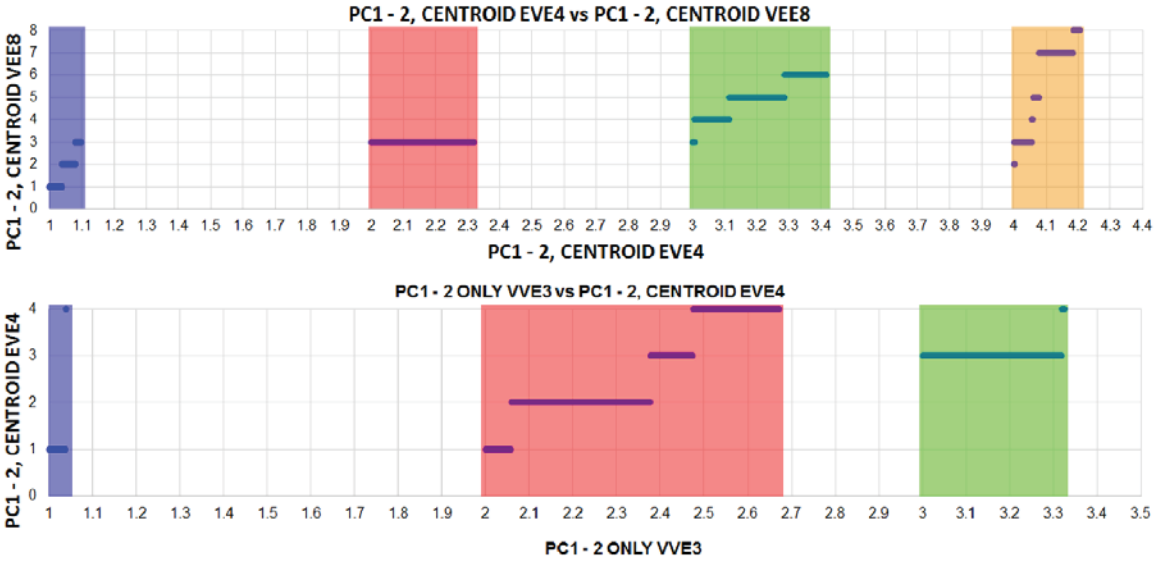


FIGURE 6.2c
A principal component analysis (PCs 1 – 2) of all sampled *Penion*, *Kelletia* and *Antarctoneptunea* shells. Colouration corresponds to clusters identified naïvely by the R model-based clustering package mclust 5.2. Mean confidence ellipses (90%; same colouration) indicate that the group means are not likely to overlap. The clusters shown were identified by the EVE4 model using the statistically significant PCs 1 – 2 and centroid size.

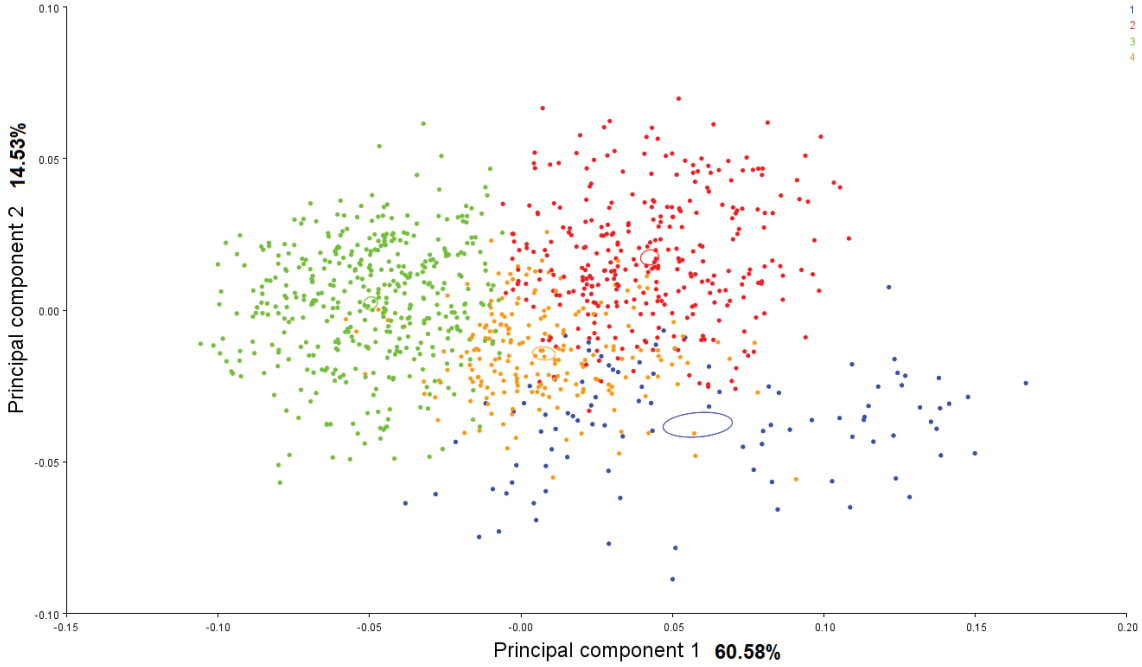


FIGURE 6.2d
A principal component analysis (PCs 1 – 2) of all sampled *Penion, Kelletia* and *Antarctoneptunea* shells. Colouration corresponds to clusters identified naïvely by the R model-based clustering package mclust 5.2. Mean confidence ellipses (90%; same colouration) indicate that the group means are not likely to overlap. The clusters shown were identified by the VEE8 model using the statistically significant PCs 1 – 2 and centroid size.

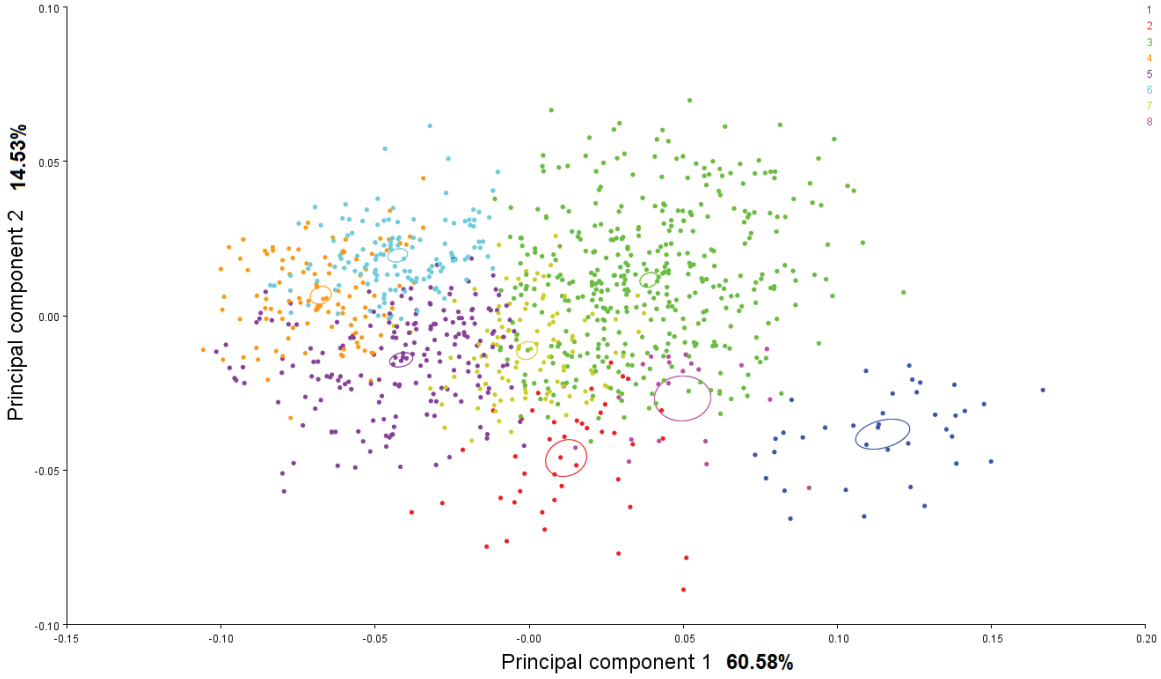


FIGURE 6.2e
A principal component analysis (PCs 1 – 2) of all sampled *Penion*, *Kelletia* and *Antarctoneptunea* shells. Colouration and confidence ellipses correspond to putative taxonomic species under maximal OTUs. Mean confidence ellipses (90%; same colouration) indicate that the means of many taxa are unlikely to overlap.

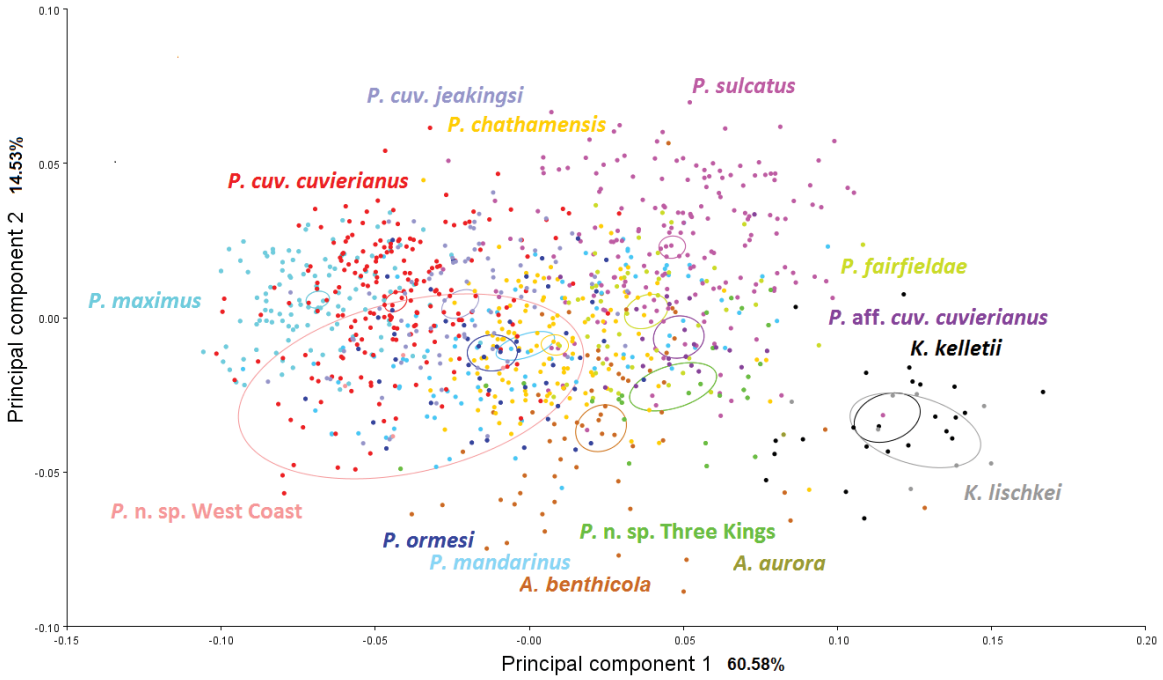


FIGURE 6.2f
A principal component analysis (PCs 1 – 2) of all sampled *Penion*, *Kelletia* and *Antarctoneptunea* shells. Colouration and confidence ellipses correspond to putative taxonomic species under revised OTUs based on results from molecular analyses (see Chapters 3 and 4). Mean confidence ellipses (90%; same colouration) indicate that the means of most taxa are unlikely to overlap.

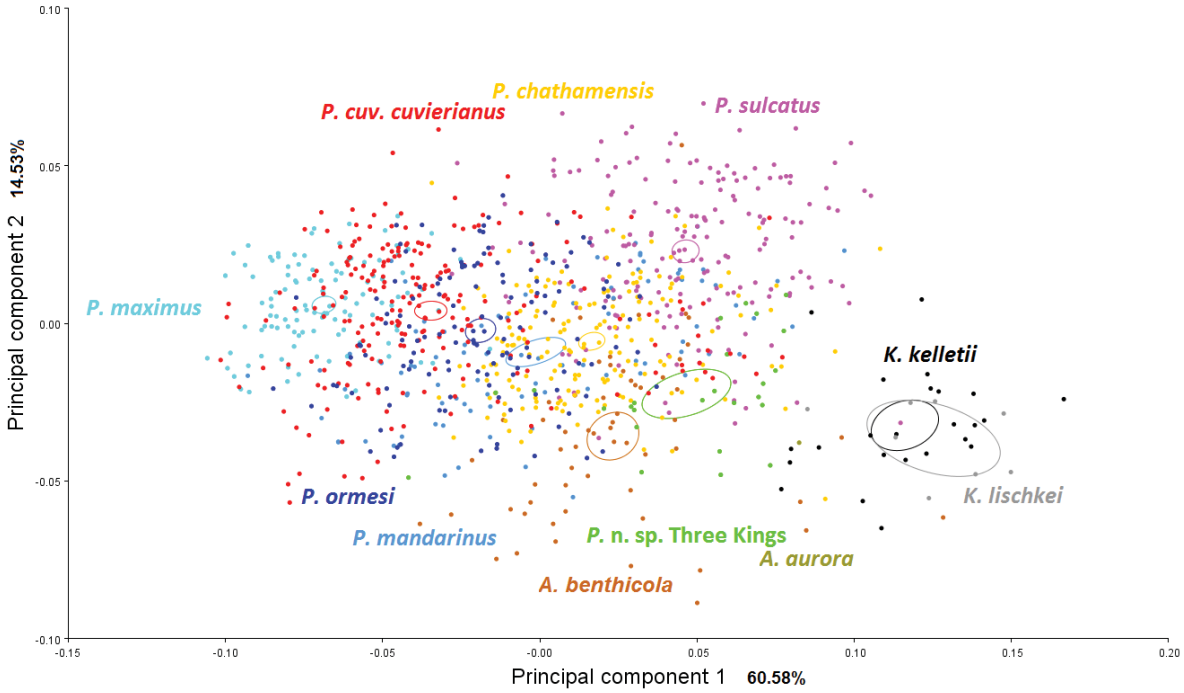


FIGURE 6.2g
A canonical variates analysis of all shells sampled from *Penion*, *Kelletia* and *Antarctoneptunea*. Mean confidence ellipses (90%; same colouration) indicate if groups overlap. Specimens are classified by putative taxonomic genera, which includes the revised reclassification of *P. benthicolus* to *Antarctoneptunea* based on previous molecular results (see Chapter 3).

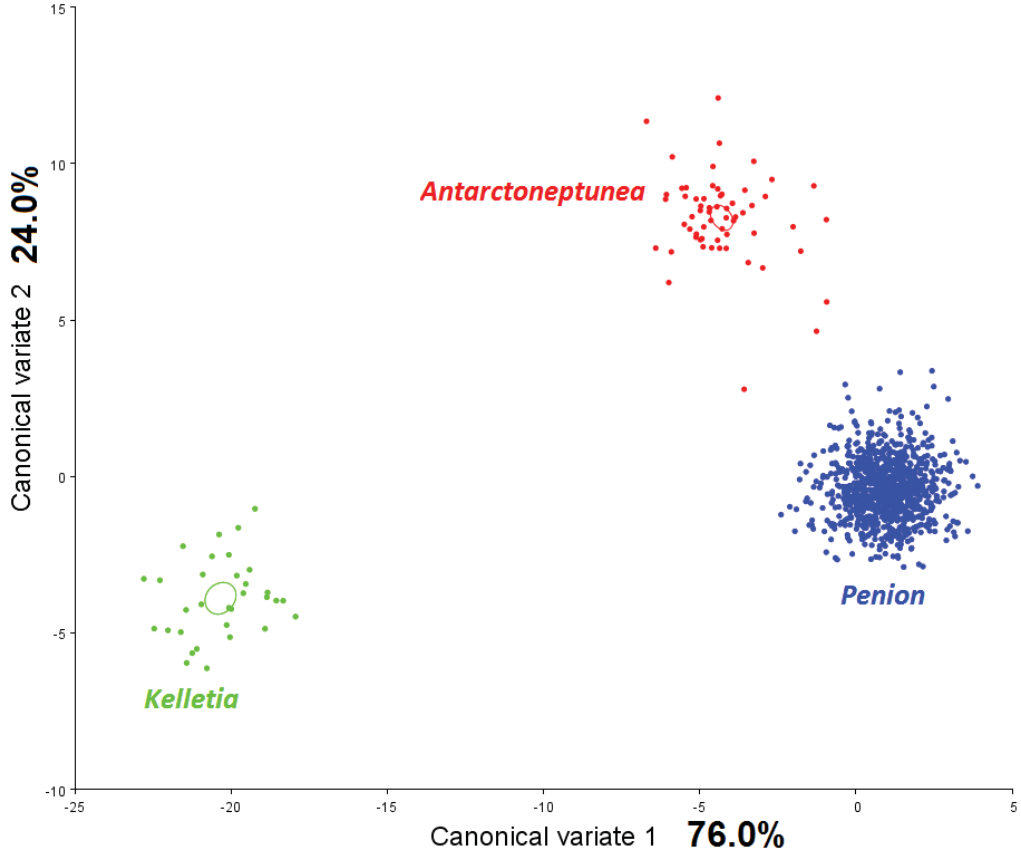
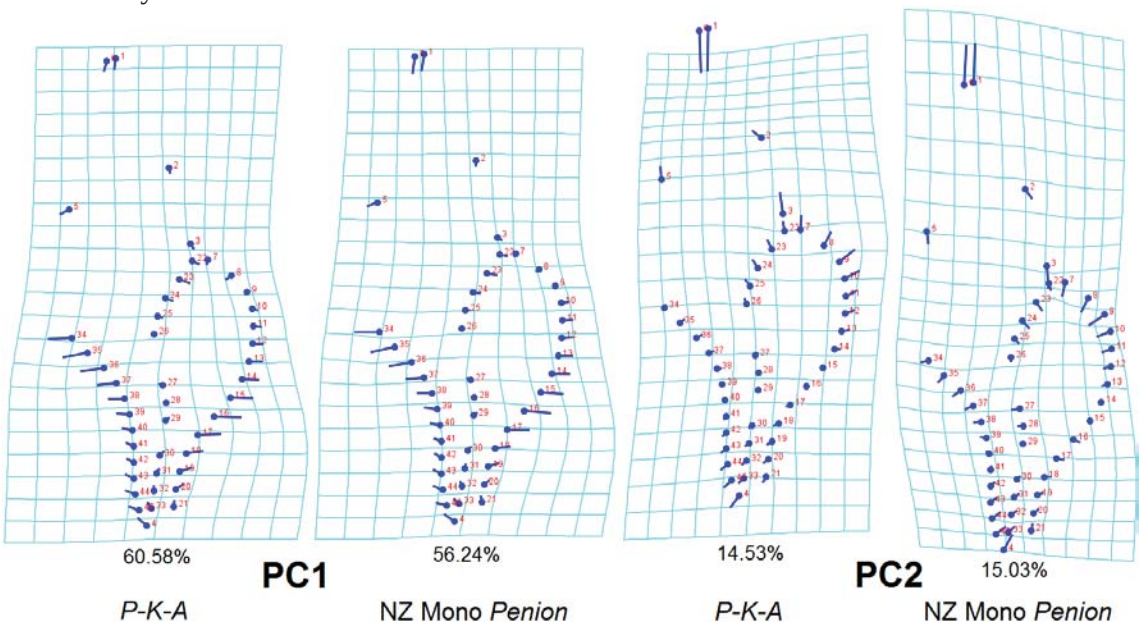


FIGURE 6.2h
Thin plate spline (TPS) diagrams with a transformation grid, showing the shape differences represented by PC1 and PC2 for two different PCAs. The first dataset (left side) is a PCA including all shells sampled from *Penion*, *Kelletia* and *Antarctoneptunea*, whereas the second dataset (right side) only includes sampling from the clade of monophyletic New Zealand *Penion*. Despite being generated from different datasets, the shape differences represented by PCs 1 – 2 in both analyses are similar.



Morphometric variation within monophyletic New Zealand *Penion*

Ordinations of PCA and CVA frequently demonstrated overlapping distributions in shape space among monophyletic New Zealand taxa, especially for maximal OTUs, in contrast to comparisons of distantly related taxa (e.g. *Penion* and *Kelletia*). Likewise, mclust exhibited lower assignment confidences for individuals of monophyletic New Zealand *Penion*. We therefore reduced our dataset to 741 shells belonging to that clade so that the significant PCs had the potential to represent optimally the variation between closely related lineages, without the interference of variation between deeper phylogenetic splits (see Supplementary Figure 6.1).

Three statistically significant PCs were identified (broken-stick test): PC1 = 56.2%; PC2 = 15.0%; PC3 = 7.1%. Based on BIC score, mclust found highest support for 3 clusters when only the significant PCs were analysed, although many models with more than 3 clusters also received high BIC support (Supplementary Figure 6.7). When centroid size was also considered 3 clusters was again the best fitting model (Supplementary Figure 6.7). Across clustering models, clusters appeared to mostly match the hierarchy of the molecular phylogeny (Figures 6.3a and 6.3b). Using the VEE3 model with PC1 – PC3 and centroid size, cluster 2 corresponded closely to the molecular clade of *P. c. cuvierianus, P. ormesi, P. c. jeakingsi* and *P.* n. sp. West Coast (89.5% of molecular clade, 93.1% of cluster 2), and cluster 1 appeared to contain most other taxa, with the exception of *P. chathamensis* that was frequently isolated within cluster 3 (Figure 6.3a). A notable exception was *P.* aff. *c. cuvierianus*, which was not clustered with *P. c. cuvierianus.* For three clusters, the inclusion of centroid size generally increased assignment confidence (Figure 6.3a). However at higher numbers of clusters, assignment probabilities varied and the identified clusters often corresponding to subgroups within putative taxa that did not conform to any molecular or taxonomic hypothesis. Consequently the results from >3 clusters were not as easy to interpret, however under most models centroid size did appear to allow for the recognition of *P.* n. sp. Three Kings Islands (Figures 6.3a – 6.3c), and it appears that some distinguished clusters reflected possible intraspecific differences among populations – such as *P. sulcatus* from the Cook Strait (Figure 6.3a). The fact that centroid size overall was less informative than for the generic-level dataset is not surprising as the monophyletic New Zealand siphon whelks overlap considerably in size.

Using PCA, species under both maximal and revised OTUs exhibited distributions with less overlap than before (based on 90% mean confidence ellipses)

(Figures 6.3d and 6.3e), implying that the significant PCs captured more relevant shape variation for these related lineages. Cross-validation via CVA was only marginally improved (Supplementary Table 6.2), but the plotted ordination was significantly clearer and most putative species could be readily separated (Supplementary Figure 6.8). A continued exception was *P.* n. sp. West Coast, however this species was sampled with very low frequency and results from CVA are potentially misleading.

FIGURE 6.3a
Bayesian assignment probability of all sampled specimens classified within the clade of monophyletic New Zealand *Penion* to clusters estimated by the R model-based clustering package mclust 5.2. Specimens are coloured by assignment probability to clusters, are organised following the molecular phylogeny (see Chapter 4), and are labelled based on putative taxonomic classification and also sampling region for more frequently sampled taxa. Colours used for each cluster are identified within a key. The EEI3 model (top) was the best support model using only the statistically significant PCs 1 – 3, and the VEE3 model (middle) received the highest BIC support when centroid size was also included. The EEE6 model (bottom), also using PCs 1 – 3 and centroid size, received the highest BIC support among alternatives when 6 clusters were considered. See Supplementary Figure 6.8 for a comparison for BIC values among clustering models.

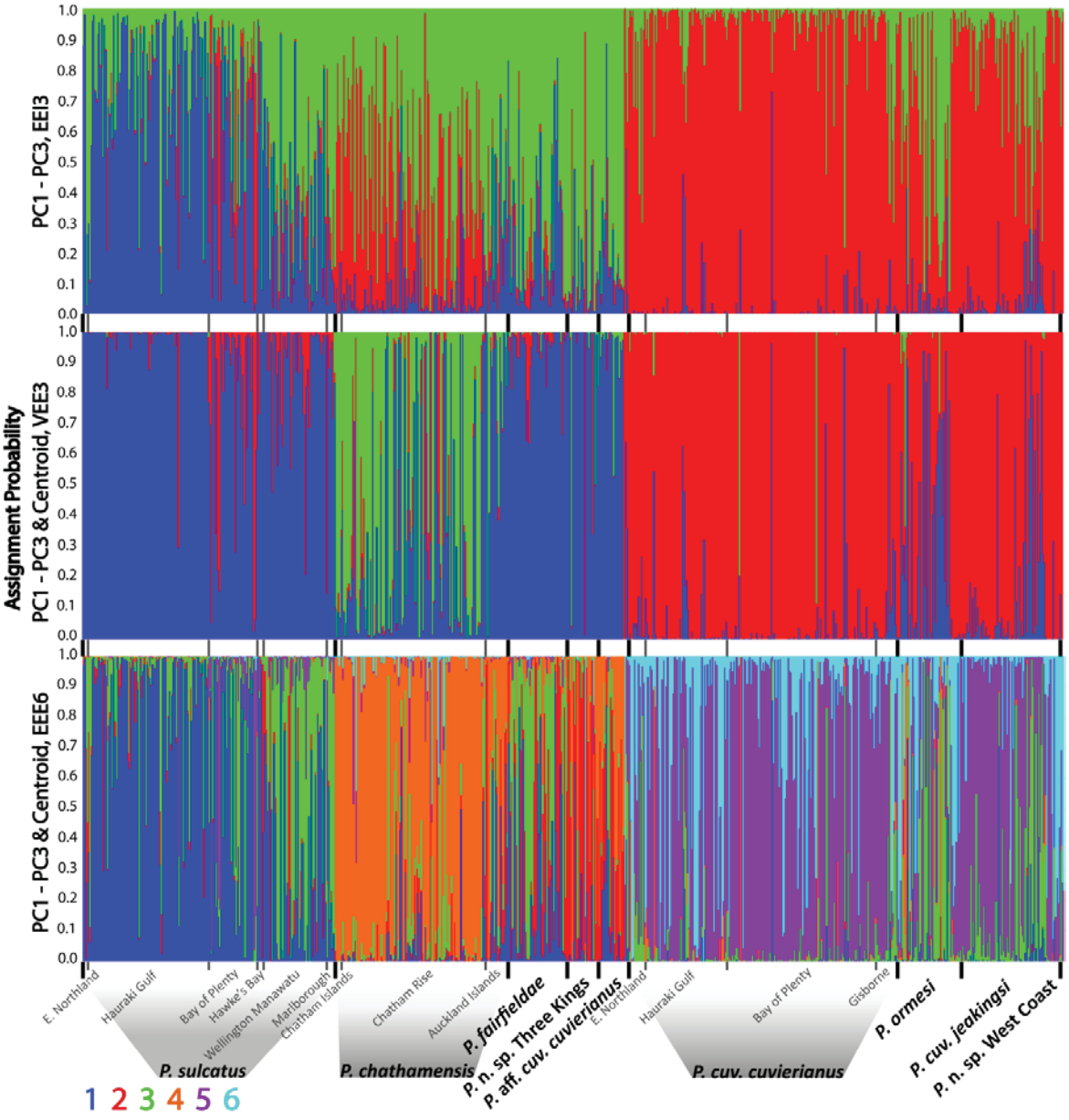


FIGURE 6.3b
A principal component analysis (PCs 1 – 3) of all sampled specimens classified within the clade of monophyletic New Zealand *Penion*. Colouration corresponds to clusters identified naïvely by the R model-based clustering package mclust 5.2. Mean confidence ellipses (90%; same colouration) indicate that the group means are not likely to overlap. The clusters shown were identified by the VEE3 model using the statistically significant PCs 1 – 3 and centroid size.

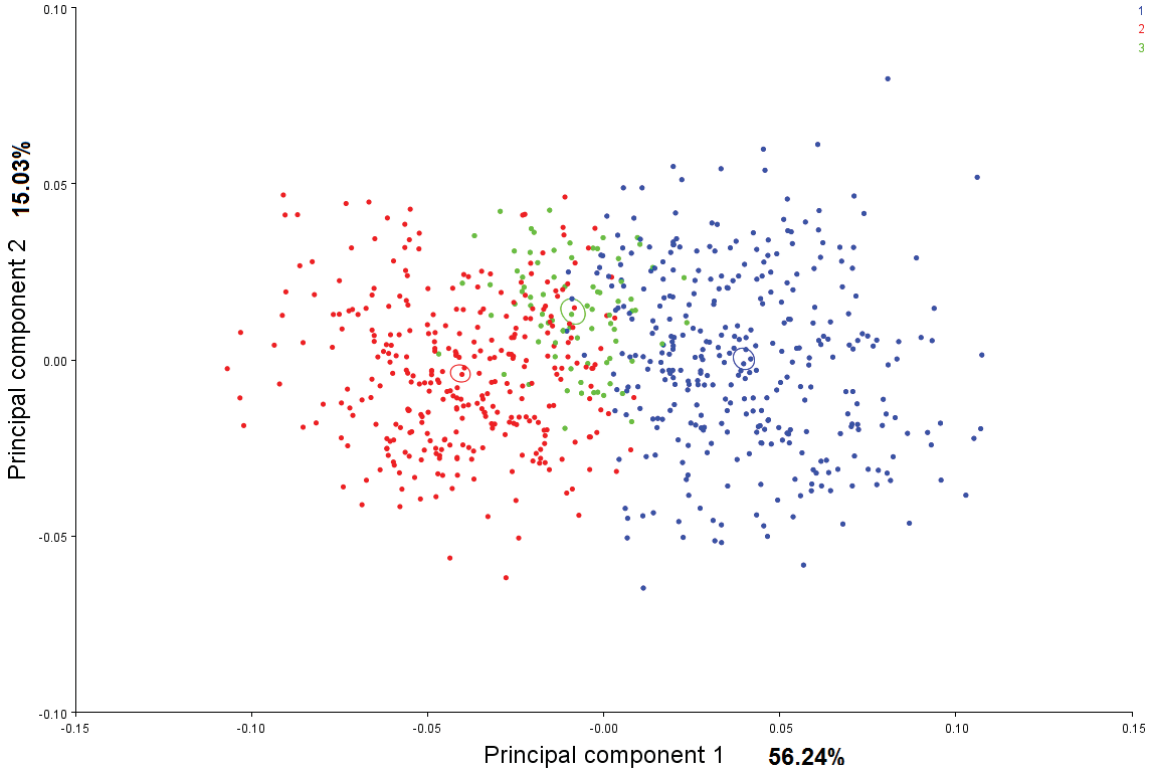


FIGURE 6.3c
A principal component analysis (PCs 1 – 3) of all sampled specimens classified within the clade of monophyletic New Zealand *Penion*. Colouration corresponds to clusters identified naïvely by the R model-based clustering package mclust 5.2. Mean confidence ellipses (90%; same colouration) indicate that the group means are not likely to overlap. The clusters shown were identified by the EEE6 model using the statistically significant PCs 1 – 3 and centroid size.

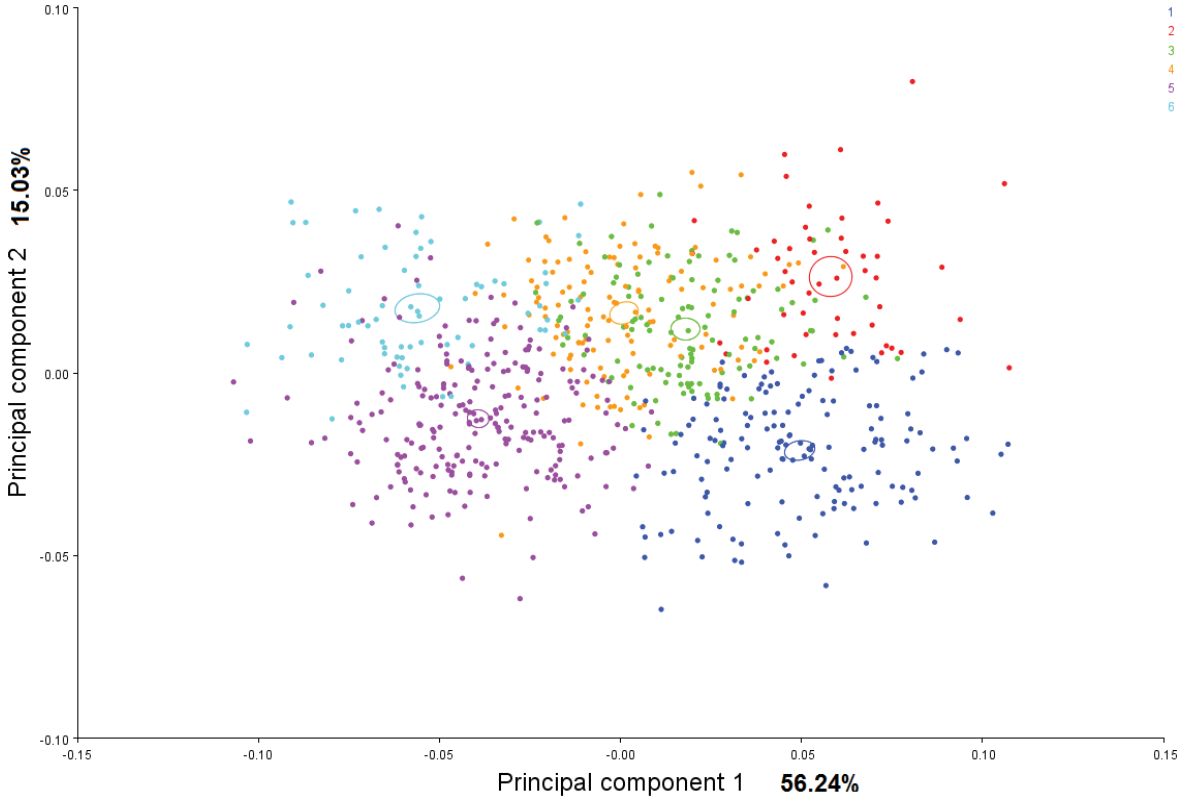


FIGURE 6.3d
A principal component analysis (PCs 1 – 2) of all sampled specimens classified within the clade of monophyletic New Zealand *Penion*. Colouration and confidence ellipses correspond to putative taxonomic species under maximal OTUs. Mean confidence ellipses (90%; same colouration) indicate that many taxa do not overlap.

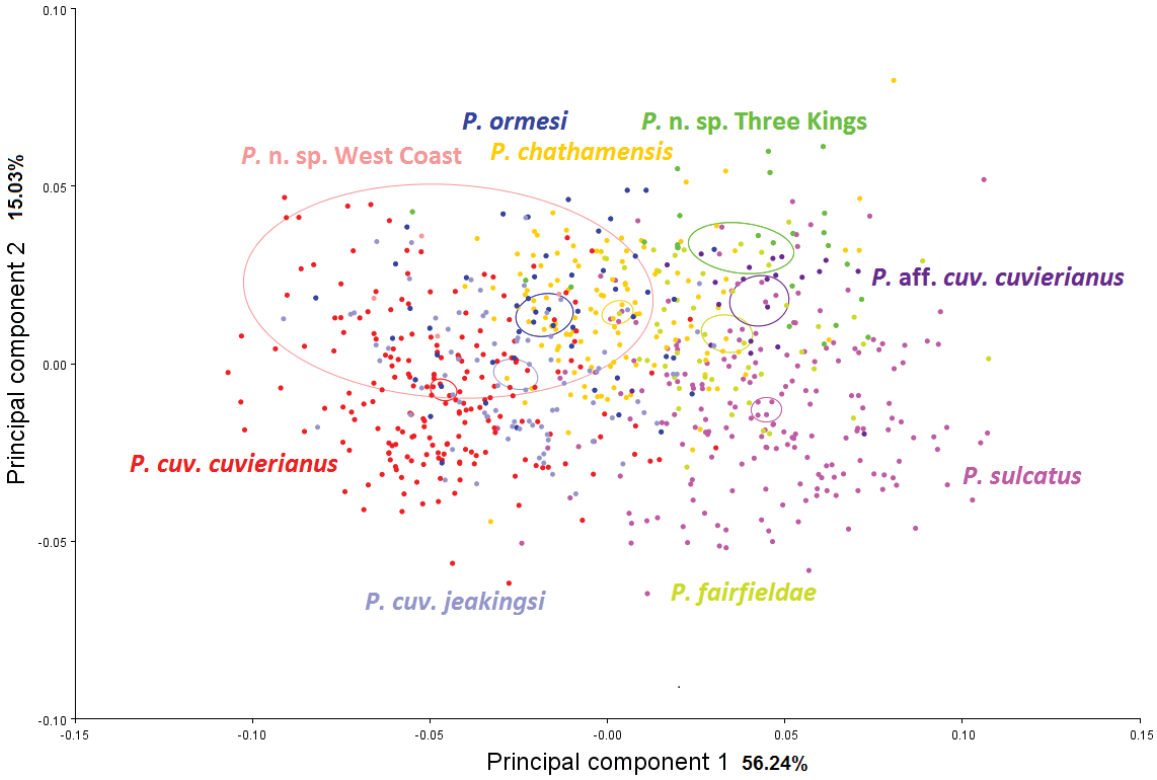
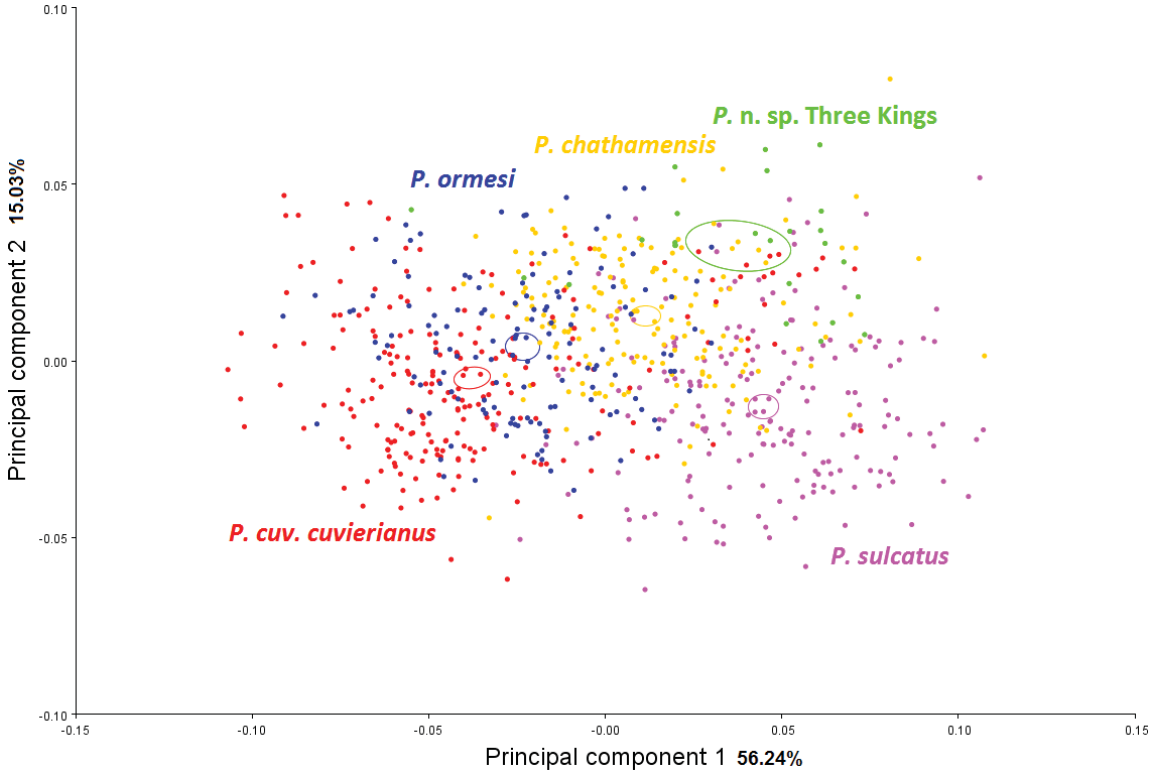


FIGURE 6.3e
A principal component analysis (PCs 1 – 2) of all sampled specimens classified within the clade of monophyletic New Zealand *Penion*. Colouration and confidence ellipses correspond to putative taxonomic species under revised OTUs based on results from molecular analyses (see Chapter 4). Mean confidence ellipses (90%; same colouration) indicate that none of the taxa overlap.



Morphometric variation among closely related lineages

Shell shape and size differences could be observed between many putative taxa included within the larger datasets above (e.g. Figures 6.2e, 6.3d; also see Chapter 5). However, we wanted to test if geometric morphometric analysis could differentiate closely related lineages within reduced datasets (Supplementary Figure 6.1), and see if patterns observed match with small-scale phylogenetic and nuclear SNP analysis results (Chapters 3 and 4).

Penion chathamensis and Penion fairfieldae

Genetic data failed to differentiate *P. chathamensis* and *P. fairfieldae*, and so we focussed on sampling from 173 specimens to investigate whether shell morphological variation could distinguish the putative species. The broken-stick model identified 4 significant PCs (PC1 = 42.5%; PC2 = 18.0%; PC3 = 12.6%; PC4 = 5.9%). Models with 1 or 2 clusters fitted the data best when these four PCs were analysed. With the inclusion of centroid size the best support was for 2 or 3 clusters (based on BIC scores using mclust; Supplementary Figure 6.9). Based on the assignment probability of individuals across the best supported models, it appears that using shape alone *P. chathamensis* and *P. fairfieldae* cannot easily be distinguished, but when shell size is considered the two species can be readily separated (Figure 6.4a). For example, the assignment for EEI2 model using centroid size closely corresponds to the taxonomic classification of individuals, although some shells from Southland and the Auckland Islands classified as *P. chathamensis* are grouped with *P. fairfieldae* (Figure 6.4a).

Although the two species overlapped in PCA morphospace, the 90% mean confidence ellipses for the species do not overlap (Figure 6.4b). However, when the geographic location of individuals was plotted instead of putative species, it seems that the observed difference between *P. chathamensis* and *P. fairfieldae* reflects geographically structured, population-level variation (Figure 6.4b). Specifically, all sampled geographic locations between both species overlap with the exception of Chatham Rise for any combination of PC1 – 3. Cross-validation via CVA did not separate *P. chathamensis* and *P. fairfieldae* with substantial support, with 16.8% and 42.86% of individuals being misassigned for each species respectively (Supplementary Figure 6.10).

Overall, it seems that there is a genuine size difference between individuals belonging to each putative species, but it is likely that *P. fairfieldae* represents

populations of smaller *P. chathamensis* rather than a distinct lineage. According to CVA and mclust results, the shape difference between the two species is minor and without size data their shells cannot readily be distinguished. These results contradict previous assertions that *P. chathamensis* more closely resembled *P. ormesi* and *A. benthicola* (Powell 1947, Dell 1956, Powell 1979). Given these results and lack of genetic difference observed (Chapter 4), we conclude that these two taxa should be treated as conspecific.

FIGURE 6.4a
Bayesian assignment probability of all sampled specimens classified as *P. chathamensis* or *P. fairfieldae* to clusters estimated by the R model-based clustering package mclust 5.2. Specimens are coloured by assignment probability to clusters, and are organised and labelled based on putative taxonomic classification and sampling region. Colours used for each cluster are identified within a key. The VEI2 model (top) was the best support model using the only the statistically significant PCs 1 – 4, and the VEI3 model (bottom) received the highest BIC support when centroid size was also included. The EEI2 model (middle), also using PCs 1 – 4 and centroid size, received the highest BIC support among alternatives when only 2 clusters were considered. See Supplementary Figure 6.10 for a comparison for BIC values among clustering models.

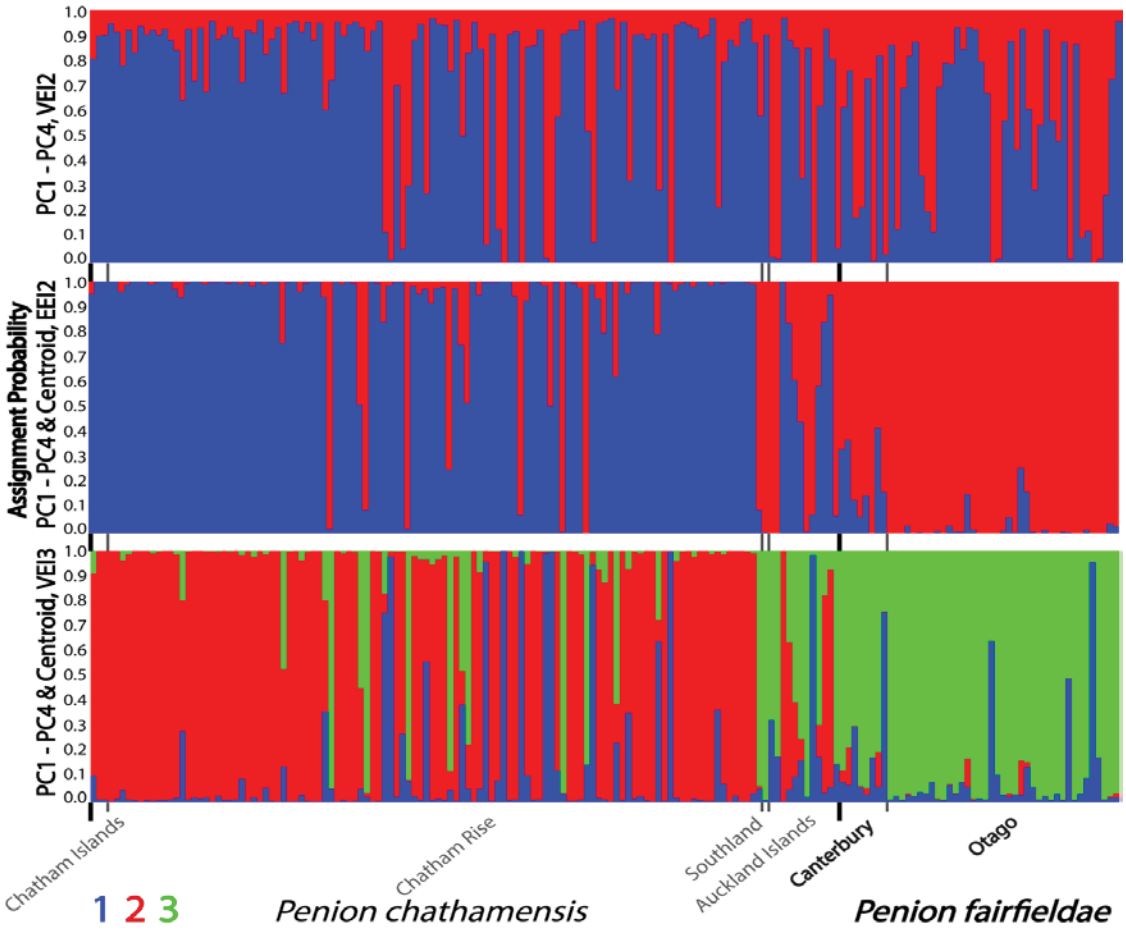
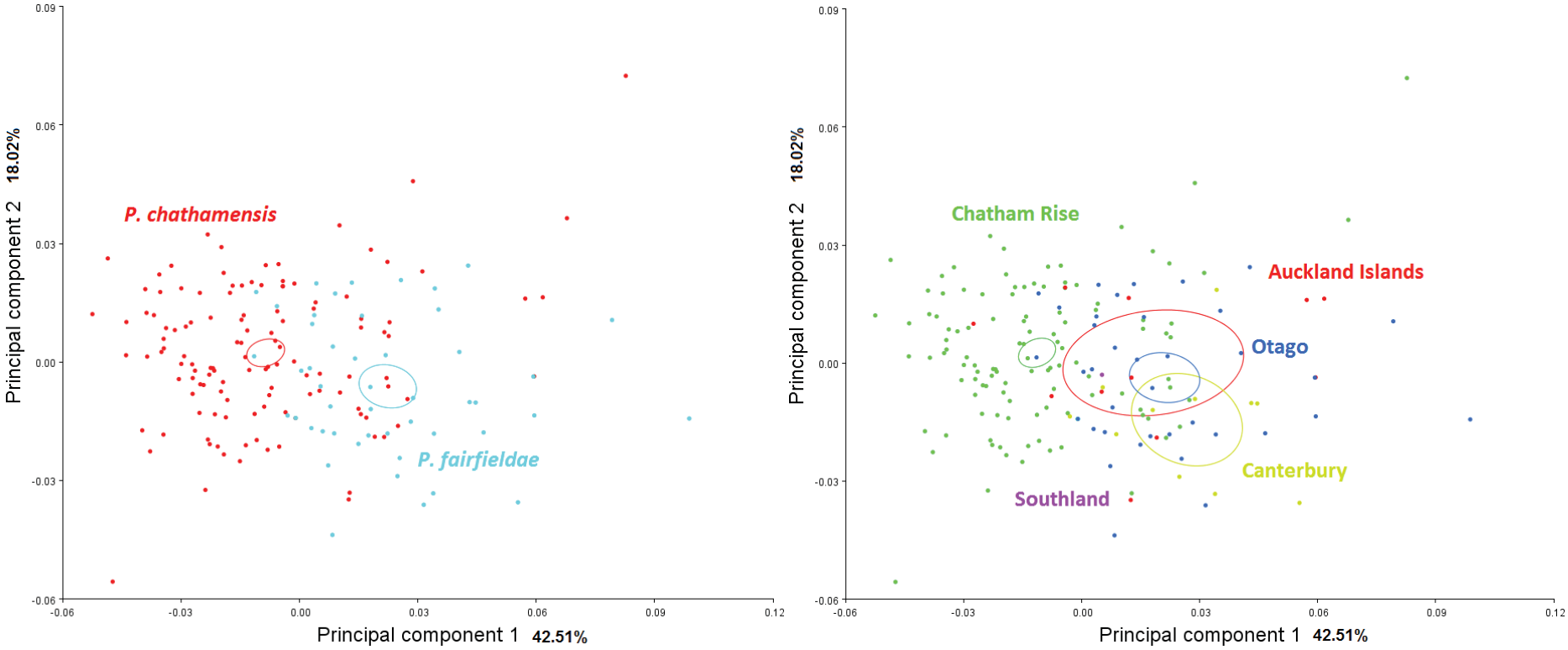


FIGURE 6.4b
Two repeated diagrams of the same principal components analysis (PC1 and 2) of our sampling of shells classified as *P. chathamensis* or *P. fairfieldae*. The left PCA diagram shows shape variation among specimens when they are taxonomically classified, whereas the right PCA diagram classifies individuals by sampled geographic region. Colouration and confidence ellipses (90% mean) correspond to each classification.



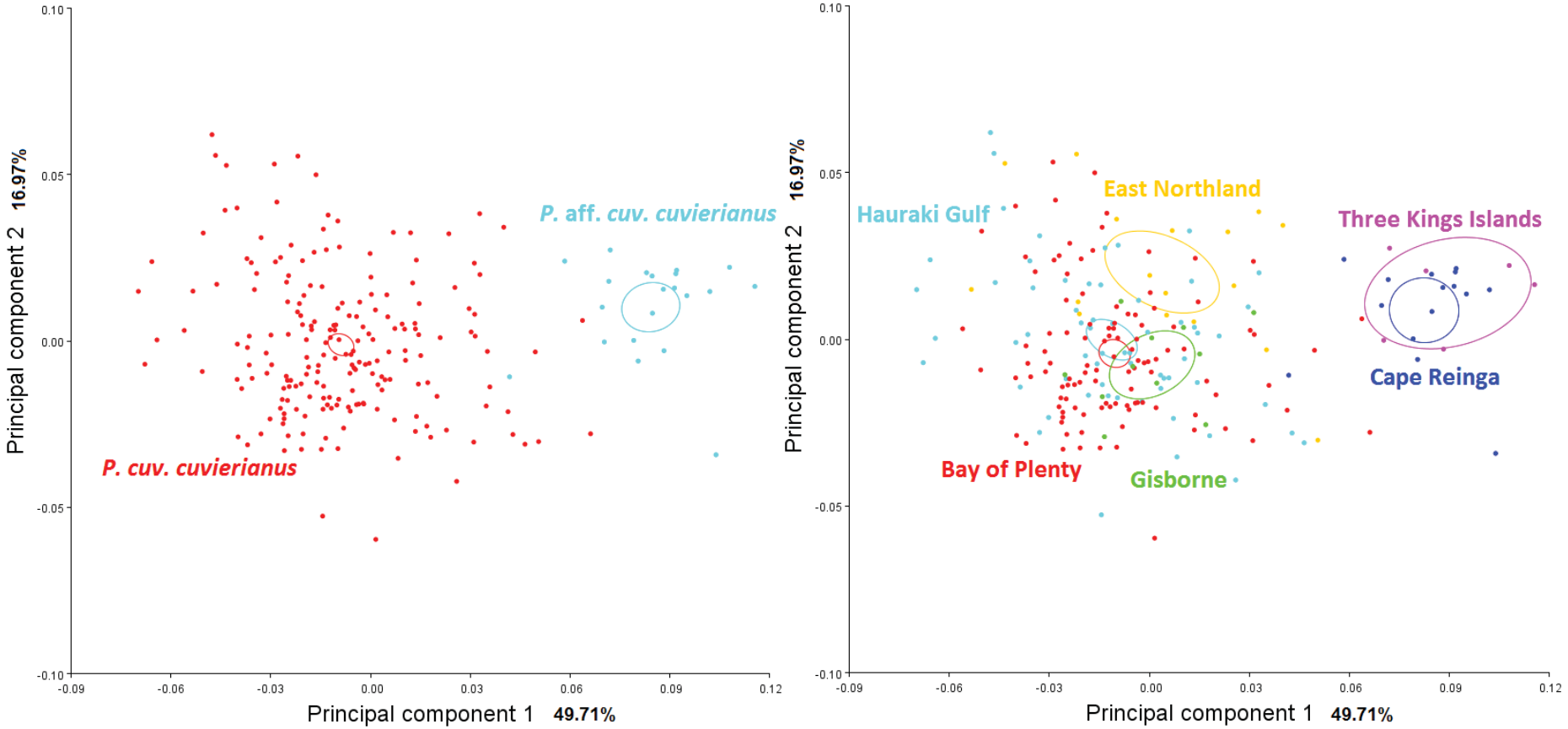
Penion c. cuvierianus and P. aff. c. cuvierianus

Although sampling was limited, genetic data did not distinguish a specimens of *P.* aff. *c. cuvierianus* from material identified as *P. c. cuvierianus*, therefore we wanted to investigate if geometric morphometric shell data agreed with this outcome using shells from 221 individuals. Four significant PCs were identified (broken-stick test): PC1 = 49.7%; PC2 = 17.0%; PC3 = 10.0%; PC4 = 5.9%. The best fitting models had 1 or 4 clusters when the significant PCs were analysed with and without the inclusion of centroid size (based on BIC scores using mclust; Supplementary Figure 6.11). Based on the assignment probability of individuals across models, the majority of *P.* aff. *c. cuvierianus* could be distinguished with high confidence from *P. c. cuvierianus* (Supplementary Figure 6.12). However the assignment of some specimens varied, especially when centroid size was included (Supplementary Figure 6.12). For the VEE2 model using only PCs 1 – 4, clusters were almost a perfect match to the classification of specimens (95.2% of *P.* aff. *c. cuvierianus*, 100.0% of cluster 1; 100.0% of *P. c. cuvierianus*, 99.5% of cluster 2). The further possible clusters identified by mclust mostly occurred within *P. c. cuvierianus*, consist of individuals distributed across the entire geographic range of the species, and typically exhibit lower assignment confidence than individuals of *P.* aff. *c. cuvierianus* assigned to their own cluster.

The earlier PCA analyses involving maximal OTUs at the deep phylogenetic and monophyletic New Zealand levels indicated that the two taxa could be readily distinguished (Figures 6.2e, 11), and the same pattern was true for the reduced dataset (Figure 6.4b). The two species hardly overlapped in morphospace with distinct 90% mean confidence ellipses (Figure 6.5). When individuals were classified instead via geographic locality, the situation was somewhat more nuanced. Individuals from the Hauraki Gulf, Bay of Plenty, and Gisborne overlapped with one another but shells from the east coast of Northland classified as *P. c. cuvierianus* could be distinguished (Figure 6.5). Likewise, shells from Cape Reinga and off the Three Kings Islands, which are mostly classified as *P.* aff. *c. cuvierianus* overlapped with each other but were readily distinguished from all other populations (Figure 6.5). Given the distances in morphospace, it is possible that for PC2 the shells from the east coast of Northland are as distinct as *P.* aff. *c. cuvierianus*, but for PC1, only *P.* aff. *c. cuvierianus* is substantially different. Lastly, the two putative taxa could very easily be differentiated using cross-validation via CVA. Only one individual of each species was missassigned to the other.

The molecular (mtDNA and rDNA) sequence data using a small number of samples found little difference between *P. c. cuvierianus* and *P.* aff. *c. cuvierianus* (Chapter 4), but here we find that the two species can be readily distinguished based on shell shape and size. It is possible that *P.* aff. *c. cuvierianus* represents a very recent evolutionary split, and therefore the mtDNA and nuclear rDNA evidence that we considered was not capable of detecting substantial differences. Alternatively, *P. c. cuvierianus* may just be a highly divergent species for shell morphology, or *P.* aff. *c. cuvierianus* may represent hybridisation between distinct taxa. Populations may occur in a cline of increasing shell difference, perhaps demonstrated by the ability to separate shells from the east coast of Northland via PCA. Further molecular data, ideally using fast-evolving nuclear markers is required to investigate the situation further. However, these morphometric results do indicate that snails from the east coast of Northland may be of interest, which demonstrates the value of geometric morphometric analyses as this region was not previously of significant interest based on traditional taxonomic investigation.

FIGURE 6.5
Two repeated diagrams of the same principal components analysis (PC1 and 2) of our sampling of shells classified as *P. c. cuvierianus* or *P.* aff. *c. cuvierianus.* The left PCA diagram shows shape variation among specimens when they are taxonomically classified, whereas the right PCA diagram classifies individuals by sampled geographic region. Colouration and confidence ellipses (90% mean) correspond to each classification.



P. ormesi, P. c. jeakingsi and P. n. sp. West Coast

Genetic evidence identified a species complex not previously recognised, consisting of the described species *P.* ormesi, the subspecies *P. c. jeakingsi*, and an unidentified *Penion* population from the New Zealand west coast. For morphometric analysis we had sampled 50, 78 and 4 shell from each taxon respectively, from which four significant PCs were identified (broken-stick test): PC1 = 37.7%; PC2 = 21.7%; PC3 = 10.5%; PC4 = 7.4%. When specimens were analysed without *a priori* classification, 2 – 3 clusters were best supported when only the significant shell shape PCs were analysed, and when centroid size was included 2 – 4 cluster received best support (based on BIC scores using mclust; Supplementary Figure 6.13). Using shape variation alone allowed for approximately a third of *P. c. jeakingsi* to be separated from the other taxa, however assignment confidence varied (Figure 6.6a). With the inclusion of centroid size, more individuals of *P. c. jeakingsi* could be separated with higher confidence, but the largest cluster across all models remained taxonomically mixed (Figure 6.6a). As well as this, both datasets allowed for a small number of shells across the entire geographic range of the complex to be separated. This additional cluster appeared to reflect very large shells, regardless of their taxonomic classification.

The earlier PCA analyses involving maximal OTUs at the deep phylogenetic and monophyletic New Zealand levels indicated that *P. ormesi* and *P. c. jeakingsi* could be distinguished readily, but that *P.* n. sp. West Coast with lower sampling could not for any combination of PC1 – 4 (Figures 6.2e). The same was true for the reduced dataset, although there was substantial overlap in the overall distribution of individuals (Figure 6.6b). When individuals were re-classified based on geographic location, the situation was very noisy with most localities overlapping with one another (Figure 6.6b). For example, shells from off Nelson could be separated from those from Marlborough, however shells from Marlborough overlapped with shells from Canterbury and sampling from all three locations overlapped with shells sampled from Northland and the West Coast. However, three putative taxa separately easily via CVA ordination (Supplementary Figure 6.14), although results may be misleading due to the low sampling frequency for *P.* n. sp. West Cost. Cross-validation via pair-wise CVA between taxa resulted in low rates of misassignment, with the exception of *P.* n. sp. West Coast that struggled to be separated from either *P. ormesi* or *P. c. jeakingsi.*

Overall, we infer that these taxa are likely to be conspecific, but there also seems to be variation among populations belonging to this complex. The species *P. ormesi* and

P. c. jeakingsi can be distinguished using the *a priori* classification of specimens in CVA (Supplementary Figure 6.9), suggesting that there is possible morphological concordance with genetic data. However, differences in shell shape appear to be minor as they are not diagnostic in naïve analyses (Figure 6.6a). Differences in shell size are somewhat informative, but again do not provide the same resolution as in comparisons of genetically distinct lineages (e.g. analyses of deeper phylogenetic splits above; comparison of *P. chathamensis* and *P. sulcatus* in Chapter 5). The additional cluster identified by mclust likely represented the product of ecological variation or perhaps ongoing speciation.

FIGURE 6.6a
Bayesian assignment probability of all sampled shells classified as *P. ormesi*, *P. c. jeakingsi* or *P.* n. sp. West Coast to clusters estimated by the R model-based clustering package mclust 5.2. Specimens are coloured by assignment probability to clusters, are organised following the molecular phylogeny (see Chapter 4), and are labelled based on putative taxonomic classification and sampling region. Colours used for each cluster are identified within a key. The VEI3 model (top) was the best support model using the only the statistically significant PCs 1 – 4, whereas the VVI2 model (bottom) received the highest BIC support when centroid size was also included. See Supplementary Figure 6.14 for a comparison for BIC values among clustering models.

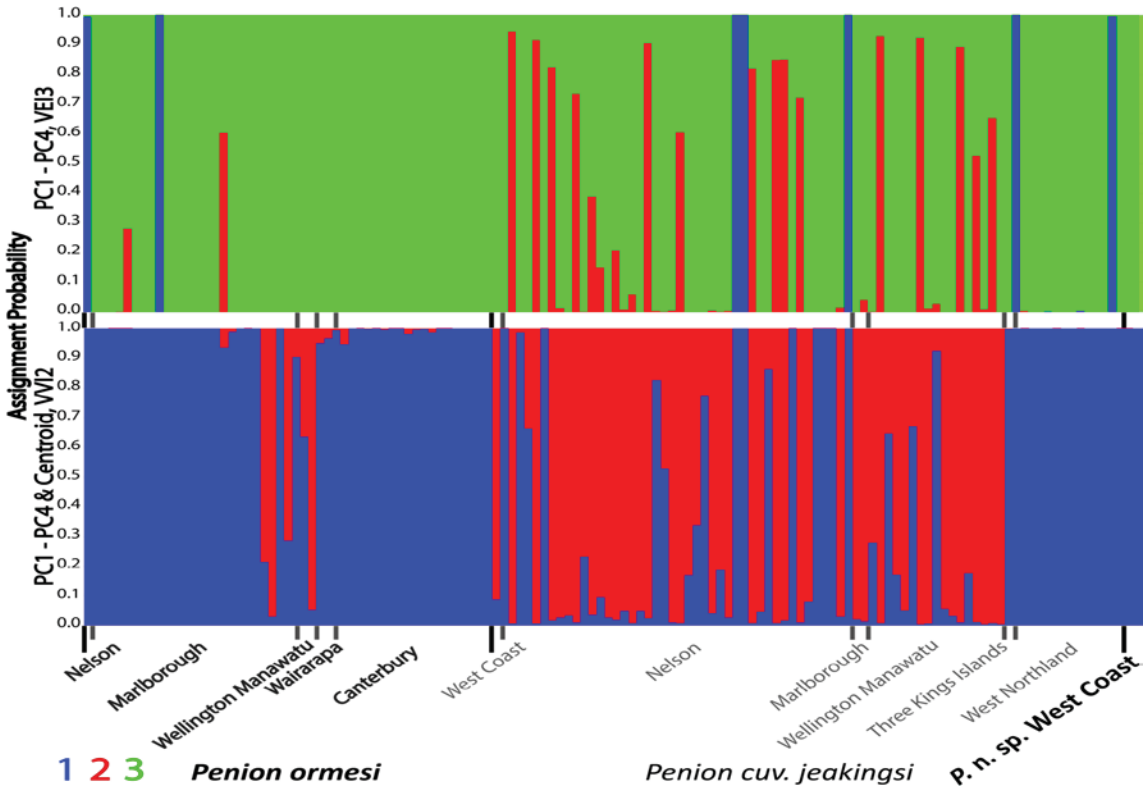
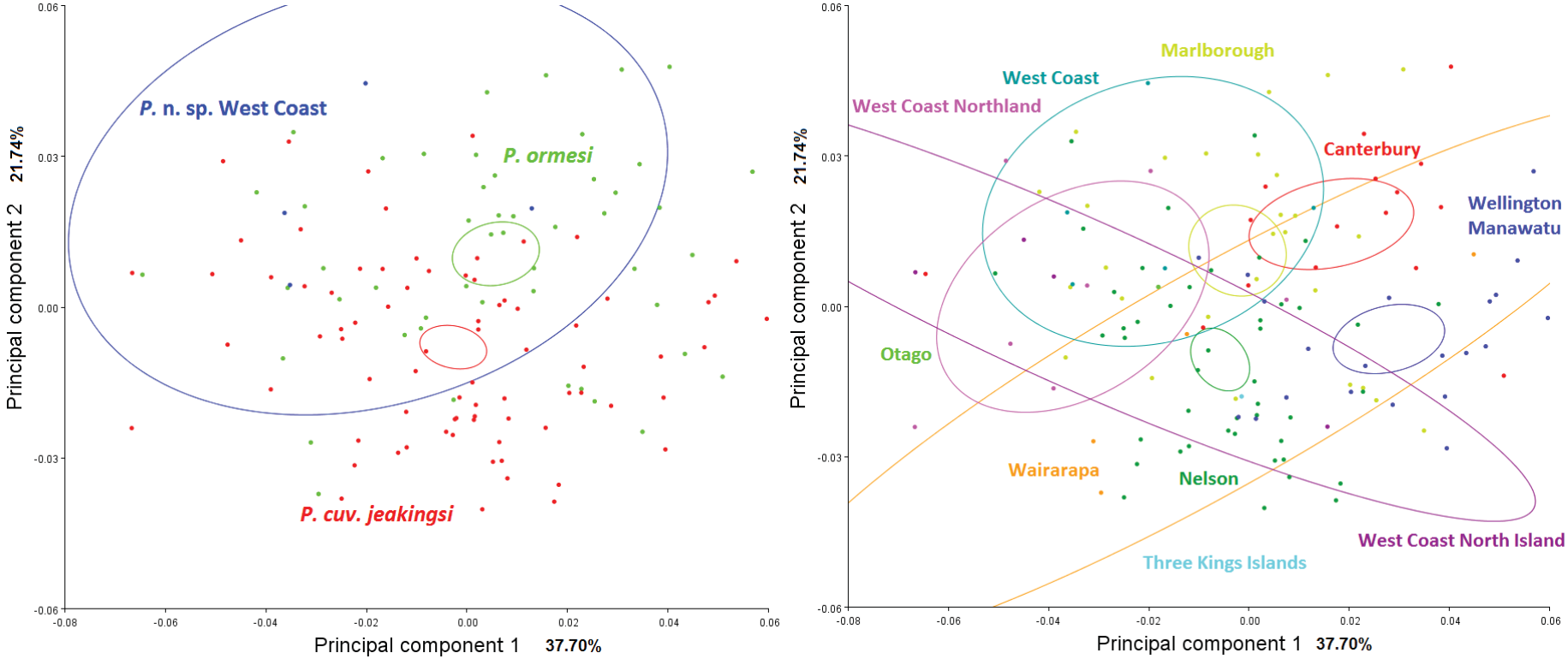


FIGURE 6.6b
Two repeated diagrams of the same principal components analysis (PC1 and 2) of all sampled shells classified as *P. ormesi*, *P. c. jeakingsi* or *P.* n. sp. West Coast. The left PCA diagram shows shape variation among specimens when they are taxonomically classified, whereas the right PCA diagram classifies individuals by sampled geographic region. Colouration and confidence ellipses (90% mean) correspond to each classification.



Conclusion

Our geometric morphometric method managed to capture biologically relevant variation in the shell morphology of the true whelk genera *Penion, Kelletia* and *Antarctoneptunea*. Crucially, the variation observed exhibited strong concordance with the molecular phylogeny of the clade. Not surprisingly, this technique resolved the same patterns identified via the traditional morphological examination of shells, but did so without reference to location or taxonomy. Variation in the shape and size of shells clearly carries a strong phylogenetic signal in siphon whelks. The congruence between molecular evidence and the apparent intra- and interspecific morphological variation within *Penion* is remarkable, especially because variation in shell morphology has previously represented a challenge for *Penion* and Buccinidae taxonomy (Ponder 1973, Powell 1979).

Admittedly, there is a risk of circularity in our investigation of taxa identified based on the examination of shell traits. The traditional taxonomic classification considers traits not captured by our two dimensional landmarks, such as protoconch morphology, presence and size of axial ribs on the teleoconch, shell thickness, and shell colouration. However it is possible that some of these features could be correlated with the morphological variation captured by our landmarks. We believe our approach is fairly robust overall though, as for each set of closely related taxa examined, molecular evidence is available for comparison (Supplementary Figure 6.1; Chapters 3 and 4), allowing for maximal and molecular-derived revised OTUs to be compared (e.g. Figures 6.2e – 6.2f, 6.3d and 6.3e), and most putative taxa have distinct distributions with little geographic overlap (see Figures 3.7, 4.1).

In summary, we were able to successfully differentiate groups separated by deep phylogenetic splits (genera of *Penion, Kelletia* and *Antarctoneptunea*), and among monophyletic New Zealand *Penion* we could identify all species under revised taxonomy supported by recent phylogenetic analysis (Chapter 4). Where uncertainty was highest, it happened to be for separation of likely conspecific or closely related lineages (e.g. *P. chathamensis* and *P. fairfieldae*; *P. ormesi* and *P. c. jeakingsi, P.* n. sp. West Coast). Likewise, when the morphological data disagreed with previously held taxonomy (e.g. *P. c. cuvierianus* and *P. c. jeakingsi*), they agreed with our phylogenetic data. We were also able to demonstrate that the novel, genetically distinct lineage of *P.* n. sp. Three Kings Islands is also morphologically distinct.

A sole exception to the concordance of our morphometric and molecular results was that *P.* aff. *c. cuvierianus* was readily differentiated from *P. c. cuvierianus*. This agrees with taxonomic examination of shells by local experts, but based on current, limited genetic sampling there is not yet evidence to separate these groups phylogenetically. Our morphometric analysis however did indicate that *P. c. cuvierianus* from the east coast of Northland are also morphologically distinct, and this suggests that future molecular studies should sequence individuals from this region.

Most taxa differed in size, although there was overlap among many species. The main variation between lineages of *Penion* primarily appeared to reflect variation in the height of the teleoconch spire and aperture, and the width of the aperture (focussed on the outer curve) and the width of the body whorl and siphonal canal (Figure 6.2h). This suggests that these traits are the most taxonomically informative in true whelks. The shape variation captured by statistically significant PCs did not tend to reflect variation in length of the siphonal canal between taxa, which may support a previous hypothesis that variation for this trait reflects phenotypic plasticity in response to water depth (Ponder 1971). It is likely though that the significant principal components for lower-level analyses between species captured an increased amount of environmental variation among populations. The increased 'noise' in some analyses where mclust models identified clusters that seemingly did not concord with taxonomy or phylogeny (e.g. in *P. ormesi, P. c. jeakingsi, P.* n. sp. West Coast complex) therefore may reflect this environmental variation. In future, it would be of benefit to investigate the relationship between shape variation observed among populations of *Penion* with environmental variables such as water depth and substrate type.

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Supplementary Data for Chapter Six

In this supplementary section we provide details regarding our investigation of experimental error and genetic representation of morphological diversity for the analysis of shell morphology in *Penion.* We also present additional figures and tables to fully illustrate the results of the main investigation.

Estimation of experimental error

All positioning, photography and subsequent digitisation detailed below was conducted by a single person in order to avoid experimenter variation (Schilthuizen and Haase 2010). We previously investigated experimental variation due to photographic (positioning of shells, height of the camera) and digitisation (placing landmarks and combs) using *P. chathamensis* and found variation between negligible (Chapter 6). In case error varied with species (some shells may be harder to digitise as accurately as others), in this study we investigated error a second time using a single shell from *P. c. cuvierianus*.

The study shell was photographed 5 times at 3 different heights (83 cm, 103 cm, 125 cm). Camera height was considered to be a potential source of variation as siphon whelks vary significantly in size and therefore cannot be photographed at a single camera height. For each photograph the specimen was removed and repositioned on the sand base. All 15 of these photographs were then given combs and digitised. This provided 3 sets of 5 photographs for investigating how camera height and positioning variation affected digitisation accuracy. Lastly, one photo from the 83 cm height set was given combs and digitised a further 4 times. This provided a measure of pure digitisation error in the absence of photographic error. Experimental error was visualised using principal components analysis (Supplementary Figure 6.2), with neither repeated photography nor digitisation appearing to have a significant effect (Supplementary Figure 6.6).

Genetic representation of morphological sampling

We wanted to know if our previous molecular sampling (Chapters 3 – 4), adequately represented total sampled variation in shell morphology. To investigate, shells that originated from individuals also sequenced in our molecular analyses (if available and suitable) were classified separately to other shells (dry collection material,

non-sequenced individuals). Using PCA ordination we then compared the distribution of the genetically-sampled and remaining shells (Supplementary Figures 6.1 – 6.3). The genetic representation of morphological variation within putative species varied, although among monophyletic New Zealand *Penion*, genetic sampling overall appeared to be a reasonable match for total sampled morphological variation (Supplementary Figure 6.3).

The difference between groups likely reflects lower sampling in the genetic data, as some species only had a single individual available for sequencing. As well, it is quite common within species for some populations to have been sequenced but the shells of those individuals were not suitable for morphometric analysis (Supplementary Figure 6.2). If those shells were available, they would likely occur in the regions of shape space under-represented by genetic sampling. In species where genetically sampled individuals frequently have suitable shells, the concordance between genetic representation and overall morphological variation was obvious (Supplementary Figure 6.4).

Supplementary References

Fraley, C., Raftery, A.E. (2012). mclust version 4 for R: normal mixture modelling for model-based clustering, classification, and density estimation. *Technical Report 597, University of Washington.*

Klingenberg, C. P. (2011). MorphoJ: an integrated software package for geometric morphometrics. *Molecular Ecology Resources* 11, 353 – 357.

Schilthuizen, M., Haase, M. (2010). Disentangling true shape differences and experimenter bias: are dextral and sinistral snail shells exact mirror images? *Journal of Zoology* 282, 191 – 200.

Supplementary Tables

SUPPLEMENTARY TABLE 6.1

Cross-validation scores estimated for the discrimination of *Penion*, *Kelletia* and *Antarctoneptunea*, compared as genera (left), and compared as species under revised (middle) and maximal OTUs (left). Cross-validation scores were estimated via canonical variates analysis (CVA) implemented in the R package MASS 7.3-26 (Venables and Ripley 2002), using principal components generated from PCA in MorphoJ 1.06c (Klingenberg 2011). Colours reflect the frequency of individuals identified within each group (green = low, orange = intermediate, red = high). Taxonomic names are abbreviated to the first three letters.

	Ant.	Kel.	Pen.
Ant.	59	0	2
Kel.	0	32	0
Pen.	0	0	944

	A. aur	K. kel	K. lis	P. ben	P. cuv	P. cha	P. TKI	P. man	P. orm	P. sul	P. max
A. aur	0	0	0	0	0	0	0	0	0	0	0
K. kel	0	23	1	0	0	0	0	0	0	0	0
K. lis	0	3	5	0	0	0	0	0	0	0	0
P. ben	0	0	0	59	1	0	0	0	0	0	0
P. cuv	0	0	0	0	200	1	0	0	18	2	0
P. cha	0	0	0	0	0	167	0	0	6	0	0
P. TKI	0	0	0	0	0	0	25	0	0	0	0
P. man	0	0	0	0	0	1	0	87	1	0	0
P. orm	0	0	0	0	9	4	1	0	117	1	0
P. sul	0	0	0	0	2	4	0	0	6	178	0
P. max	0	0	0	0	0	0	0	0	0	0	114

	A. aur	K. kel	K. lis	P. ben	P. cuv	P. cha	P. jea	P. TKI	P. man	P. orm	P. sul	P. aff	P. fai	P. wes	P. max
A. aur	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
K. kel	0	23	1	0	0	0	0	0	0	0	0	0	0	0	0
K. lis	0	3	5	0	0	0	0	0	0	0	0	0	0	0	0
P. ben	0	0	0	58	0	0	1	0	0	1	0	0	0	0	0
P. cuv	0	0	0	0	181	1	4	0	0	12	1	0	0	1	0
P. cha	0	0	0	0	0	105	0	0	1	6	1	0	12	0	0
P. jea	0	0	0	0	3	0	65	1	0	9	0	0	0	0	0
P. TKI	0	0	0	0	0	0	0	25	0	0	0	0	0	0	0
P. man	0	0	0	0	0	1	0	0	87	1	0	0	0	0	0
P. orm	0	0	0	0	1	4	2	0	0	40	2	0	0	1	0
P. sul	0	0	0	0	1	2	4	0	0	2	177	2	2	0	0
P. aff	0	0	0	0	0	0	0	0	0	2	1	18	0	0	0
P. fai	0	0	0	0	0	8	0	0	0	0	0	0	40	0	0
P. wes	0	0	0	0	0	0	0	0	0	3	0	1	0	0	0
P. max	0	0	0	0	0	0	0	0	0	0	0	0	0	0	144

SUPPLEMENTARY TABLE 6.2

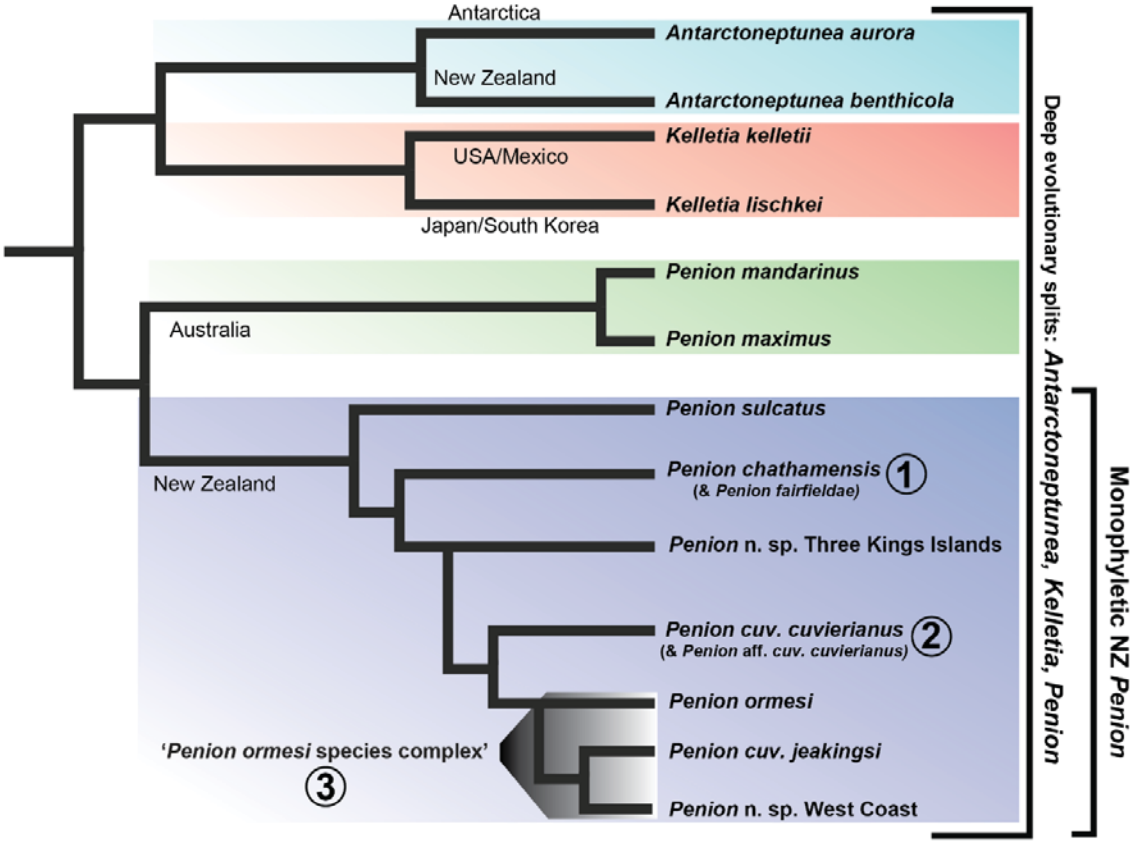
Cross-validation scores estimated for the discrimination of monophyletic New Zealand *Penion* species under revised (left) and maximal OTUs (right). Cross-validation scores were estimated via canonical variates analysis (CVA) implemented in the R package MASS 7.3-26 (Venables and Ripley 2002), using principal components generated from PCA in MorphoJ 1.06c (Klingenberg 2011). Colours reflect the frequency of individuals identified within each group (green = low, orange = intermediate, red = high). Taxonomic names are abbreviated to the first three letters.

	P. cuv	P. cha	P. TKI	P. orm	P. sul
P. cuv	199	2	0	17	3
P. cha	0	170	0	2	1
P. TKI	0	0	25	0	0
P. orm	9	4	1	116	2
P. sul	3	3	0	6	178

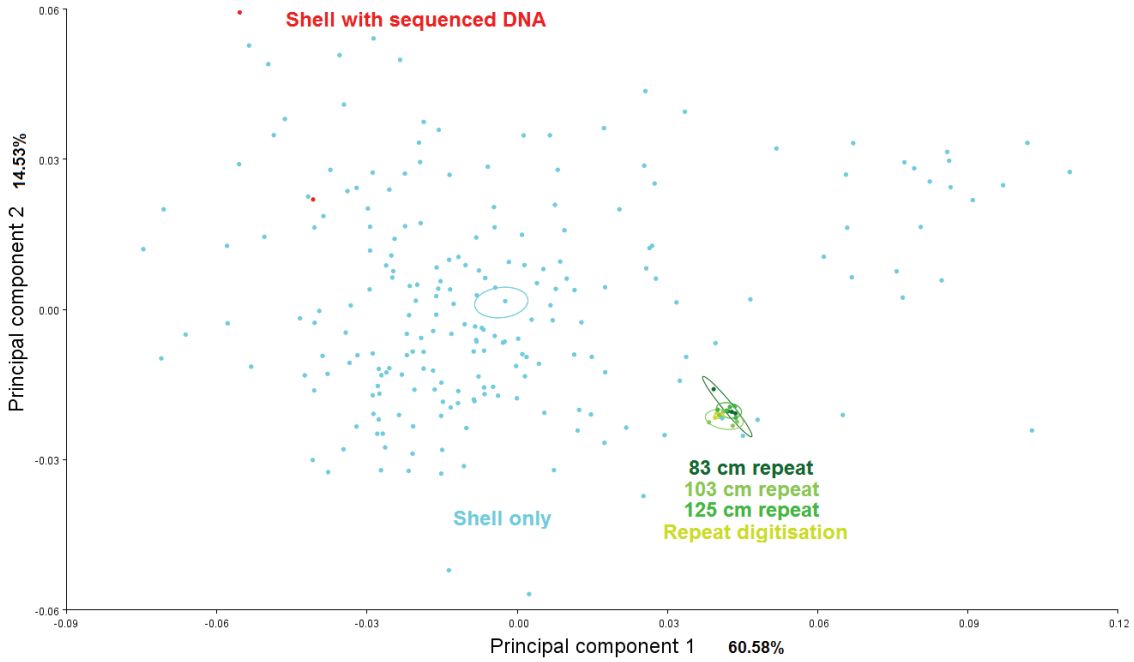
	P. cuv	*P. cha*	*P. jea*	*P. TKI*	*P. orm*	*P. sul*	*P. aff*	*P. fai*	*P. wes*
P. cuv	184	1	2	0	11	2	0	0	0
P. cha	0	110	0	0	2	2	0	11	0
P. jea	3	0	65	1	8	1	0	0	0
P. TKI	0	0	0	25	0	0	0	0	0
P. orm	1	3	2	0	41	2	0	0	1
P. sul	1	3	3	0	3	177	2	1	0
P. aff	0	0	0	0	1	1	18	1	0
P. fai	0	7	0	0	0	0	0	41	0
P. wes	0	0	0	0	3	0	1	0	0

Supplementary Figures

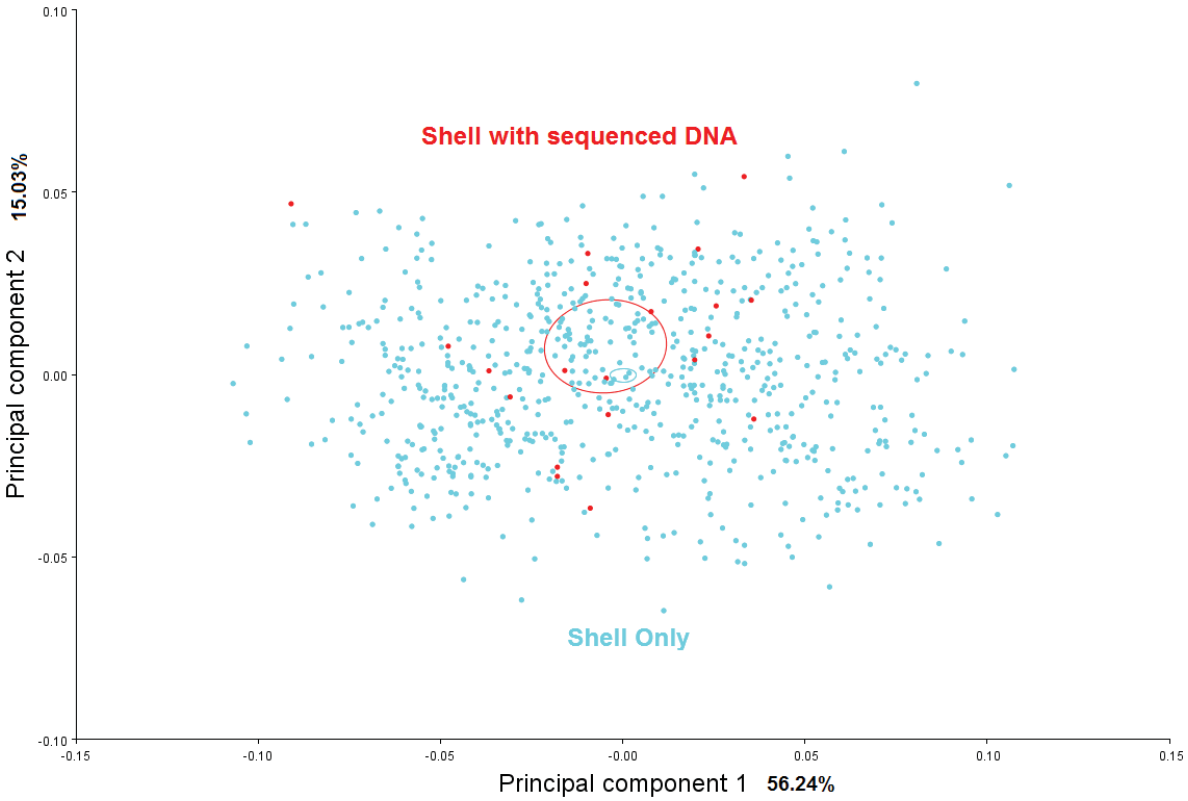
SUPPLEMENTARY FIGURE 6.1
A diagram showing the hypothesised evolutionary relationships among extant *Antarctoneptunea*, *Kelletia* and *Penion* species, which should help to follow our hierarchical progression through the geometric morphometric shell data. The coloured blocks represent evolutionary lineages that are considered to be separated by 'deep' phylogenetic splits, which corresponds to the distinction of all three genera, as well as the split of New Zealand and Australian *Penion*. Brackets on the left delineate which individuals were included for our overall *Antarctoneptunea*, *Kelletia* and *Penion* dataset and monophyletic New Zealand *Penion* dataset. Numbers 1 – 3 mark the phylogenetic placement of closely related lineages that were analysed last, which were: 1) *P. chathamensis* and *P. fairfieldae*; 2) *P.* aff. *c. cuvierianus* and *P. c. cuvierianus*; and 3) *P. ormesi*, *P. c. jeakingsi* and *P.* n. sp. West Coast.



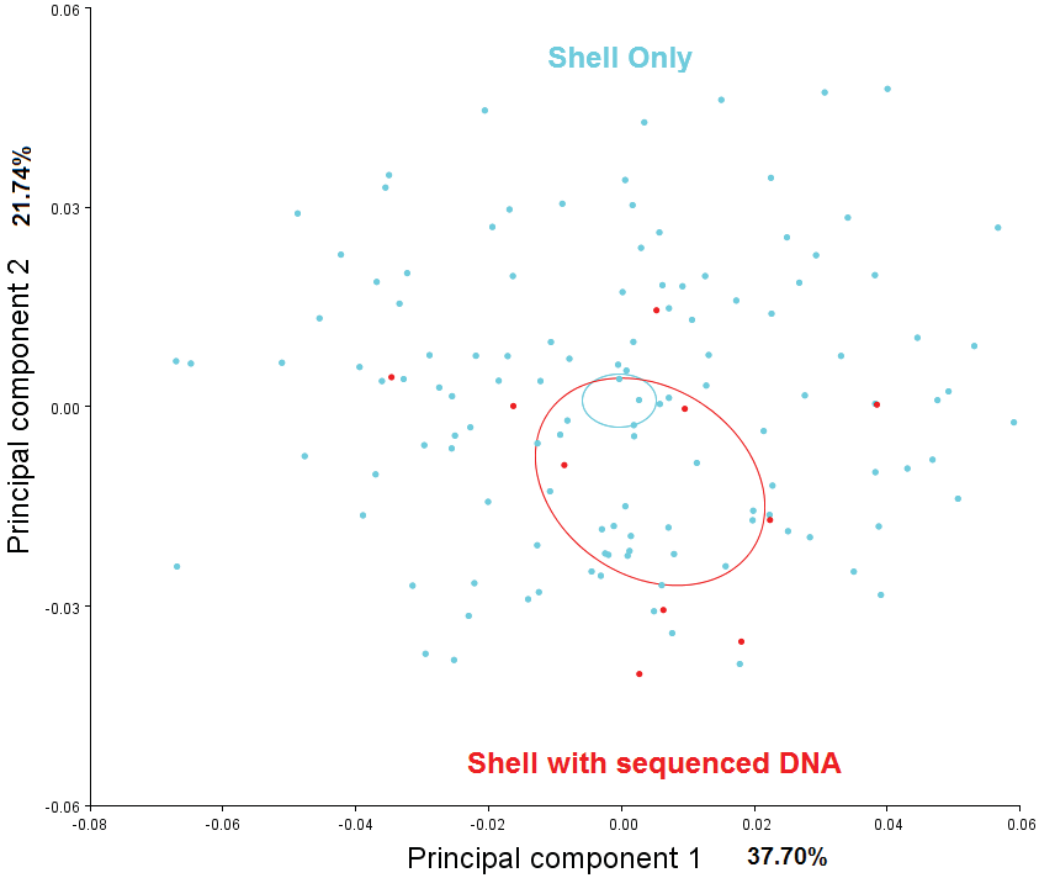
SUPPLEMENTARY FIGURE 6.2
A principal component analysis (PCs 1 – 2) of all sampled shells classified as *P. c. cuvierianus* or *P.* aff. *c. cuvierianus*. Mean confidence ellipses (90%; same colouration) indicate if groups overlap. The four groups of specimens coloured in different shades of green correspond to each camera height (83, 103, 125 cm) and repeated digitisation test conducted for our error study. Specimens coloured in cyan are shells solely held within a dry collection, whereas specimens coloured in red also had tissue available for genetic sequencing (see Chapters 3 and 4).



SUPPLEMENTARY FIGURE 6.3
A principal component analysis (PCs 1 – 2) of all sampled specimens classified within the clade of monophyletic New Zealand *Penion*. Mean confidence ellipses (90%; same colouration) indicate if groups overlap. A principal component analysis (PC1 and PC2) of our sampling of monophyletic New Zealand *Penion*. Specimens coloured in cyan are shells solely held within a dry collection, whereas specimens coloured in red also had tissue available for genetic sequencing (see Chapters 3 and 4).

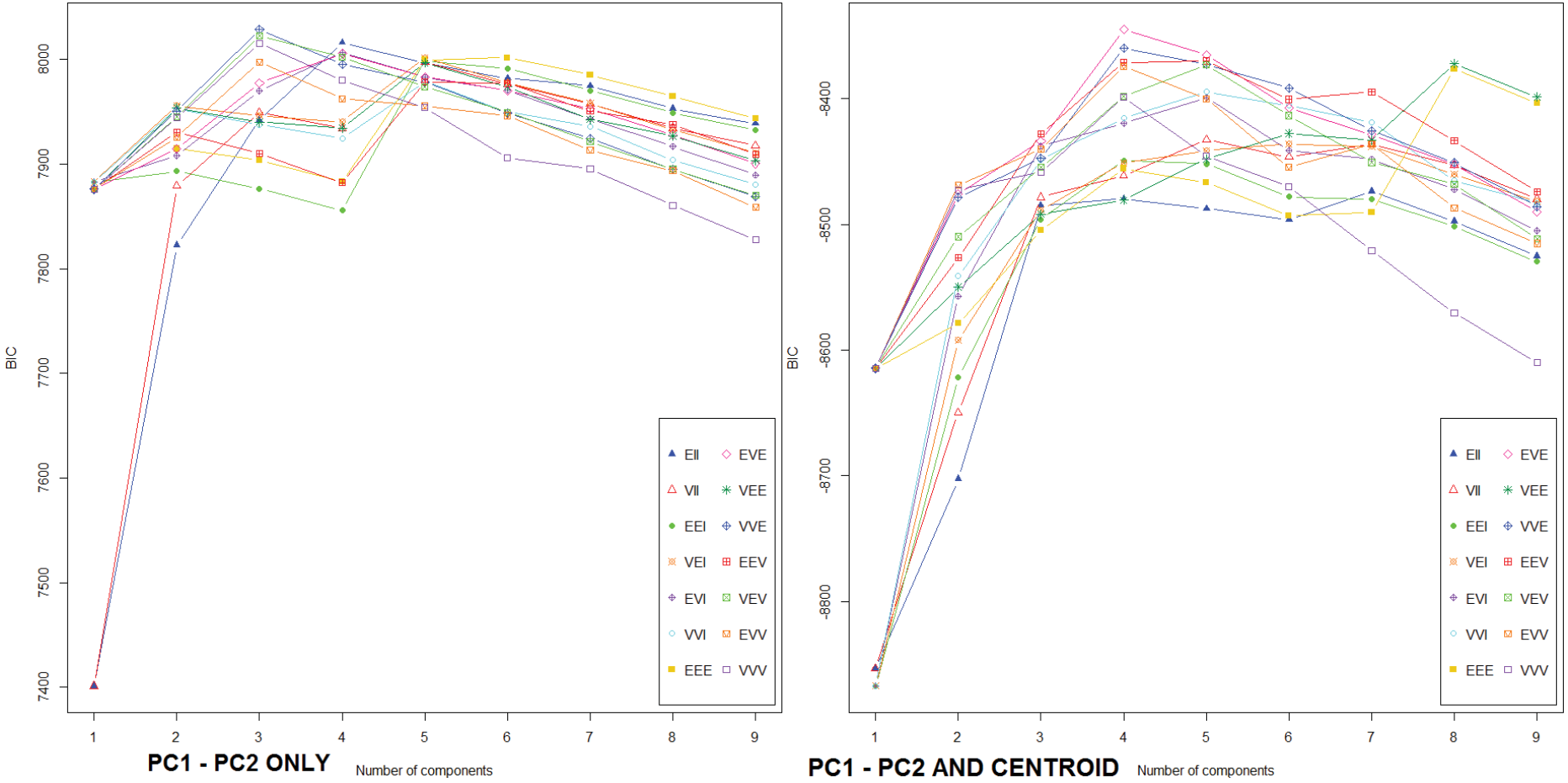


SUPPLEMENTARY FIGURE 6.4
A principal component analysis (PCs 1 – 2) of all sampled specimens classified as *P. ormesi*, *P. c. jeakingsi* or *P.* n. sp. West Coast. Mean confidence ellipses (90%; same colouration) indicate if groups overlap. A principal component analysis (PC1 and PC2) of our sampling of monophyletic New Zealand *Penion*. Specimens coloured in cyan are shells solely held within a dry collection, whereas specimens coloured in red also had tissue available for genetic sequencing (see Chapter 4).

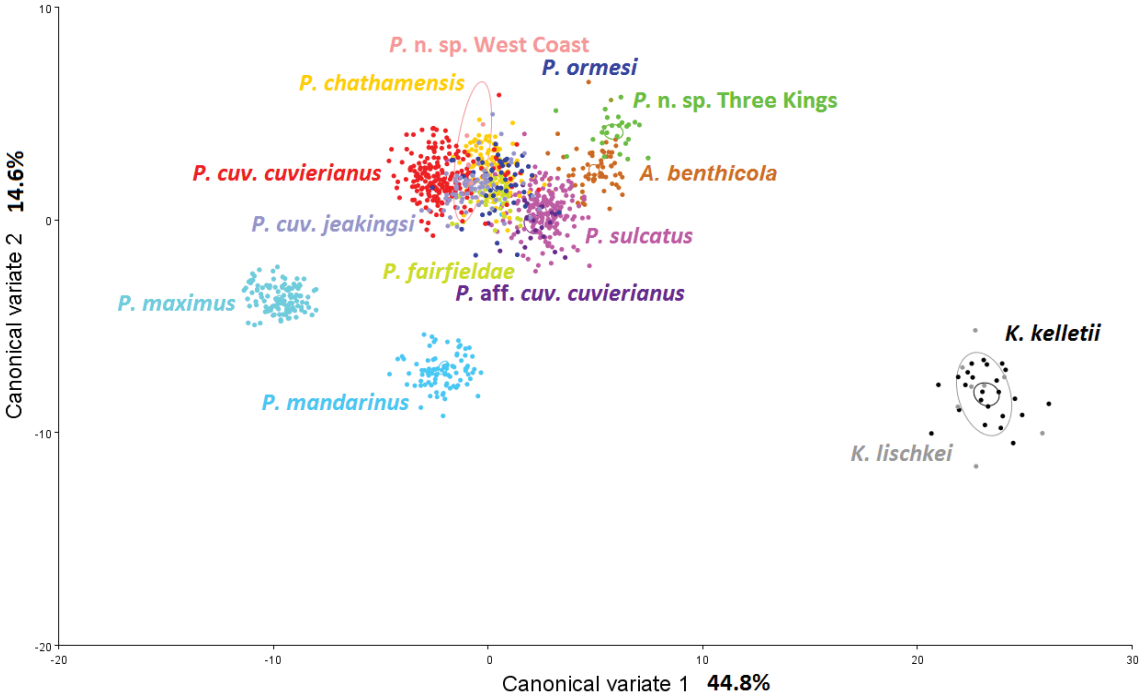


SUPPLEMENTARY FIGURE 6.5

A plot of Bayesian information criterion (BIC) scores for fitting various clustering models to variation among all sampled shells classified as *Penion*, *Kelletia* and *Antarctonetpunea* using mclust 5.2. On the left, the BIC scores are shown using only the statistically significant PCs 1 – 2, and on the right the analysis is repeated when centroid size is also incorporated. See Fraley and Raftery (2012), for an explanation of the models listed.

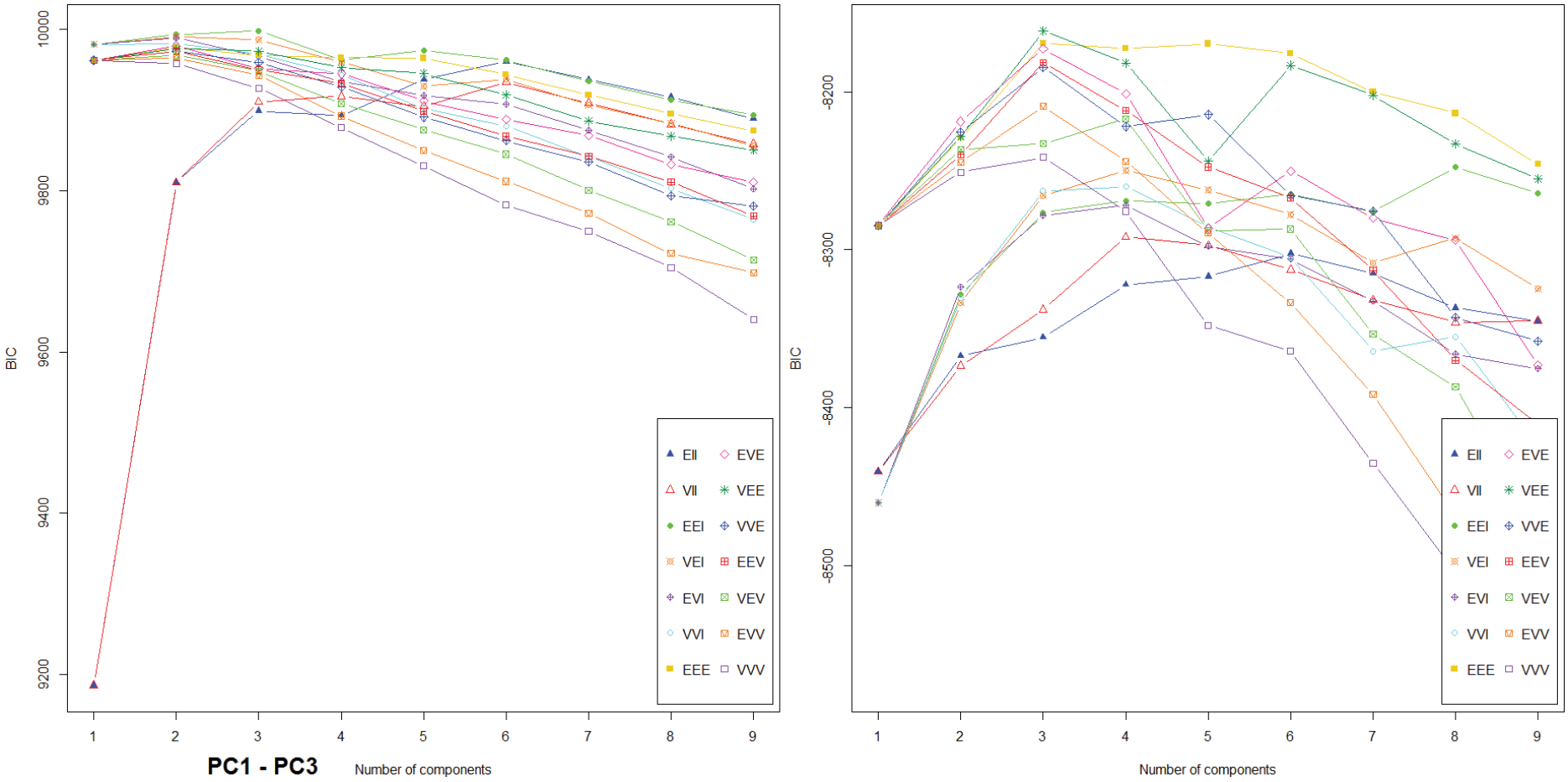


SUPPLEMENTARY FIGURE 6.6
A canonical variates analysis of all shells sampled from *Penion*, *Kelletia* and *Antarctoneptunea*. Mean confidence ellipses (90%; same colouration) indicate if groups overlap. Specimens are coloured by putative taxonomic classification under maximal OTUs.

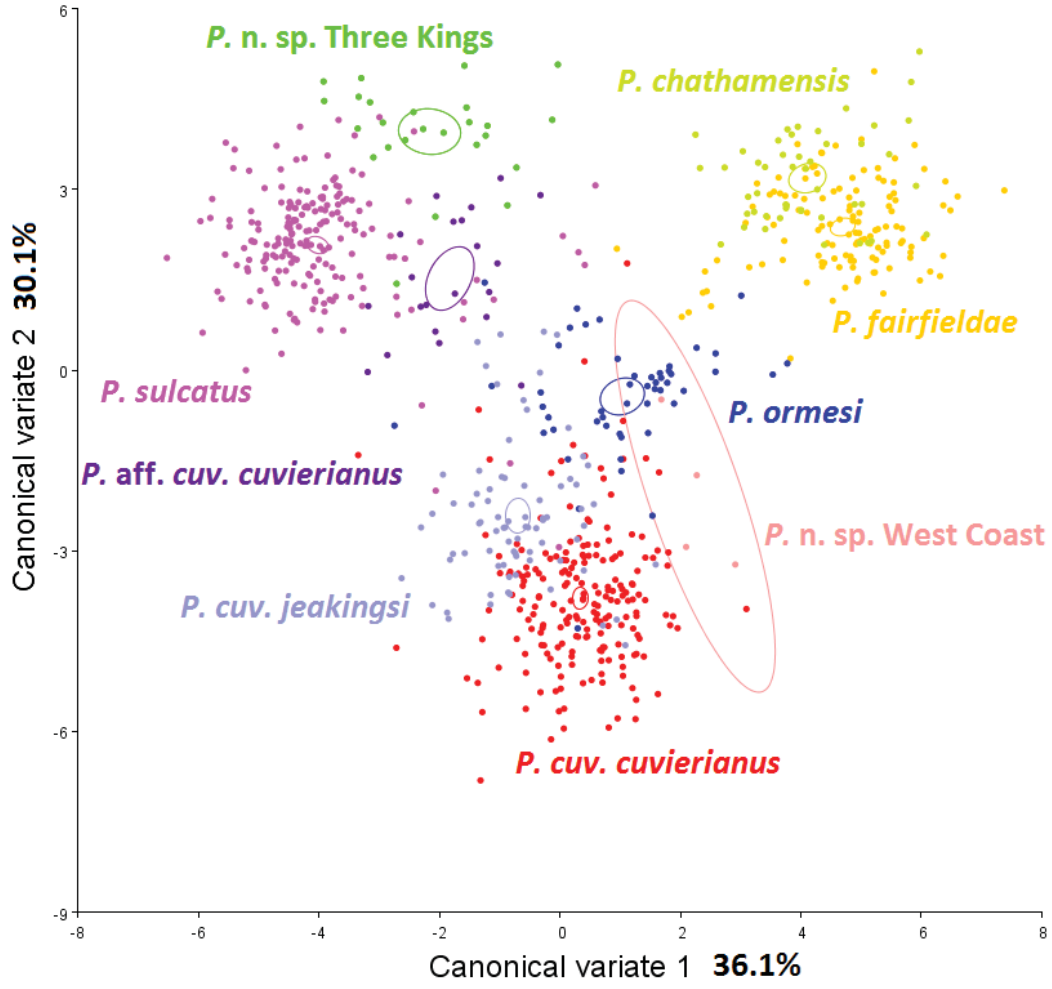


SUPPLEMENTARY FIGURE 6.7

A plot of Bayesian information criterion (BIC) scores for fitting various clustering models to variation among all sampled shells classified within the monophyletic New Zealand *Penion* clade using mclust 5.2. On the left, the BIC scores are shown using only the statistically significant PCs 1 – 3, and on the right the analysis is repeated when centroid size is also incorporated. See Fraley and Raftery (2012), for an explanation of the models listed.

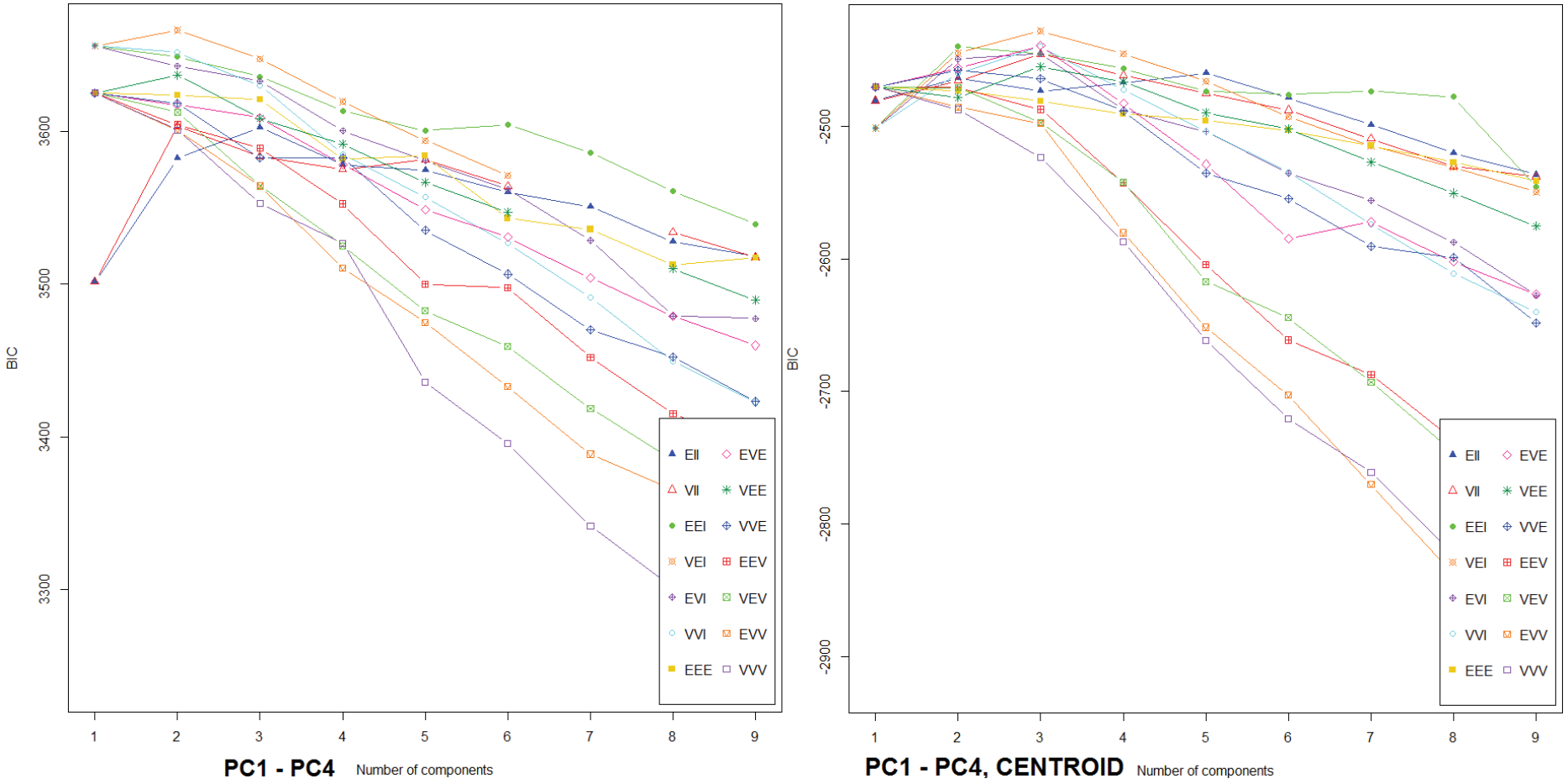


SUPPLEMENTARY FIGURE 6.8
A canonical variates analysis of all shells sampled from within the clade of monophyletic New Zealand *Penion*. Mean confidence ellipses (90%; same colouration) indicate if groups overlap. Specimens are coloured by putative taxonomic classification under maximal OTUs.

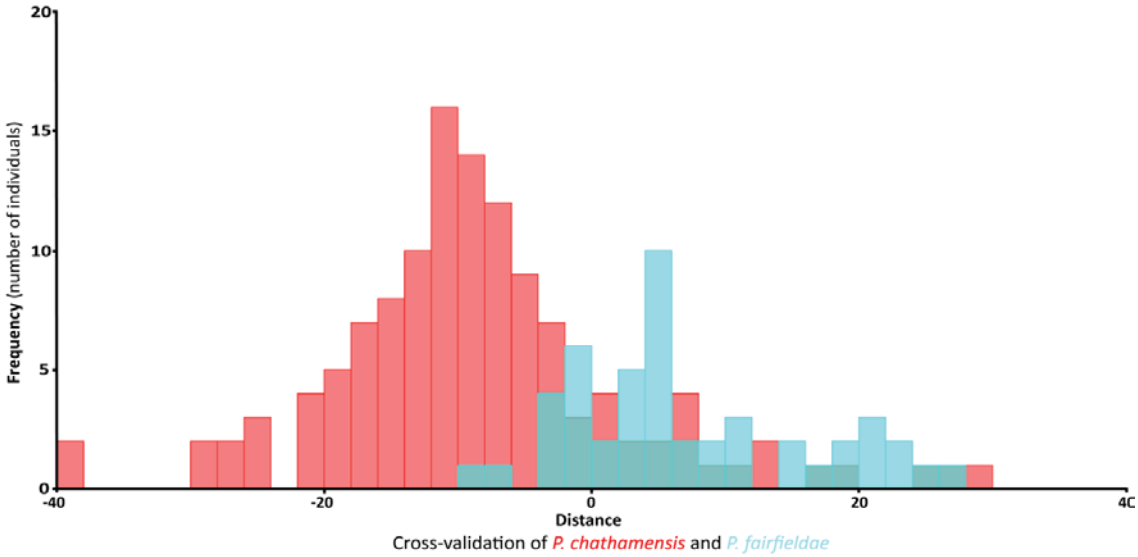


SUPPLEMENTARY FIGURE 6.9

A plot of Bayesian information criterion (BIC) scores for fitting various clustering models to variation among all sampled shells classified as *P. chathamensis* or *P. fairfieldae* using mclust 5.2. On the left, the BIC scores are shown using only the statistically significant PCs 1 – 4, and on the right the analysis is repeated when centroid size is also incorporated. See Fraley and Raftery (2012), for an explanation of the models listed.

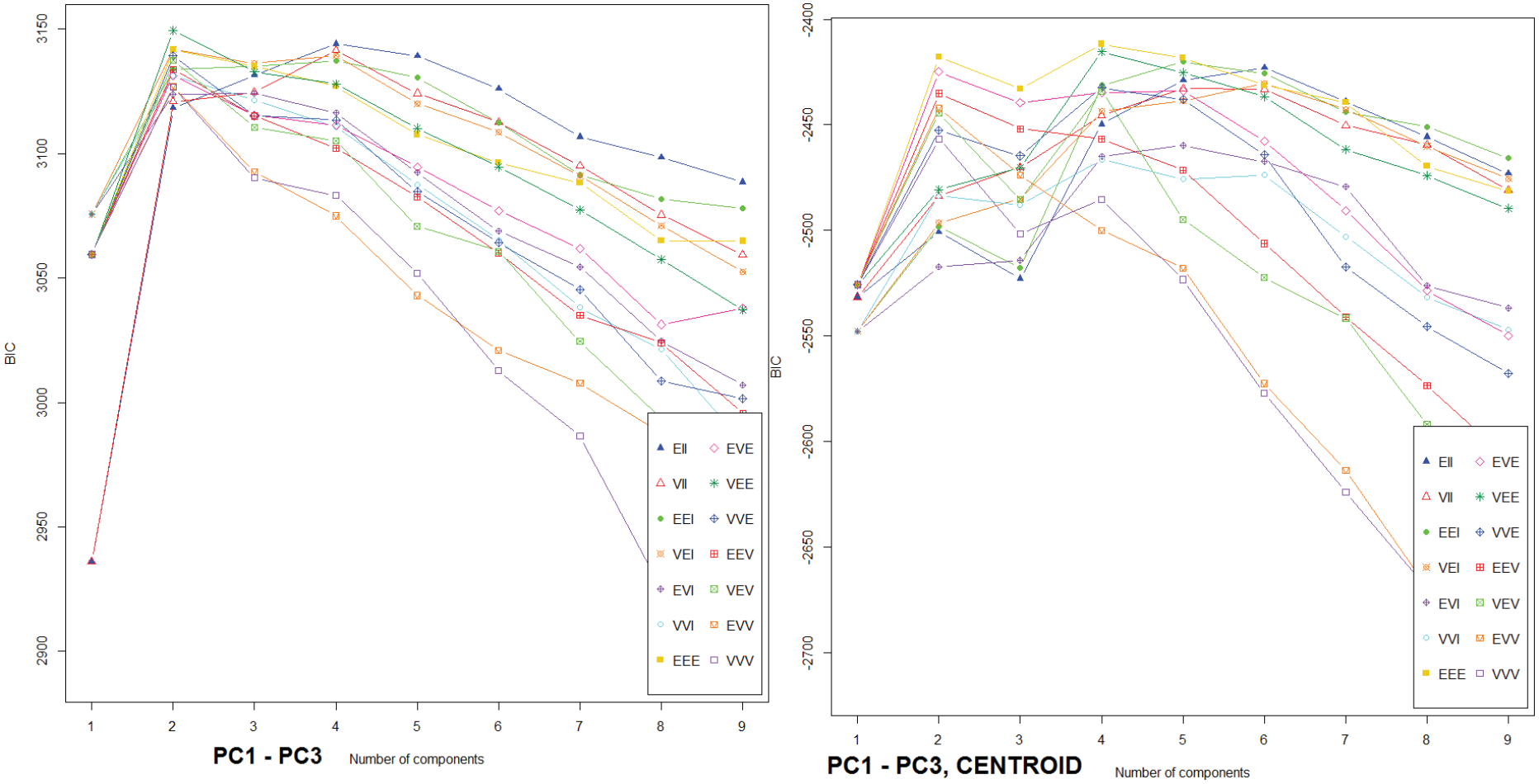


SUPPLEMENTARY FIGURE 6.10
Pair-wise canonical variates analysis of sampling of shells classified as *P. chathamensis* and *P. fairfieldae.* Discrimination is estimated via cross-validation scores using MorphoJ 1.06c (Klingenberg 2011). Specimens of *P. chathamensis* are coloured in red, and shells of *P. fairfieldae* are cyan. As can be observed, *P. fairfieldae* is not readily distinguished from *P. chathamensis*.

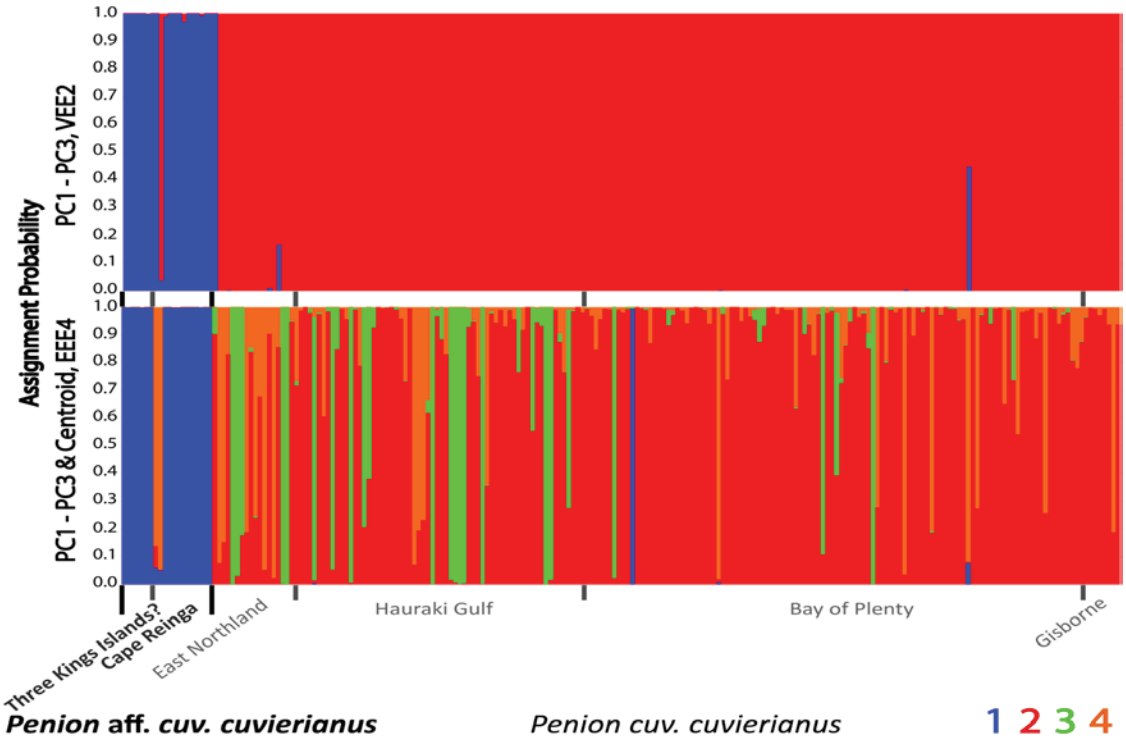


SUPPLEMENTARY FIGURE 6.11

A plot of Bayesian information criterion (BIC) scores for fitting various clustering models to variation among all sampled shells classified as *P. c. cuvierianus* or *P.* aff. *c. cuvierianus* using mclust 5.2. On the left, the BIC scores are shown using only the statistically significant PCs 1 – 3, and on the right the analysis is repeated when centroid size is also incorporated. See Fraley and Raftery (2012), for an explanation of the models listed.

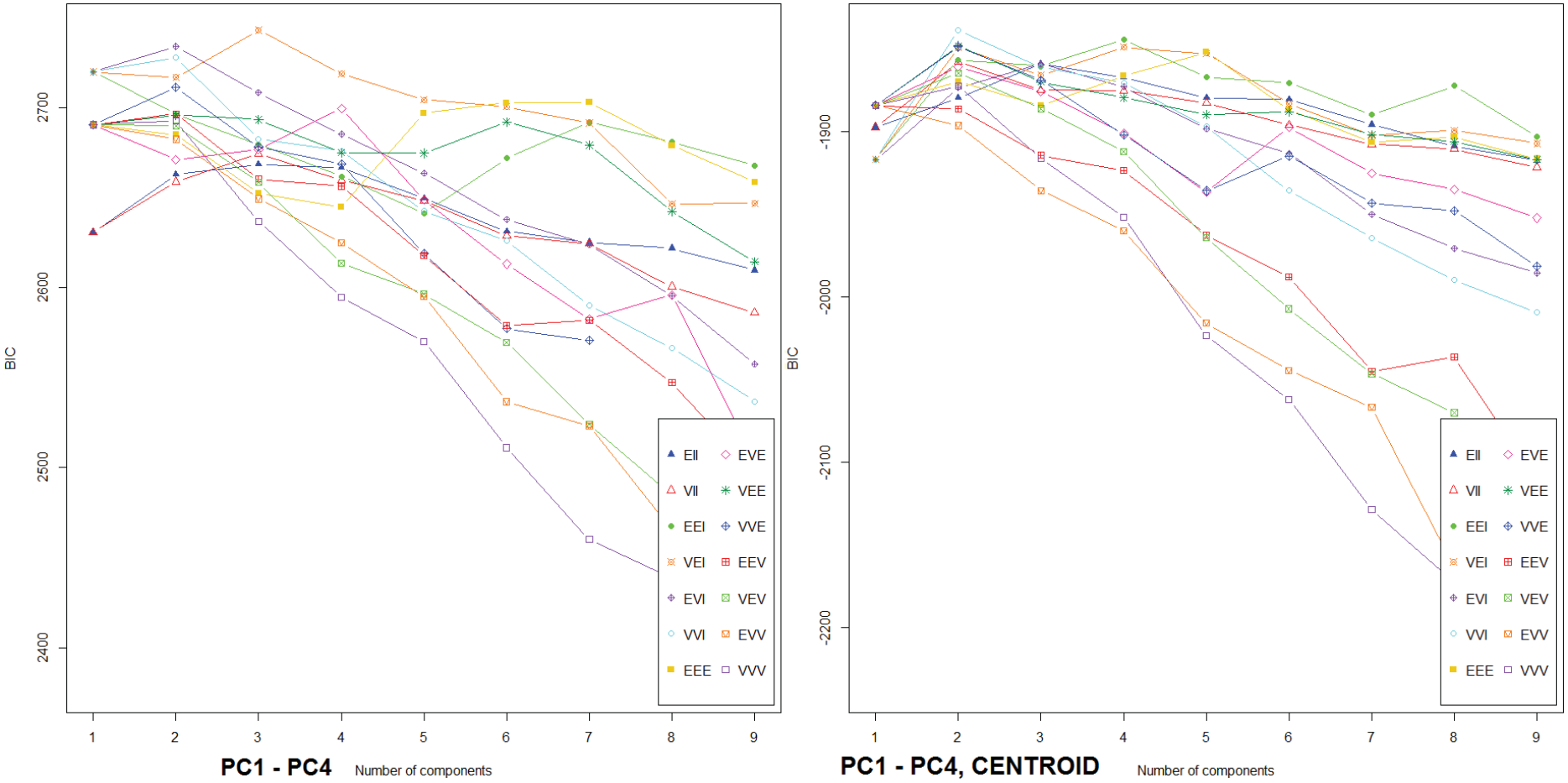


SUPPLEMENTARY FIGURE 6.12
Bayesian assignment probability of all sampled shells classified as *P. c. cuvierianus* or *P.* aff. *c. cuvierianus* to clusters estimated by the R model-based clustering package mclust 5.2. Specimens are coloured by assignment probability to clusters, are organised following the molecular phylogeny (see Chapter 4), and are labelled based on putative taxonomic classification and sampling region. The Three Kings Islands locality is listed with a question mark for *P.* aff. *c. cuvierianus* because these specimens have uncertain provenance data (may be from Cape Reinga). Colours used for each cluster are identified within a key. The VEE2 model (top) was the best support model using the only the statistically significant PCs 1 – 3, whereas the EEE4 model (bottom) received the highest BIC support when centroid size was also included. See Supplementary Figure 6.14 for a comparison for BIC values among clustering models.

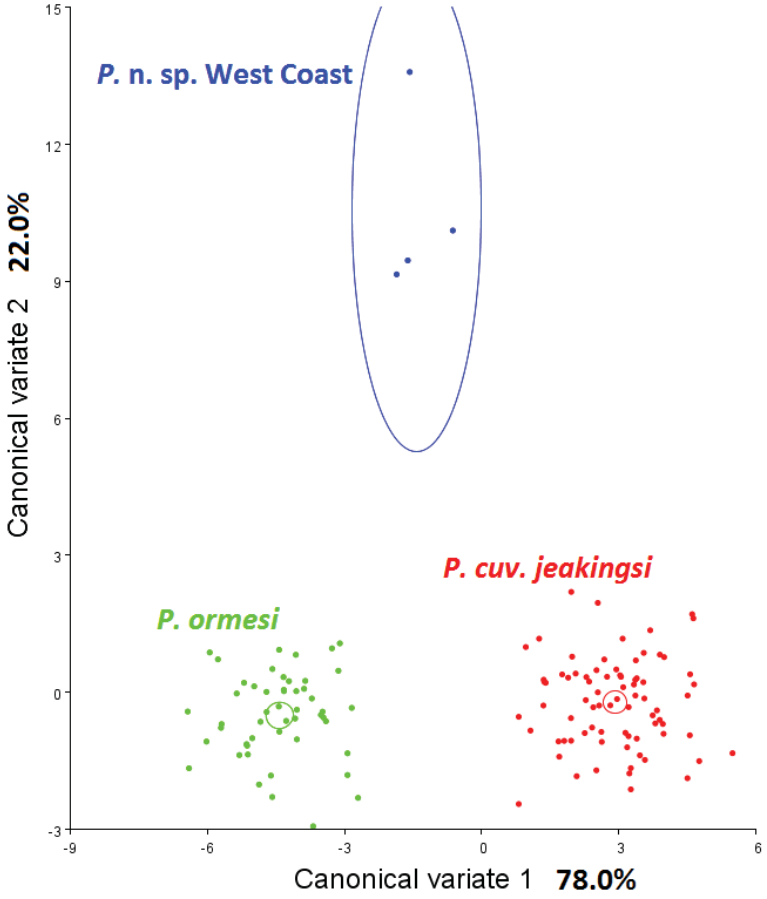


SUPPLEMENTARY FIGURE 6.13

A plot of Bayesian information criterion (BIC) scores for fitting various clustering models to variation among all sampled shells classified *P. ormesi*, *P. c. jeakingsi* or *P.* n. sp. West Coast using mclust 5.2. On the left, the BIC scores are shown using only the statistically significant PCs 1 – 4, and on the right the analysis is repeated when centroid size is also incorporated. See Fraley and Raftery (2012), for an explanation of the models listed.



SUPPLEMENTARY FIGURE 6.14
A canonical variates analysis of all sampled shells putatively classified as *P. ormesi*, *P. c. jeakingsi* or *P.* n. sp. West Coast. Mean confidence ellipses (90%; same colouration) indicate if groups overlap. Specimens are coloured by putative taxonomic classification under maximal OTUs.



Chapter Seven

Time and relative dimensions in shape: a geometric morphometric investigation of the siphon whelk (*Penion*) fossil record

Excavation at Kai Iwi beach (left), a fossil of *Penion crawfordi*† (Hutton, 1873) at Hurupi Stream, Cape Palliser (middle), and a suspicious police telephone box near GNS in Naenae (right).

Introduction

"If we are to follow Darwin's lead and make progress toward a synthetic understanding of the evolution of species, a necessary priority will be to develop tools and datasets that permit full integration of observations from the fossil record with those from living biota." (Hunt 2010)

The fossil record provides the most direct observation of the evolutionary history of life on Earth. Interpretation of the fossil record has long been recognised as challenging, primarily due to the unevenness of preservation among organisms across time and space. Debate is often directed toward large-scale morphological change, but the devil is in the detail (e.g. Darwin 1859, Gould 1977, Woodruff 1980, Foote 1997, Benton *et al.* 2000). On a practical basis, although the ability to retrieve molecular data from the fossil record has improved drastically in recent decades (e.g. Huynen *et al.* 2003, Prüfer *et al.* 2014, Delsuc *et al.* 2016, Hartl *et al.* 2015), most palaeontological studies are restricted to analysis of fossil morphology. There is no doubting the value of evidence provided by morphological variation in cellular (e.g. Bomfleur *et al.* 2014, Matzke-Karasz *et al.* 2014, Hartl *et al.* 2015), tissue and limb (e.g. Xing *et al.* 2016), whole body (e.g. Thewissen *et al.* 1994, Liu *et al.* 2014, McCoy *et al.* 2016), and trace fossils (e.g. Seilacher *et al.* 1998, Varricchio *et al.* 2007, Szrek *et al.* 2016). However the key challenge from an evolutionary perspective, is that morphological variation is not necessarily informative of evolutionary relationships because of factors such as convergence, plesiomorphy, and phenotypic plasticity. Integration of high quality fossil data with molecular data from extant taxa is an ideal (Hunt 2010), but the correct assignment of fossils from different times and places to particular evolutionary lineages is difficult.

Morphological information routinely identifies the general phylogenetic placement of an organism, and morphological and molecular change can be concordant (e.g. Hooge and Tyler 2006, Moussalli *et al.* 2009, Doddala *et al.* 2015). However, morphological difference alone cannot easily distinguish evolutionary change that occurs before or after a lineage-split (Chapter 1: Vaux *et al.* 2016). This ambiguity causes the differentiation of closely related lineages to be difficult, and it makes the distinction of intra- and interspecific variation, and wider analyses of diversity, divergence and lineage-splitting challenging in the fossil record (Chapter 1: Vaux *et al.*

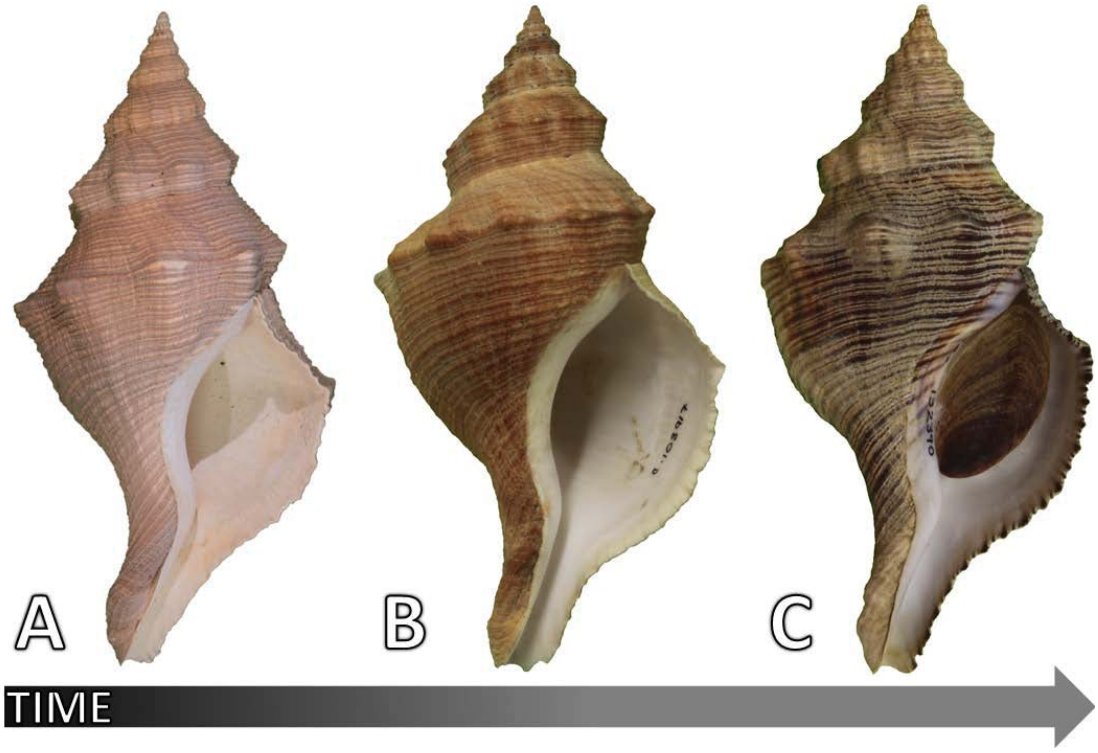
2016). Taxonomic over- or under-splitting is inevitable and unquantifiable (Charlesworth *et al.* 1982, Chapter 2: Vaux *et al.* 2016, Allmon 2016).

In a situation where molecular data are not available from the fossil record, the best theoretical approach would be to demonstrate a close concordance of morphological and molecular variation among extant representatives, and extrapolate the relationship to estimate evolutionary relationships among putative taxa in the fossil record. This approach has several requirements:

1) Any used morphological traits need to be readily preserved and accessible in fossils, both in abundance and quality of preservation, so that fossil and modern specimens are comparable.
2) The molecular phylogeny of living representatives needs to be comprehensive, reliable and demonstrate monophyly. This means that mitochondrial (mtDNA) and nuclear DNA sequence data should be used to avoid confusion due to differences in cytoplasmic and nuclear inheritance, as well as conserved and fast-evolving genes. Ideally more than one individual per species should be sequenced and population genetic analyses should be conducted to estimate gene flow and the boundaries of intraspecific genetic variation. Even with ideal molecular data however, the classification of species for a study will remain arbitrary as the treatment of inter- and intra-population genetic variation is dependent upon the taxonomic paradigm used and hypothesis of interest (Chapter 1: Vaux *et al.* 2016).
3) A concordant relationship needs to be demonstrated between the morphological trait(s) of interest preserved in the fossil record and the molecular phylogeny. A trait does not necessarily need to accurately reflect every detail of the genetic interpretation, but it needs to be informative to a scale relevant to the fossil record and hypothesis of interest (e.g. to investigate putative populations in the fossil record, a trait must be capable of distinguishing most genetic populations).
4) Where possible the effect of genetics and environment on phenotypic variation should be determined. If a trait shows ontogenetic variation, then analyses must control for organism age (with attendant uncertainties in the estimation of organismal age). Significant secondary sexual dimorphism or fluctuating asymmetry should not be exhibited by a trait, or the limits of its effect should be estimated. The presence of ontogenetic variation, sexual dimorphism or fluctuating asymmetry is not necessarily fatal to an analysis however, as these

factors, even if undetected, will inflate estimates of trait variance and reduce statistical power.

FIGURE 7.1
Three shells classified as *Penion sulcatus* (Lamarck, 1816), demonstrating that modern shells and fossil can be readily compared due to pristine preservation. A: MA36575 [AM] fossil from Te Piki, Cape Runaway, age estimated to Nukumaruan Stage (2.40 – 1.63 Ma); B: C.103917 [AUS] a recent empty shell, collected from sediment off of Moutohora Island, Bay of Plenty; it is possible that this shell is thousands of years old as seashells can persist for a long time even without fossilisation (see discussion in method); C: M.132390 [MNZ] a shell belonging to a live-caught specimen collected from off Leigh, Auckland, shown with operculum.



In this study, we investigate the fossil record of the true whelk genus *Penion* Fischer, 1884 (Neogastropoda: Buccinoidea: Buccinidae). Commonly known as siphon whelks, *Penion* species are large, benthic marine snails with extant species endemic to New Zealand and Australia (Ponder 1973, Powell 1979, Spencer *et al.* 2017). The genus has a rich fossil record in New Zealand (Beu and Maxwell 1990), Australia (Ponder 1973), Chile and Argentina (Frassinetti 2000, Nielsen 2003, Reichler 2010), and Antarctica (Stilwell and Zinsmeister 1992, Beu 2009; Chapter 3). We investigate the ability to distinguish extinct putative fossil taxa, and to determine the accuracy of their taxonomic classification. We also investigate whether fossils classified as representatives of extant species are sufficiently similar to modern populations, and observe what model of morphological evolution best fits the lineages investigated. Based on previous work using *Penion* (Chapters 3 – 6), we believe that this study system satisfies at least some of the requirements listed above.

1) Shell morphology is the trait of interest. Crucially, gastropod shells are readily preserved in the fossil record due to their calcareous structure, and the frequent occurrence of marine species on soft-sediment substrates. Shells of *Penion* often fossilise in good condition, meaning fossils that are millions of years-old can be compared readily with shells collected from living specimens (Figure 7.1). A key limitation of morphological analyses using large marine snails such as *Penion* however, is that large fossil shells are more susceptible to damage, meaning that the number of intact shells per geological locality can be lower than desired.
2) We produced phylogenies derived from mtDNA genomic and nuclear ribosomal DNA sequence data sampled from individuals belonging to all extant species of *Penion* (although sample sizes were small; Chapters 3 and 4). *Penion benthicolus* Dell, 1956 was found to be sister to *Antarctoneptunea aurora* (Hedley, 1916), and the remaining New Zealand species form a monophyletic clade (Chapter 3). Analysis of single nucleotide polymorphic (SNP) variation for anonymous nuclear loci among multiple individuals of some species helped delimit species boundaries, and was concordant with mtDNA and rDNA phylogenetic results (Chapter 4).
3) Using a two-dimensional landmark-based geometric morphometric method, we demonstrated that there was a relatively close concordance between variation in shell morphology and molecular phylogeny for *Penion* (Chapter 6). Variation in

shell shape and size could readily separate the sister genera of *Antarctoneptunea* Dell, 1972, *Kelletia* Bayle, 1884 and *Penion*. Shell morphology could identify particular species with high accuracy as data were investigated hierarchically across the phylogeny. Furthermore, geometric morphometric results mostly showed concordance with molecular results, even where molecular data suggested alternative relationships to the current taxonomy (Chapter 6). Shell morphology could also identify apparent differences in shell shape and size between some intraspecific populations (Chapter 6).

4) Siphon whelks appear to exhibit high intraspecific variation in shell morphology, alongside variation in other traits such as body size and colouration. It seems likely that this variation is largely due to environmental plasticity. Although our morphometric analysis indicated that there was significant intraspecific variation, results suggested that *Penion* species that are closely related have shell shapes that are more similar to each other, than to distantly related species (Chapter 6). The results of our sampling of extant species indicate that it is important to sample across the entire range of a species with as high replication as possible. Gastropod shells do vary in shape and size with age, and therefore we controlled for the age of specimens by only analysing shells with at least 6 teleoconch whorls, which appears to be terminal shell growth in most *Penion* (Chapters 5 and 6). In a separate geometric morphometric study, results indicated that secondary sexual dimorphism is not exhibited in the shells of *P. chathamensis* (Powell, 1938) (Chapter 5). We therefore assume for this study that secondary sexual dimorphism is also not exhibited in the shells of other siphon whelk species or that it has little, if any, impact in morphometric comparisons of taxa.

Molluscs have an abundant, widely studied fossil record extending over approximately 500 million years (e.g. Steiner *et al.* 2007, Parkhaev and Demidenko 2010), of which marine snails represent a substantial proportion (Foote *et al.* 2015). New Zealand in particular has been noted for its rich fossil record for marine snails with well-studied stratigraphy (Crampton *et al.* 2006, Allmon and Smith 2011), and there is a high diversity of extant, endemic species in waters off New Zealand (Powell 1979). New Zealand marine snail lineages like *Penion* have been cited as good targets to investigate models of evolutionary change and speciation (Gould 1991).

The siphon whelk fossil record provides the opportunity to investigate patterns of morphological change in putative evolutionary lineages. To achieve this we tested the fit of morphological variables through time against three different models of evolutionary change, using the R (R Core Team 2016) package paleoTS 0.4.4 (Hunt 2006, Hunt 2007). For this study, the morphological variables are statistically significant (and mathematically independent) principal components (PCs) and centroid size (explanation in methods) estimated from extant and fossil samples from multiple time periods. The models tested were 1) evolutionary stasis, meaning limited fluctuations from a mean state through time; 2) an unbiased random walk (URW), representing stochastic change in a trait through time; and 3) a generalised random walk (GRW), representing directional change in a trait with some stochastic variation considered. Under stasis, drift is more limited than expected under the URW model, indicating that constraining (stabilising) selection or gene flow are limiting fluctuations. We deliberately consider these simplistic evolutionary models, rather than more elaborate possibilities, so that fewer statistical assumptions are made. The intention is to, "limit ourselves to the more modest but attainable goal of inferring something about the aggregate qualities of a set of evolutionary changes, i.e., their directionality and volatility," (Hunt 2006). PaleoTS also does not make an explicit assumption about species classification; instead models are fitted against variation exhibited through time within a putative evolutionary lineage (Hunt 2006, Hunt 2007, Hunt 2010, Monnet *et al.* 2011). This means that it is legitimate to combine data from multiple species if there is evidence to hypothesise that they belong to the same evolutionary lineage. The prediction of an evolutionary lineage itself is dependent on many other assumptions such as the concordance of phenotypic divergence and lineage-splitting (Chapter 1: Vaux *et al.* 2016), but statistical methods such as cluster analysis can at least help to consistently interpret variation in morphological data among groups (see below). Marine invertebrates are frequently the topic of large-scale paleoTS analyses considering evolutionary change earlier than the Quaternary Period (<2.58 Ma; e.g. Hunt 2006, Hunt 2007, Novack-Gottshall and Lanier 2008, Hunt *et al.* 2010, Monnet *et al.* 2011, Hopkins and Lidgard 2012, Payne *et al.* 2013, Sigurdsen and Øyvind 2016), although fossil sequences from a wide diversity of organisms have been investigated using the statistical package (e.g. Hunt 2008, Hopkins and Lidgard 2012, Piras *et al.* 2012, Prothero *et al.* 2012, Huttenlocker 2014, Pandolfi *et al.* 2015, Sansalone *et al.* 2015).

Methods

Taxonomy

As with the previous phylogenetic (Chapters 3 and 4), and morphometric studies (Chapter 6), specimens were assigned to putative taxa based primarily on traditional examination of shell traits such as shell size, protoconch morphology and the presence of features such as axial ribs (Ponder 1973, Powell 1979, Beu and Maxwell 1990). Taxonomic treatment of extant taxa occasionally refers to body parts, including the morphology of radula and opercula (Dell 1956, Ponder 1973, Powell 1979). All sampled specimens were identified by experienced molluscan taxonomists: Bruce A. Marshall (Collection Manager Sciences, Museum of New Zealand Te Papa Tongarewa) and Alan G. Beu (Palaeontologist, GNS). Current taxonomy and the operational taxonomic units (OTUs) used for this thesis are reviewed in Chapter 8.

Sampling of shells

We sampled fossil shells classified as *Penion* from localities in New Zealand and Australia (Figure 7.2), with the oldest specimens dated to 27.5 Ma (Table 7.1). This sampling was supplemented with existing data including individuals of all extant species of *Antarctoneptunea, Kelletia* and *Penion* (Chapter 6). It is desirable from a statistical viewpoint for the number of samples per group exceed the number of landmarks used (45). For downstream analyses adequate sampling ensures that the degrees of freedom exceed the shape dimensionality of the data; meaning that there are an adequate number of principal components for later analyses. We attempted to sample individuals from more than one location per species in order to capture population-level variation. However, from the fossil record only the extinct species *P. marwicki* was represented by more than 45 individuals, because the number of fossils in adequate condition for geometric morphometric analysis was low.

The majority of shells were sourced from museum and university collections (listed in Acknowledgements). Complete or near-complete shells (specimens with intact edges and points encompassed by landmarks) with reliable provenance data were used. Only conchologically mature shells were photographed, with maturity being estimated by the presence of at least six teleoconch whorls, thickening of the outer aperture lip, and ascent of the end of the last whorl. Although sexual maturity can occur earlier

(Jones 1938), shell maturity is usually treated as a proxy for adulthood in snails as it indicates when a snail is no longer growing in size (Goodfriend 1986).

Potentially many of the empty shells sampled from modern benthic sediments via trawling could have been thousands of years old (Powell and Davies 1990, Kidwell 2013). Although this is a very brief moment of evolutionary time and it is unlikely to influence comparisons between separate evolutionary lineages, it might mean variation due to environmental plasticity through time is falsely combined (compared to modern-day variation). From a palaeontological perspective however, fossil shell bed samples typically undergo time-averaging to a range between 100 and 1000 years (Kowaleski *et al.* 1998).

FIGURE 7.2
Fossil localities from New Zealand and Australia sampled for this study, with the distribution of extant *Penion* species highlighted. At each fossil location the putative classification of specimens (whether representatives of an extant or extinct species) is listed. Most sites contain only one species, however, Wanganui (1 on map) and Te Piki (2 on map) are each purported to contain a mixture of species. Wanganui appears to contain a mixture *P. sulcatus* and *P. ormesi* fossils, and samples from Te Piki are hypothesised to be a mixture of *P. sulcatus* and *P. c. cuvierianus* (Powell, 1927).

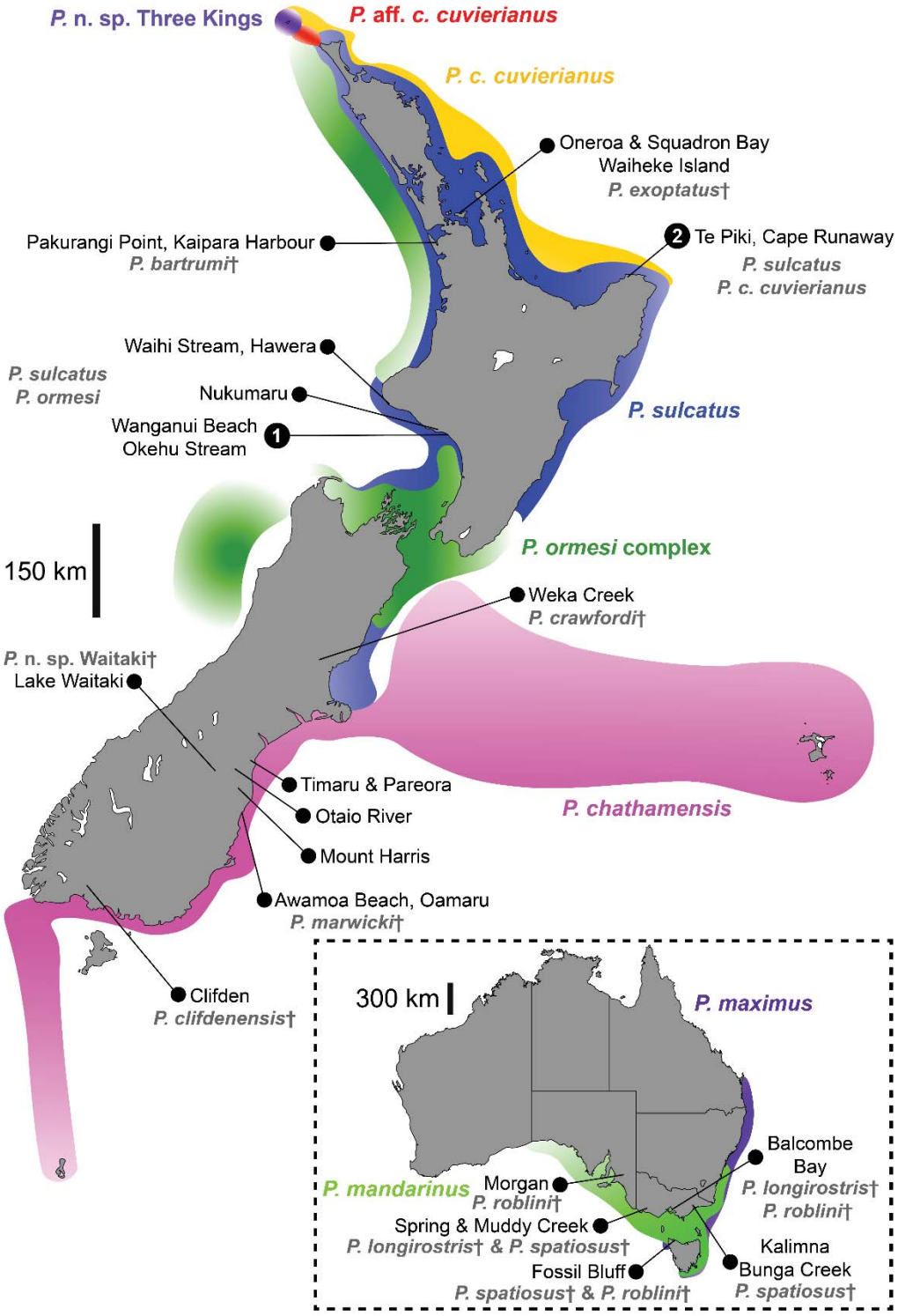


TABLE 7.1
Sampling of fossils classified as *Penion* organised by pre hoc classified species, grouped within taxa by geological site. Classification was based on traditional analysis of shell morphology, focussing on features such as siphonal canal length, teleoconch spire height, protoconch morphology, and the presence and size of axial ribs. For the Castlecliff locality, occurrences across multiple strata for a species are combined as a single range for simplicity in this table.

Classification	Fossil sites	Age (Ma)	Count
P. bartrumi†	Pakurangi Point, Kaipara Harbour, Auckland	18.70 – 15.90	1
P. clifdenensis†	Clifden, Southland	15.90 – 15.10	7
P. crawfordi†	Weka Creek, Canterbury	18.70 – 15.90	1
P. c. cuvierianus	Te Piki, Cape Runaway, Bay of Plenty	2.40 – 1.63	17
P. exoptatus†	Fossil Bay (Oneroa), Waiheke Island, Auckland	21.70 – 18.70	3
P. exoptatus†	Squadron Bay, Waiheke Island, Auckland	21.70 – 18.70	1
P. c. jeakingsi	Castlecliff, Wanganui Beach, Manawatu-Wanganui	0.65 – 0.38	1
P. longirostris†	Balcombe Bay, Mornington, Victoria	15.50 – 15.00	5
P. longirostris†	Spring Creek, Minhamite, Victoria	5.00 – 4.30	1
P. ormesi	Castlecliff, Wanganui Beach, Manawatu-Wanganui	0.97 – 0.45	8
P. ormesi	Okehu Stream, Wanganui Beach, Manawatu-Wanganui	0.97	3
P. ormesi	Nukumaru, Manawatu-Wanganui	0.97	2
P. marwicki†	Awamoa Beach, Oamaru, Otago	15.90 – 18.70	50
P. marwicki†	Mount Harris, Canterbury	15.90 – 18.70	11
P. marwicki†	Otaio River, Canterbury	15.90 – 18.70	1
P. marwicki†	Pareora, Canterbury	15.90 – 18.70	1
P. marwicki†	Timaru, Canterbury	15.90 – 18.70	1
P. spatiosus†	Bunga Creek, Victoria	4.30 – 3.40	1
P. spatiosus†	Fossil Bluff, Table Cape, Tasmania	27.50 – 16.50	1
P. spatiosus†	Kalimna, Victoria	4.30 – 3.40	1

P. spatiosus†	McDonalds Bank, Muddy Creek, Hamilton, Victoria	4.300 – 3.40	1
P. sulcatus	Castlecliff, Wanganui Beach, Manawatu-Wanganui	0.96 - 0.38	21
P. sulcatus	Nukumaru, Manawatu-Wanganui	0.97	1
P. sulcatus	Te Piki, Cape Runaway, Bay of Plenty	2.40 – 1.63	19
P. sulcatus	Waihi Steam, Hawera, Taranaki	3.00 – 2.40	1
P. roblini†	Balcombe Bay, Mornington, Victoria	15.50 – 15.00	2
P. roblini†	Fossil Bluff, Table Cape, Tasmania	27.50 – 16.50	1
P. roblini†	Morgan, South Australia	15.50 – 15.00	1
P. n. sp. Waitaki†	Lake Waitaki, Canterbury	27.30 – 25.20	1
		TOTAL	**166**

Geometric morphometric analysis of shells

Variation in shell morphology was analysed using the two-dimensional, landmark-based geometric morphometric method described in previous investigations of extant *Penion* (for detailed method see Chapters 5 and 6). Although landmark-based geometric morphometrics can be used to investigate fluctuating asymmetry (Klingenberg 2015), we have not addressed the issue as a potential source of variation in *Penion.* In summary, shells were photographed with the aperture facing upward using a Canon EF-S 600D camera with an 18 – 55 mm IS II lens (see Figure 5.1), and the positioning of shells was controlled carefully (see discussion by Webster and Sheets 2010, Chapters 5 and 6). Combs, aligned to the central axis of the shell, were added to photographs in Adobe Photoshop CS6 so that semi-landmarks could be placed consistently. We used 45 landmarks to summarise shell shape (Figure 5.1). Six fixed landmarks captured biologically homologous points such as the top of the teleoconch, and 39 semi-landmarks described the inner and outer curves of the aperture and siphonal canal. Following the interpretation of Gunz *et al.* (2005), all of our landmarks are Type I as defined by Bookstein (1991). Landmarks and semi-landmarks were digitised and scale-calibrated using tpsUtil, tpsDig (Rohlf 2013), and the IMP program CoordGen7 (Sheets 2014), yielding X – Y Procrustes coordinates. Semi-landmarks were 'slid' to minimise the effect of the arbitrary placement of points on the curves of interest. Sliding was achieved by minimising Procrustes distances (Bookstein 1996, Zelditch *et al.* 2004, Perez *et al.* 2006), using the IMP program Semiland7 (Sheets 2014).

Partial Procrustes superimposition was conducted using MorphoJ 1.06c (Klingenberg 2011), which aligns and superimposes landmarks for all specimens to remove confounding variation due to differences in the size, translation (position) and orientation of objects (Webster and Sheets 2010, Mitteroecker *et al.* 2013, Monteiro 2013, Polly *et al.* 2013). Procrustes superimposition is the preferred method when morphological variation is relatively small (Perez *et al.* 2006). A covariance matrix was generated from the X – Y coordinates of the superimposed landmarks, providing input for principal components analysis (PCA) in MorphoJ 1.06c (Klingenberg 2011). The PCs reflect variation in the shape of objects, whereas centroid size acts as a proxy for size variation (independent of shape). Statistically significant PCs were identified using the broken-stick test on eigenvalues, implemented in the R (R Core Team 2016) package vegan 2.2-1 (Jackson 1993, Oksanen *et al.* 2015). We used PCA ordinations to

estimate the separation of *a priori* groups (e.g. monophyletic clades, taxonomic species, populations). We used 90% mean confidence ellipses of group means to determine if groups were likely to overlap. Canonical variates analysis (CVA) was used to statistically test the ability to differentiate these *a priori* groups, with the success of discrimination determined using cross-validation scores (the number of individuals correctly assigned to each *a priori* group). CVA was conducted using either MorphoJ 1.06c (Klingenberg 2011), analysing the original X – Y landmark coordinates, or the R package MASS 7.3-26 (Venables and Ripley 2002) using PCs generated from PCA in MorphoJ. For taxa with fewer specimens than the number of landmarks used, we used PCA as a dimensionality-reducing method to allow *a priori* groups to be tested with CVA.

We also wanted to investigate which groupings could be naïvely identified using the shell morphological data (shape and size) alone, without relying on *a priori* hypotheses based on other data such as genetics, taxonomy, or geography. To investigate naïve groupings, we conducted model-based cluster analysis using the R package mclust 5.2 (Fraley and Raftery 2002). Mclust can analyse both PCs (shell shape) and centroid size (shell size), and attempts to identify the clustering model that most efficiently explains variation in a dataset without any prior classification of specimens. The fit of a model is tested with an iterative expectation-maximisation (EM) method using Gaussian mixture modelling (Fraley and Raftery 2012). The models used by mclust differ in the expected distribution of data, as well as the volume, shape and orientation of the covariance matrices generated from observed data (parameters for mclust models listed in Supplementary Table 5.3; Fraley and Raftery 2012). Bayesian information criterion (BIC) scores were used to determine the relative support for competing clustering models. In mclust, BIC scores are multiplied by -1 so that higher BIC values indicate higher support. Where centroid size was included with PCs for mclust analyses, variables were scaled (using the base function in R) because centroid size is expressed on a much larger numerical scale than the PCs. Different numerical scales are problematic for mclust analysis as multiple models tested assume the same variance across all variables or estimated clusters (Fraley and Raftery 2012).

We investigated patterns of morphological change in potential evolutionary lineages of *Penion* using the R (R Core Team 2016) package paleoTS 0.4.4 (Hunt 2006, Hunt 2007), which tests the fit of variables (statistically significant PCs and centroid size) against three different models of evolutionary change (stasis, URW, GRW –

discussed above). Support for models is determined using the Aikaike information criterion with correction for finite sample sizes (AICc), utilising Aikaike weights. We did not attempt to fit more complex mode-shift models (Hunt *et al.* 2015), as none of our datasets had an adequate number of geological time slices.

Results and Discussion

Discrimination and classification of extinct fossil species

We first investigated whether extinct, putative species of *Penion* in the New Zealand and Australian fossil record could be distinguished from each other and all extant species. We included sampling from the closely related lineages *Aeneator* Finlay, 1926, *Antarctoneptunea*, and *Kelletia* so that the analysis could provide an indication of whether the *Penion* fossils were accurately classified to begin with.

The broken-stick test identified three statistically significant PCs: PC1 (56.82%), PC2 (17.15%), and PC3 (6.26%). Based on BIC score, mclust found highest support for five to seven clusters when only the significant PCs were analysed (Supplementary Figure 7.1), and when shell size was considered alongside shape variation by including centroid size (Supplementary Figure 7.1). As with the previous morphometric investigation of extant *Penion* (Chapter 6), it appeared that the clusters identified were hierarchical (Figure 7.3a). The clustering models with best BIC score typically identify significant morphological differences that appear to reflect deeper phylogenetic splits (e.g. differences between genera), whereas models with lower BIC values often related to particular species or groups of closely related lineages (Figure 7.3a). For this fossil analysis however, the exact clusters identified are not our main concern – instead, what matters is the placement of the fossil taxa relative to the extant sampling. Under the best supported mclust models the most striking result was the clear separation of *P. spatiosus* (Tate, 1888) (from Australia) from most other *Penion*, and its placement in clusters dominated by sampling from *Aeneator* (Figure 7.3a). A similar result was observed for the two other Australian fossil species *P. longirostris* (Tate, 1888) and *P. roblini* (Tenison Woods, 1876) that were clustered with *P. spatiosus* and *Aeneator*, or formed a separate grouping (Figure 7.3a). Under a few models some individuals of the New Zealand species *P. marwicki* were placed in a cluster dominated by *Antarctoneptunea*, however alternative models with high BIC support placed the species in clusters comprised of *Penion* (Figure 7.3a). Other New Zealand fossil taxa, *P. bartrumi* (Laws, 1941), *P. clifdenensis* (Finlay, 1930), *P. crawfordi* (Hutton, 1873), *P.*

exoptatus (Powell & Batrum, 1929) and *P.* n. sp. Waitaki, sampled with low frequency (Table 7.1) were consistently clustered with extant *Penion* (Figure 7.1).

FIGURE 7.3a
Bayesian assignment probability of all sampled fossils classified as extinct *Penion* species, with all extant samples of *Penion, Kelletia* and *Antarctoneptunea,* to clusters estimated by the R model-based clustering package mclust 5.2. Specimens are coloured by assignment probability to clusters. Extant specimens are organised following the molecular phylogeny (see Chapters 3 and 4), and fossil taxa are placed next to possible extant relatives (see Chapter 8). Specimens are labelled based on putative taxonomic classification and sampling region or geological site. The VEI7 model (top) was the best supported model using only the statistically significant PCs 1 – 3, whereas the VEV5 model (middle) received the highest BIC support when centroid size was also included. When 7 clusters were considered, the EEV7 model (bottom) using PCs 1 – 3 and centroid size received the highest support among alternative models. See Supplementary Figure 7.1 for a comparison for BIC values among clustering models.

FIGURE 7.3b
A principal component analysis ordination (PCs 1 and 2) of all sampled fossils classified as extinct *Penion* species, with all extant sampling from *Penion, Kelletia* and *Antarctoneptunea*. Extant specimens are coloured by genus (blue for *Penion*, black for *Kelletia,* brown for *Antarctonetpunea* inclusive of *P. benthicolus* (see Chapter 3). Fossils are coloured according to putative taxonomic classification. Mean confidence ellipses (90%; same colouration) indicate whether group means of species (fossil or extant) are likely to overlap. Specific extant species are not labelled for clarity. See Supplementary Figures 7.4 and 7.5 for ordinations including the third statistically significant principal component.

FIGURE 7.3a

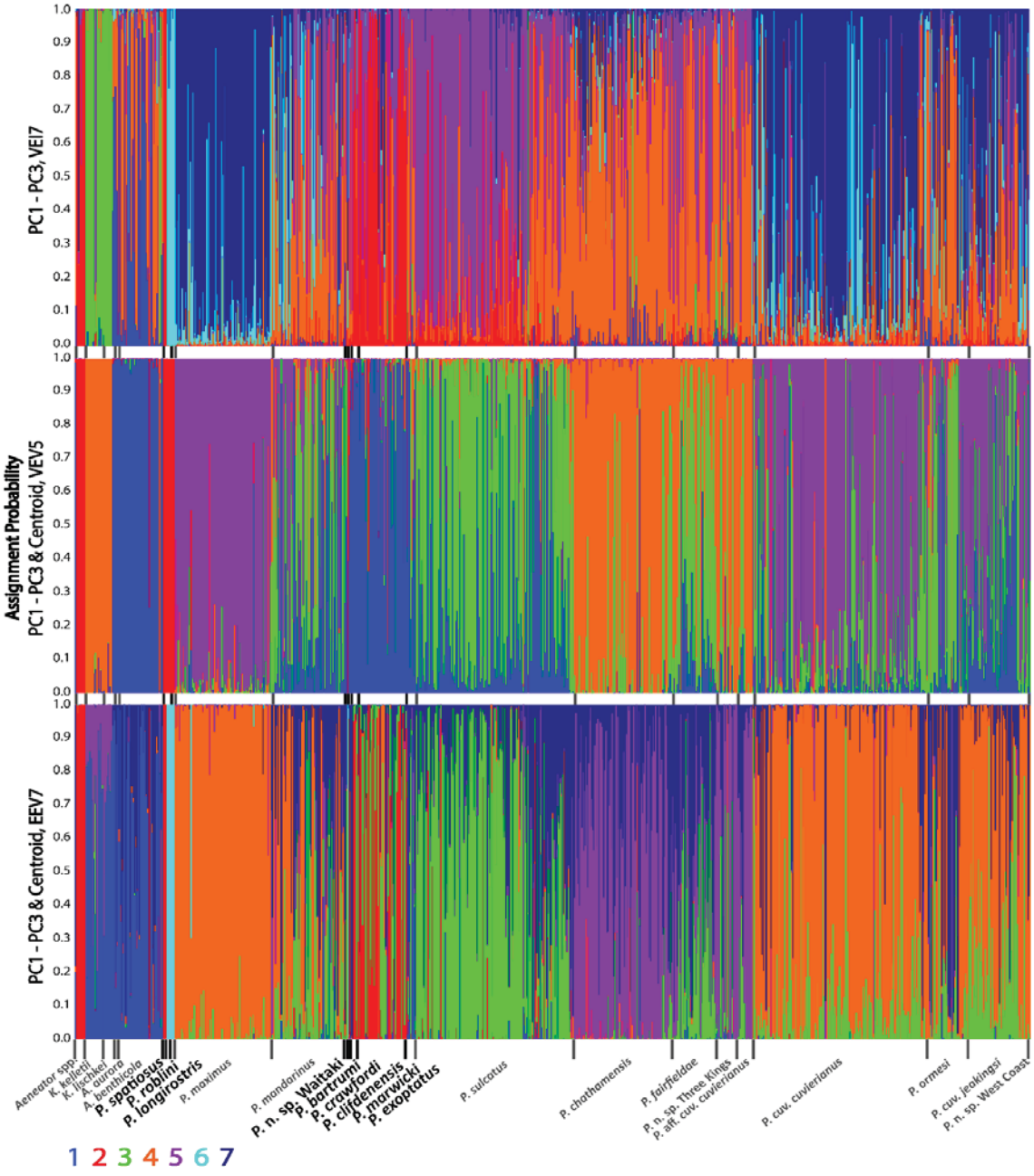
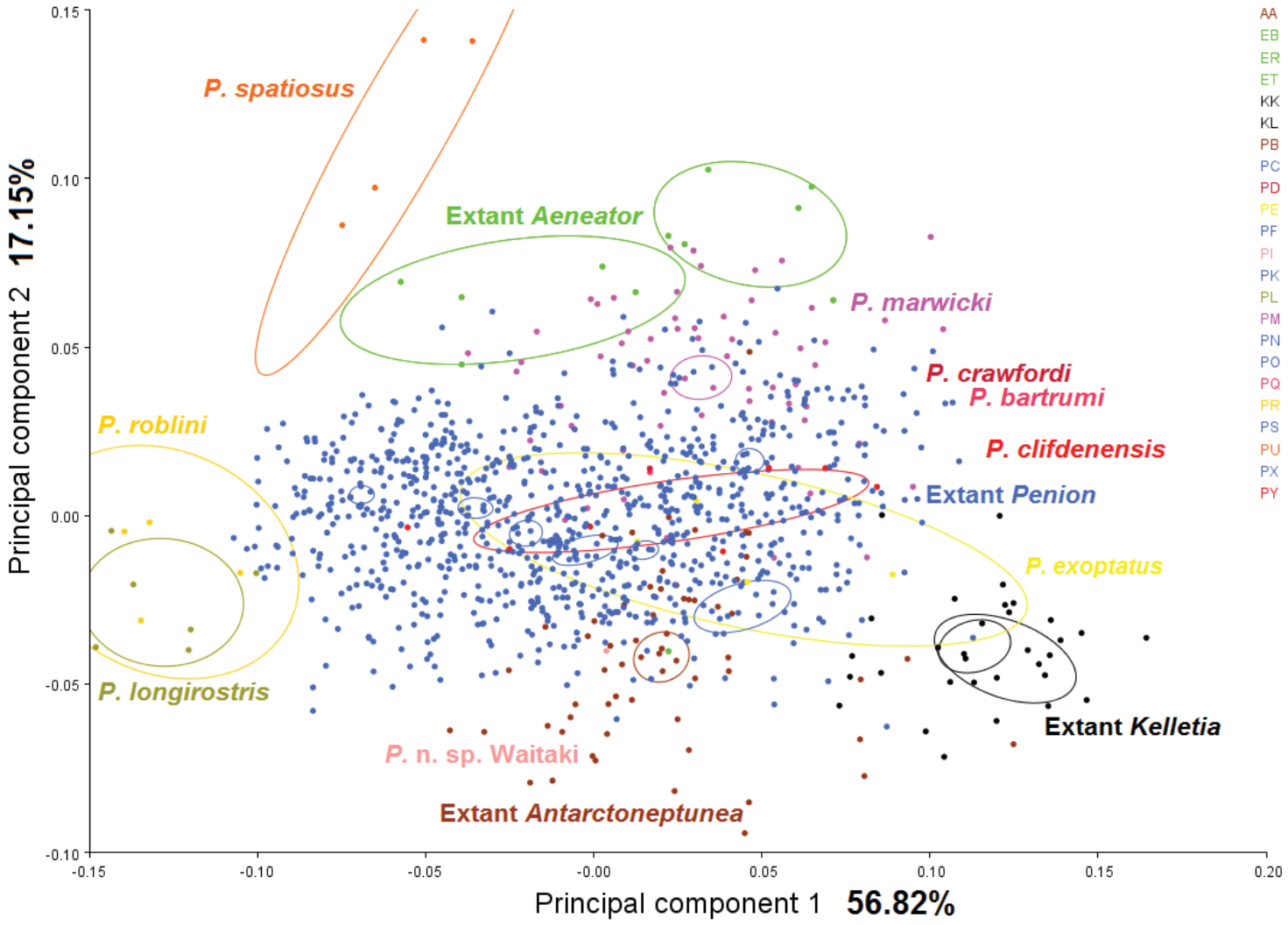

PC1 - PC3, VEI7
Assignment Probability
PC1 - PC3 & Centroid, VEV5
PC1 - PC3 & Centroid, EEV7
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Aeneator spp.
K. kelleti
K. lischkei
A. aurora
A. benthicola
P. spatiosus
P. roblini
P. longirostris
P. maximus
P. mandarinus
P. n. sp. Waitaki
P. bartrumi
P. crawfordi
P. clifdenensis
P. marwicki
P. exoptatus
P. sulcatus
P. chathamensis
P. fairfieldae
P. n. sp. Three Kings
P. aff. cuv. cuvierianus
P. cuv. cuvierianus
P. ormesi
P. cuv. jenkinsi
P. n. sp. West Coast
1 2 3 4 5 6 7

FIGURE 7.3b


P. spatiosus
Extant Aeneator
P. marwicki
P. crawfordi
P. bartrumi
P. clifdenensis
Extant Penion
P. exoptatus
P. roblini
P. longirostris
P. n. sp. Waitaki
Extant Kelletia
Extant Antarctoneptunea
Principal component 2 17.15%
Principal component 1 56.82%
0.15
0.10
0.05
0.00
-0.05
-0.10
-0.15
-0.10
-0.05
-0.00
0.05
0.10
0.15
0.20
AA
EB
ER
ET
KK
KL
PB
PC
PD
PE
PF
PI
PK
PL
PM
PN
PO
PQ
PR
PS
PU
PX
PY

Ordination of PCA scores shows that the clusters identified by mclust correspond with the classification of multiple species and reflect generic differences (Supplementary Figures 7.2 and 7.3). When individuals were re-classified by taxonomic identification, it became apparent that individuals of *P. spatiosus* occur outside the range of morphospace occupied by other fossil and extant *Penion, Aeneator, Antarctoneptunea,* and *Kelletia* (Figure 7.3b, Supplementary Figures 7.4 and 7.5). Although sampling was limited, it appears that *P. longirostris* and *P. roblini* overlap in morphospace. The means of this pair of species are some distance from other *Penion*, but some individuals overlap with the distribution of the extant Australian species *P. maximus* (Tryon, 1881) (Figure 7.3b). Some *P. marwicki* shells overlapped with the distributions of modern *Aeneator,* but most were centred on a mean position close to extant species of *Penion*, or overlapped with modern *Penion* specimens (Figure 7.3b). Results from CVA also indicated that *P. marwicki* could be readily distinguished from extant *Penion* taxa with similar sampling (Supplementary Table 7.1). Akin to results from mclust, all other sampled fossil taxa occurred within the range of modern *Penion* (Figure 7.3b, Supplementary Figures 7.4 and 7.5).

These results indicate that most fossils classified as *Penion* are part of the *Penion* clade in terms of shell morphology, however *P. spatiosus* has probably been assigned to the wrong genus. This accords with the observation that *P. spatiosus* exhibits many unique shell traits, but disagrees with the hypothesis of *P. spatiosus* being related to the extant *P. mandarinus* (Duclos, 1832) and *P. maximus* from Australia (Ponder 1973). Based on PCA the species was more different in shell shape from other *Penion* than specimens of *Aeneator, Antarctoneptunea* and *Kelletia*. With and without the inclusion of shell size, the naïve analysis using mclust consistently placed *P. spatiosus* in a cluster containing *Aeneator* (Figure 7.3a). *Penion longirostris* and *P. roblini* cannot be distinguished using shell morphology, and so they may be conspecific. These fossil species share some fossil localities, but were previously differentiated by the angle of teleoconch spire whorls, spiral sculpture and the size and growth of axial ribs in some specimens (Ponder 1973). Both species, like *P. spatiosus*, were often naïvely placed within clusters separate from other *Penion*, but since individuals overlapped in morphospace with the distribution of extant *P. maximus* specimens, we will investigate their relationship to this living species further.

Discrimination of fossils classified as extant species

We next used a geometric morphometric approach to see if it could assist with the classification of fossils from two geological localities: 1) combined strata from Wanganui Beach, Manawatu-Wanganui including nearby locations of Okehu Stream, Waihi Stream and Nukumaru, and 2) Te Piki, Cape Runaway in the Bay of Plenty. These sites are of key interest because they are two of the most abundant sources of New Zealand *Penion* fossils, but the classification of the fossils has been contentious (pers. comm. Alan G. Beu, GNS Science 2016).

Wanganui localities with P. sulcatus and the P. ormesi species complex

At Wanganui Beach, most fossils have been identified as *P. sulcatus* (Lamarck, 1816) and *P. ormesi* (Powell, 1927) (Beu and Maxwell 1990). Many fossils were also formerly classified as *P. c. cuvierianus* (Powell, 1927) (Beu and Maxwell 1990). Following our previous phylogenetic results (Chapter 4), repeated analysis of traditional shell characters, and consideration of the modern extant range of the species (Figure 7.2), we treat these latter specimens as *P. c. jeakingsi* (Powell, 1947) fossils (see Chapter 8). In addition, some fossils from Wanganui have been identified as the extinct putative species *P. hiatulus* (Powell, 1947) (Beu and Maxwell 1990), which we test here. Using geometric morphometric analysis of shells, we want to identify fossils and determine whether distinct groupings exist to support the hypotheses of additional extant or extinct species.

We analysed the fossil samples using a dataset including modern shells of *P. sulcatus, P. ormesi* and *P. c. jeakingsi* from localities in the Cook Strait (south coast of North Island, north coast of South Island; Figure 7.2). We restricted the extant sampling to this range as the morphology of both species in distant regions (e.g. Northland) can differ and could confound the analysis. Since specimens sampled from Wanganui are between 0.97 and 0.424 Ma in age, it is most likely that they represent populations ancestral to modern populations within the same broad region.

Using 359 shells (323 extant, 36 fossil), four statistically significant PCs were identified (broken-stick test): PC1 (53.20%), PC2 (13.54%), PC3 (7.47%) and PC4 (5.46%). Without *a priori* classification of specimens, two clusters were resolved (based on BIC score using mclust; Supplementary Figure 7.6). The two clusters broadly corresponded to extant *P. sulcatus* and sampling from the *P. ormesi* species complex, with most fossils being placed within a cluster dominated by extant *P. sulcatus* (Figure

7.4a). All but two fossil shells were assigned with high confidence to a cluster closely corresponding to modern *P. sulcatus*, despite our sample including numerous specimens previously identified as *P. ormesi* and *P. c. jeakingsi* (Figure 7.4a). When centroid size was included, mclust identified a third cluster dominated by extant specimens of *P. ormesi* from Marlborough and *P. c. jeakingsi* sampled north of Manawatu-Wanganui, but fossil assignment was unaffected (Figure 7.4a). No additional clusters were identified to support the classification of any putative extinct species such as *P. hiatulus.*

Ordination of PCA scores shows that clusters identified naïvely by mclust closely matched the shape distribution of the extant *P. ormesi* complex and *P. sulcatus* (Figure 7.4b and 7.4c). Based on the distribution of specimens within morphospace and 95% mean confidence ellipses, most fossils, regardless of taxonomic classification, lay within the range of *P. sulcatus* rather than of the *P. ormesi* species complex (Figure 7.4c, Supplementary Figure 7.7). Overall these results therefore strongly indicate the majority of Wanganui fossils sampled in this study belong to *P. sulcatus*. Given the rarity of material supporting classification as *P. ormesi*, these results further suggest the possibility that only *P. sulcatus* is present at the Wanganui locality overall.

FIGURE 7.4a
Bayesian assignment probability of all sampled *Penion* fossils from Wanganui Beach and nearby geological localities, with all extant samples of *P. sulcatus, P. ormesi,* and *P. c. jeakingsi* from nearby geographic regions, to clusters estimated by the R model-based clustering package mclust 5.2. Fossils classified as *P. c. jeakingsi* are marked with an asterisk as these specimens could alternatively classified as the fossil species *P. hiatulus*. Specimens are coloured by assignment probability to clusters. Fossil specimens are organised based on putative classification to each of the extant species, and specimens overall are organised following the molecular phylogeny (see Chapter 4). Specimens are labelled based on putative taxonomic classification and sampling region or geological site. The VEE2 model (top) was the best supported model using only the statistically significant PCs 1 – 4, whereas the VEE3 model (bottom) received the highest BIC support when centroid size was also included. See Supplementary Figure 7.6 for a comparison for BIC values among clustering models.

FIGURE 7.4b
A principal component analysis ordination (PCs 1 and 2) of all sampled *Penion* fossils from Wanganui Beach and nearby geological localities, with all extant samples of *P. sulcatus, P. ormesi,* and *P. c. jeakingsi* from nearby geographic regions. Colouration corresponds to clusters identified naïvely by the R model-based clustering package mclust 5.2. Mean confidence ellipses (90%; same colouration) indicate that the group means are unlikely to overlap. The clusters shown were identified by the VEE2 model using all four statistically significant PCs.

FIGURE 7.4a

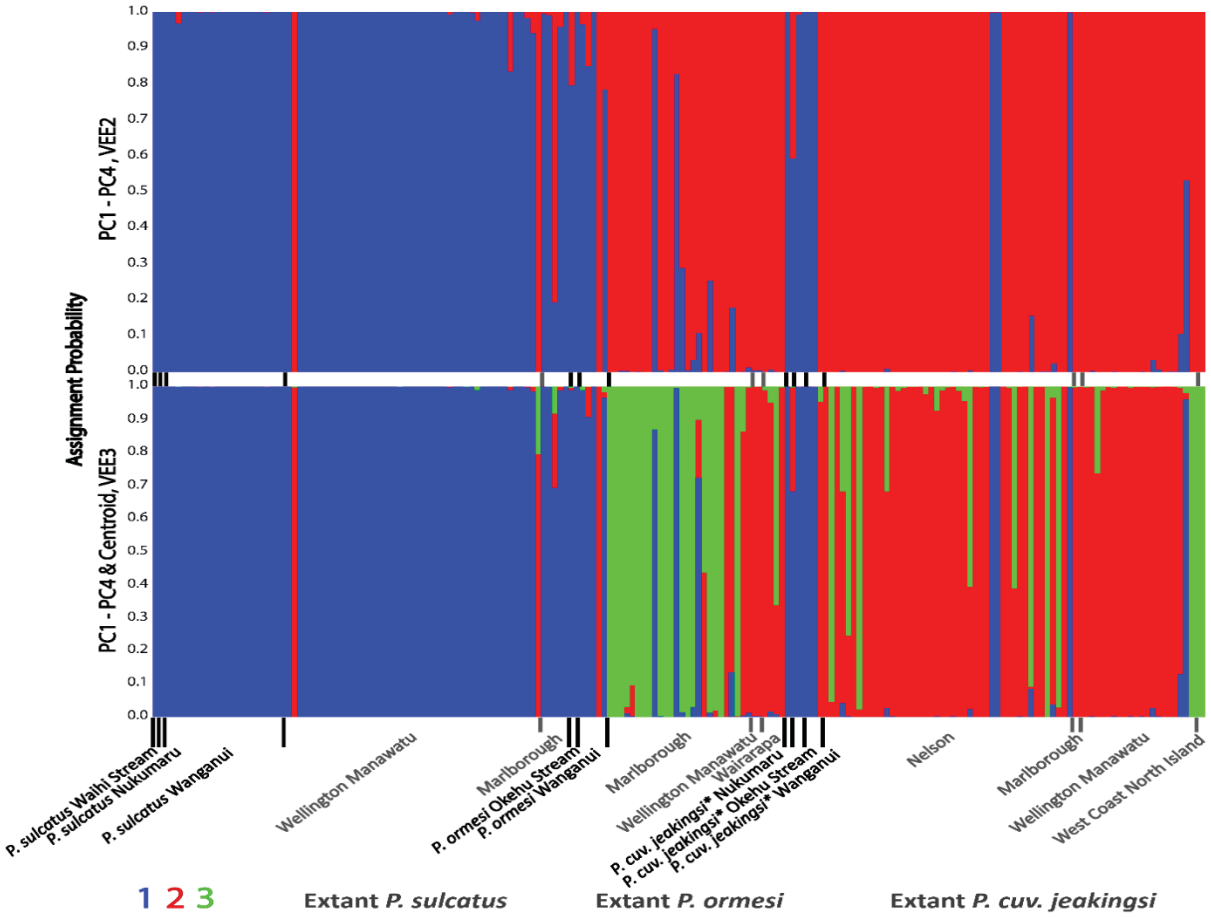


FIGURE 7.4b

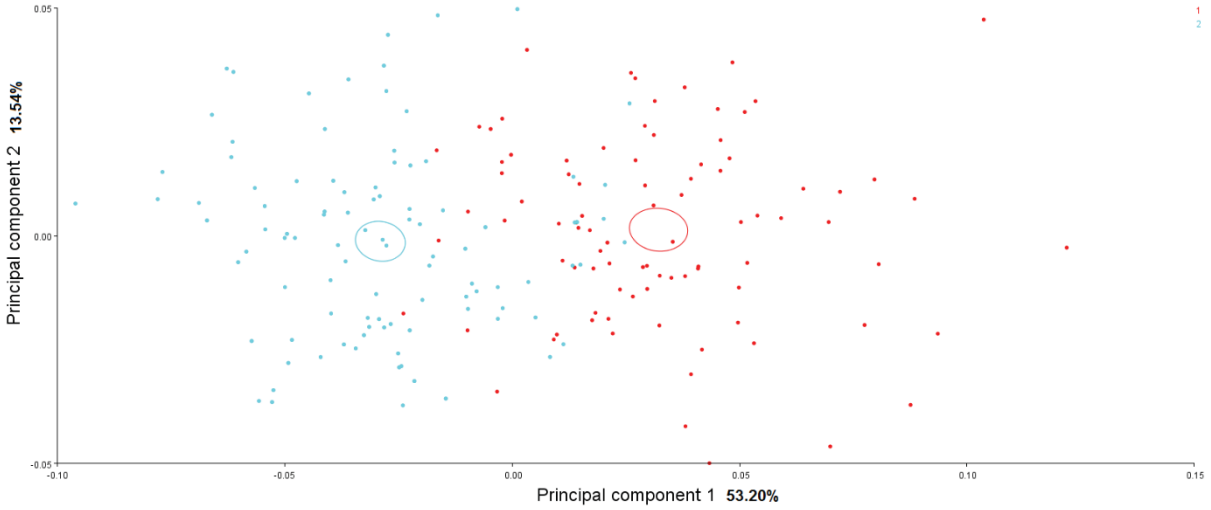
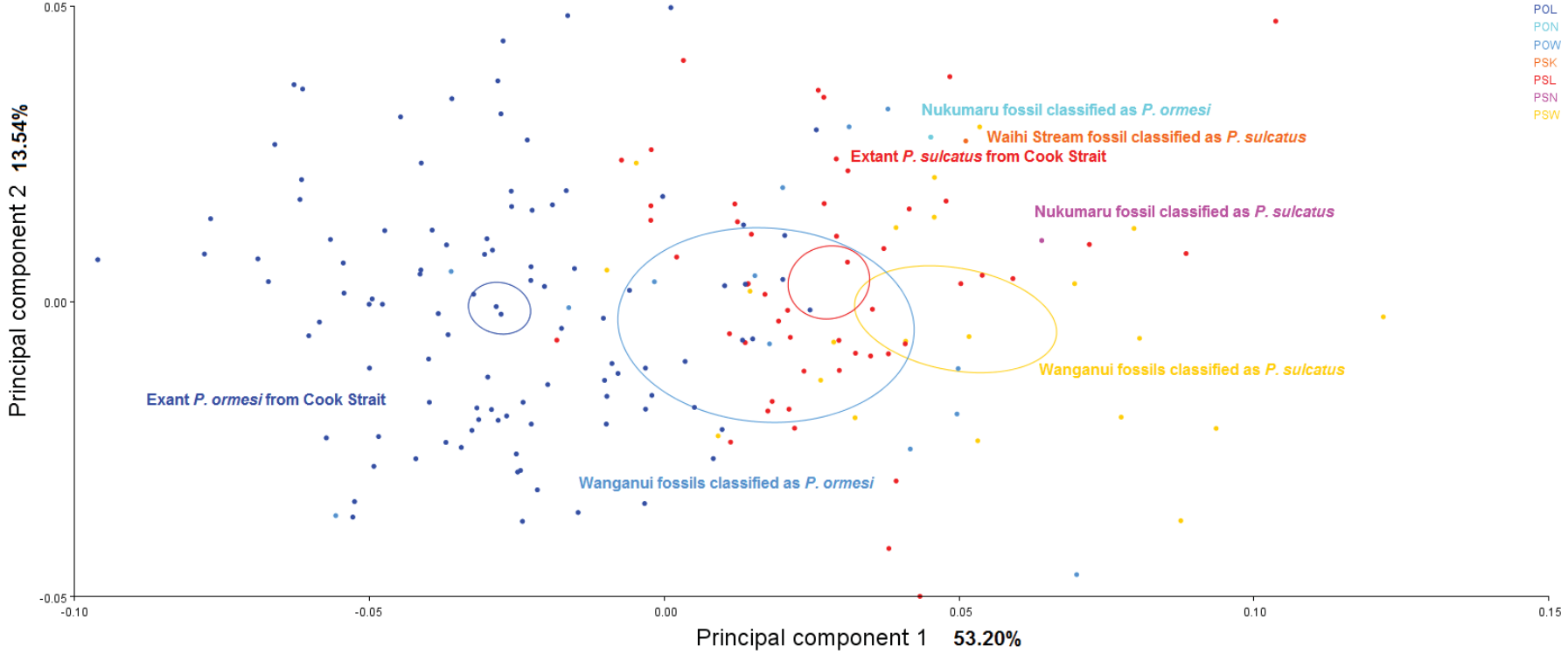


FIGURE 7.4c
A principal component analysis ordination (PCs 1 and 2) of all sampled *Penion* fossils from Wanganui Beach and nearby geological localities, with all extant samples of *P. sulcatus, P. ormesi,* and *P. c. jeakingsi* from nearby geographic regions. Fossils are coloured according to putative taxonomic classification of specimens from each geological locality, and extant shells are coloured according to putative taxonomic classification. Mean confidence ellipses (90%; same colouration) indicate that the group means of fossils from Wanganui regardless of classification are likely to overlap with modern *P. sulcatus* from nearby locations.



Te Piki shellbed with P. sulcatus and P. c. cuvierianus

At Te Piki, the shellbed is believed to contain specimens of both *P. c. cuvierianus* and *P. sulcatus*, which are sympatric in the area today (Figure 7.2). These two species are difficult to differentiate, to the point that molluscan taxonomists have hypothesised that the taxa are conspecific (Ponder 1975). We analysed the fossil samples from Te Piki using modern shells of *P. sulcatus* and *P. c. cuvierianus* from the eastern coast of Northland to Hawke's Bay. This region encompasses most of the distribution of *P. c. cuvierianus*, except populations in the far north of Northland, some of which are classified as *P.* aff. *c. cuvierianus* (Figure 7.2). These were excluded as the *P.* aff. *c. cuvierianus* morphotype would likely be a confounding source of variation (see results of Chapter 6), that could mislead analysis of the Te Piki fossils. Te Piki specimens are dated to the Nukumaruan Stage, 2.40 – 1.63 Ma, but modern populations present in the area are probably related.

Using 374 shells (338 extant, 36 fossil), three statistically significant PCs were identified (broken-stick test): PC1 (66.51%), PC2 (11.06%), and PC3 (6.30%). Without *a priori* classification of specimens, two clusters were identified using only the significant PCs, and with inclusion of centroid size models with four to five clusters were supported (based on BIC score using mclust; Supplementary Figure 7.8). The two clusters identified using shell shape variation corresponded with the taxonomic classification of extant *P. c. cuvierianus* and *P. sulcatus* (Figure 7.5a). For extant shells results were similar to previous analyses (see Chapters 5 and 6), with only 7 (3.50%) and 8 (5.79%) shells of *P. c. cuvierianus* and *P. sulcatus* respectively assigned to the alternative cluster. Under this regime, the majority of fossils, regardless of previous identification, were assigned to the cluster dominated by *P. sulcatus* (Figure 7.5a). The four clusters identified with the inclusion of centroid size appeared to be hierarchically nested within each of the former clusters (Figure 7.5a), effectively resulting in both species being split into two groups.

The two clusters identified by mclust (using the three statistically significant PCs) showed close correspondence to the taxonomic classification of extant specimens (Figures 7.5b and 7.5c, Supplementary Figure 7.9). When the four clusters identified with the inclusion of centroid size were mapped onto specimens, it seemed that PC2 causes both species to be subdivided at similar values of PC2 (Supplementary Figure 7.10). Whether fossils were classified by putative species or as a single geological site, the majority of individuals occurred within the shape range of both species but the 90%

mean confidence ellipses was closer to the mean of extant *P. sulcatus* (Figure 7.5c, Supplementary Figure 7.9). The mean confidence ellipse of the Te Piki fossils overall was also intermediate to the two *P. sulcatus* dominated clusters identified by mclust using PCs and centroid size.

Based on these results, we infer that the majority of *Penion* fossils sampled from Te Piki belong to *P. sulcatus*. However, unlike the Wanganui fossils, many exceptions indicate that historical sympatry at Te Piki. It also seems that there is a sampling bias for the Te Piki material, as fossils classified as *P. c. cuvierianus* are more often broken and too poorly preserved for digitisation. This is probably because shells recognised as *P. c. cuvierianus* are typically larger with thinner shell walls than specimens of *P. sulcatus* within the same region. The results of this investigation demonstrate however that geometric morphometrics can be used to aid with the uncertain identification of specimens.

FIGURE 7.5a
Bayesian assignment probability of all sampled *Penion* fossils from Te Piki geological locality, and all extant samples of *P. sulcatus* and *P. c. cuvierianus* from nearby geographic regions, to clusters estimated by the R model-based clustering package mclust 5.2. Specimens are coloured by assignment probability to clusters. Fossil specimens are organised based on putative classification to each of the extant species, and specimens overall are organised following the molecular phylogeny (see Chapter 4). Specimens are labelled based on putative taxonomic classification and sampling region or geological site. The EEI2 model (top) was the best supported model using only the statistically significant PCs 1 – 3, whereas the EEE4 model (bottom) received the highest BIC support when centroid size was also included. See Supplementary Figure 7.8 for a comparison for BIC values among clustering models.

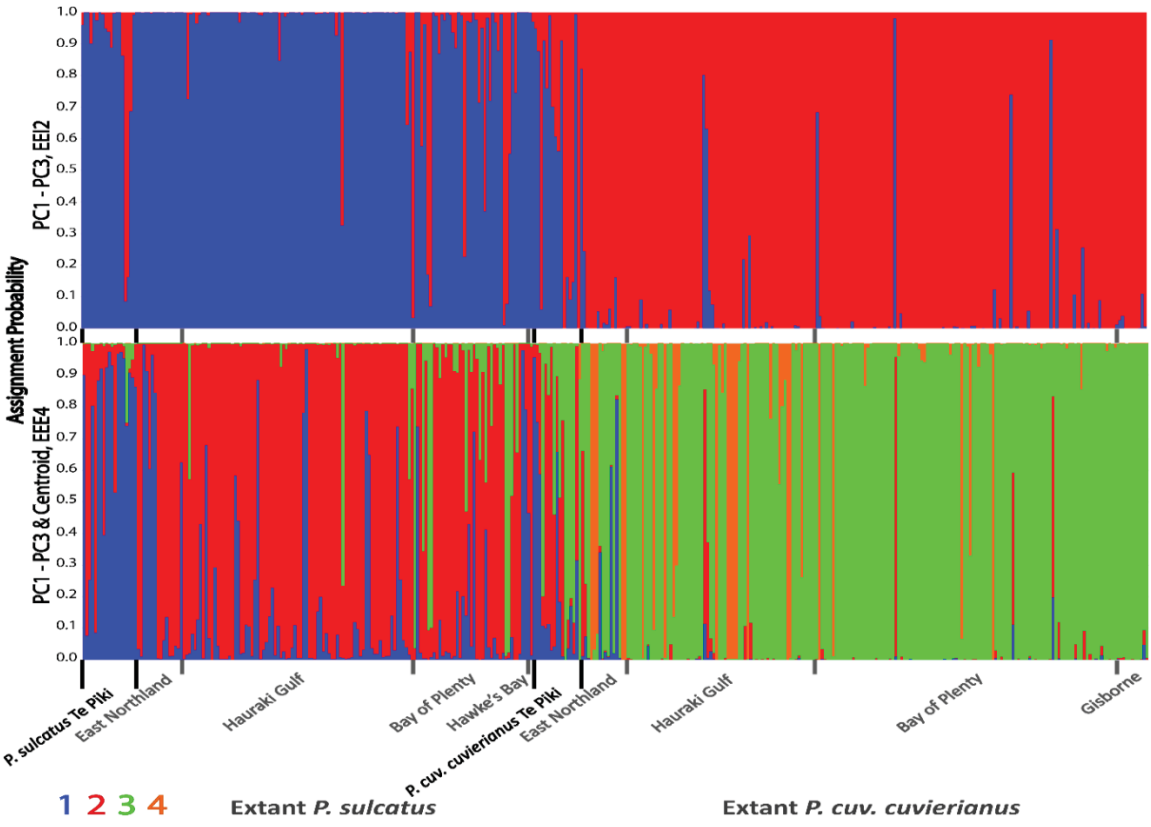


FIGURE 7.5b
A principal component analysis ordination (PCs 1 and 2) of all sampled *Penion* fossils from Te Piki geological locality, and all extant sampling from *P. sulcatus* and *P. c. cuvierianus* from nearby geographic regions. Colouration corresponds to clusters identified naïvely by the R model-based clustering package mclust 5.2. Mean confidence ellipses (90%; same colouration) indicate that the group means are unlikely to overlap. The clusters shown were identified by the EEI2 model using only the statistically significant PCs 1 – 3.

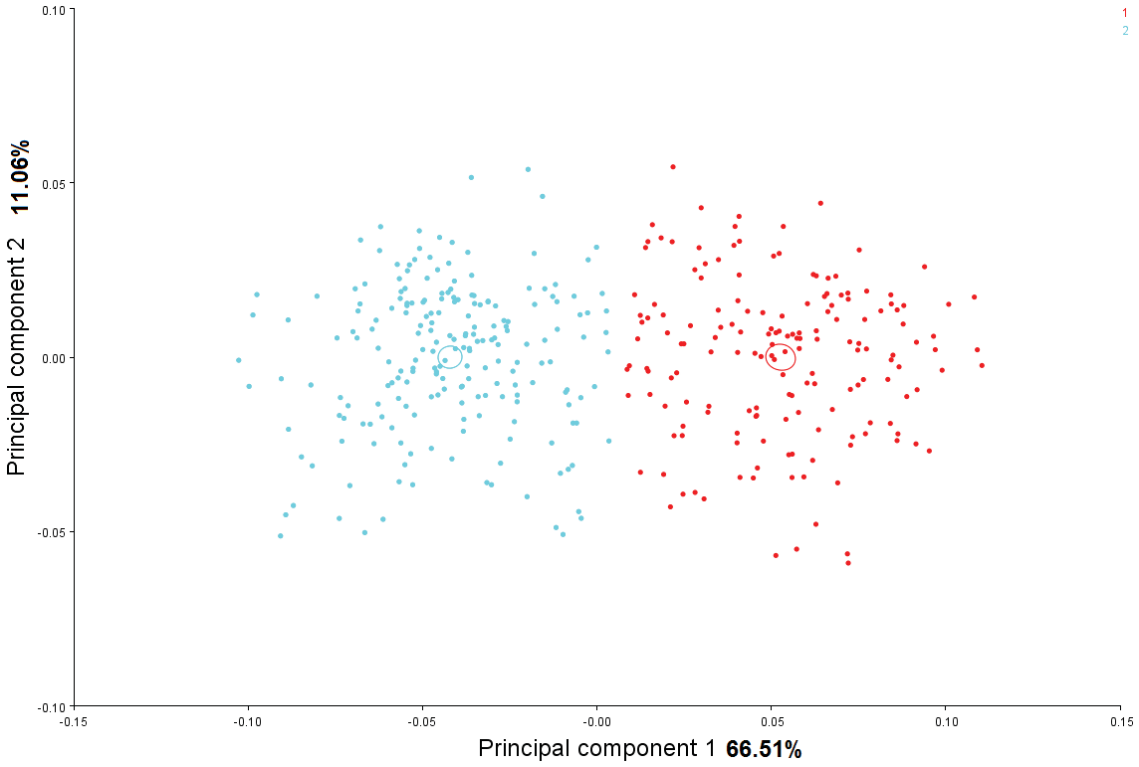
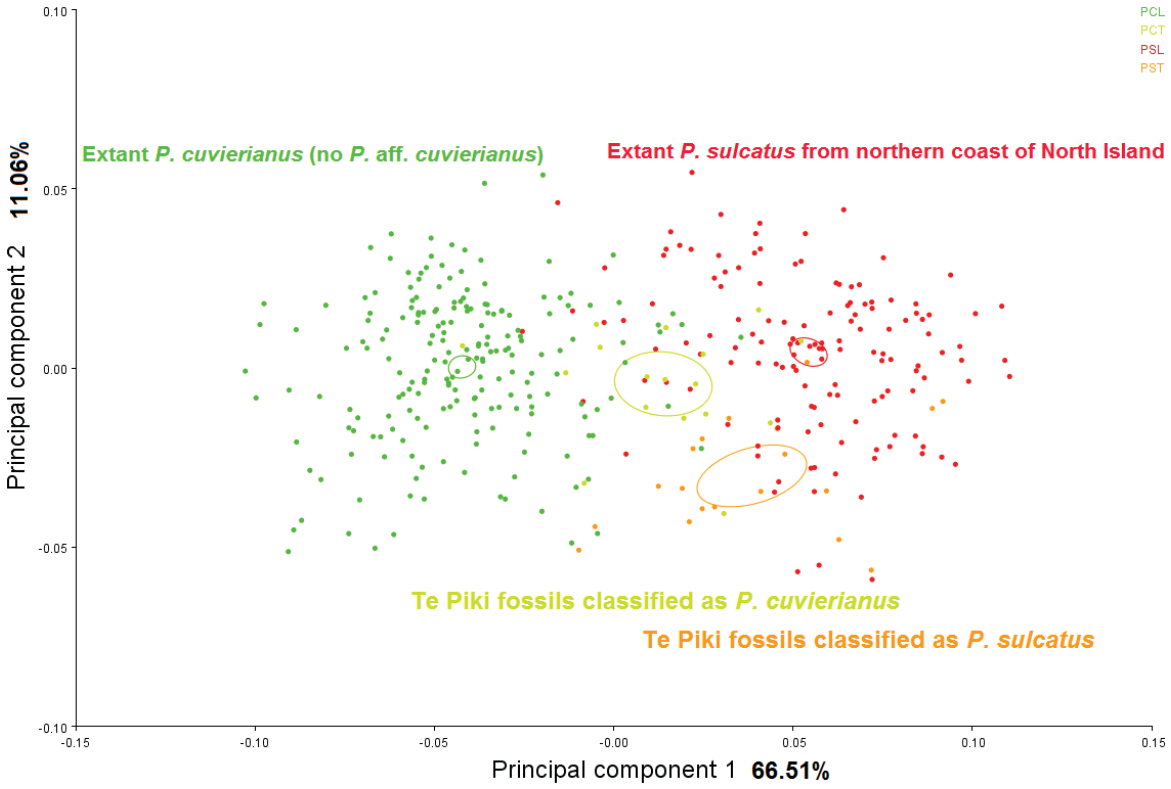


FIGURE 7.5c
A principal component analysis ordination (PCs 1 and 2) of all sampled *Penion* fossils from Te Piki geological locality, and all extant sampling from *P. sulcatus* and *P. c. cuvierianus* from nearby geographic regions. Fossils are coloured according to putative taxonomic classification of specimens from each geological locality, and extant shells are coloured according to putative taxonomic classification. Mean confidence ellipses (90%; same colouration) indicate that none of the group means are likely to overlap.



Identification of possible evolutionary lineages and morphological change through time

Lastly, we investigated whether geometric morphometric evidence could yield insight towards possible evolutionary relationships between extant and fossil taxa – making the assumption that similarity in morphology reflects underlying phylogenetic similarity.

Penion maximus, Penion longirostris, Penion roblini

Previous taxonomic reviews noted that shells of *P. roblini* and *P. maximus* are similar, and *P. longirostris* and *P. roblini* have each been compared to *P. c. cuvierianus* (Ponder 1973), which can be challenging to differentiate from *P. maximus* in the absence of geographic data (see Chapter 6). We found that *P. longirostris* and *P. roblini* were similar in shell shape and size, and that they occurred mostly outside of the morphological range occupied by other species of *Penion* (Figure 7.3b, Supplementary Figures 7.4 and 7.5). However, the morphometric distribution of both of these taxa overlapped with the distribution of *P. maximus*. Small or juvenile specimens of *P. maximus* resemble the fossil species and it is notable all three species are from Australia.

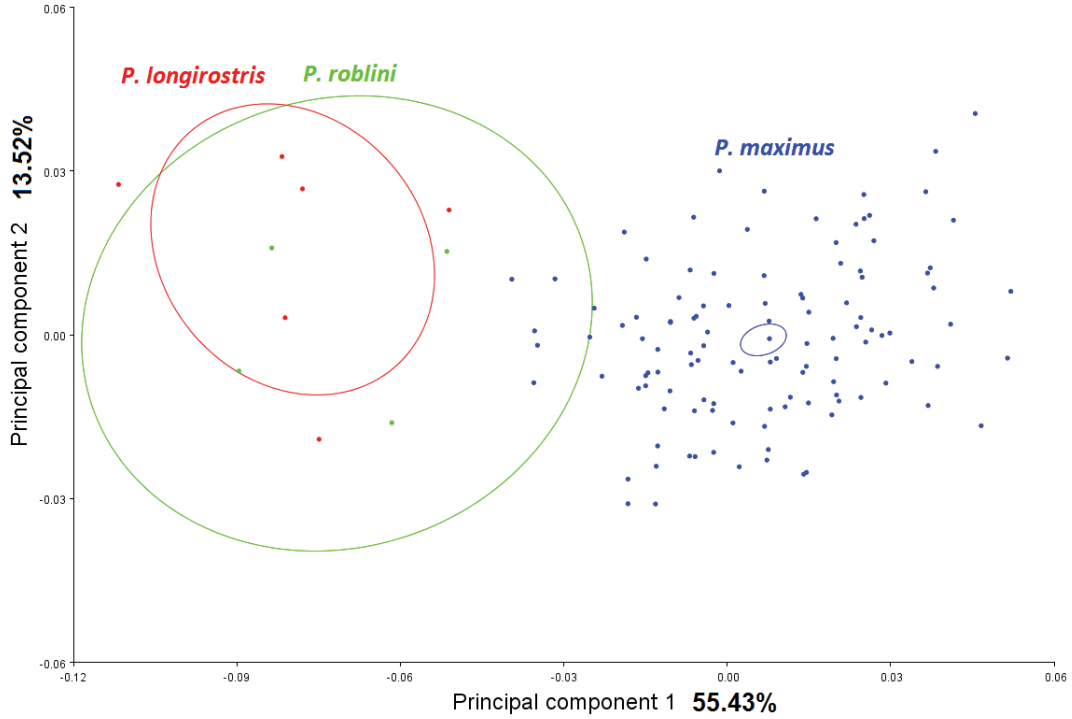
Using a dataset containing all modern samples of *P. maximus*, and specimens of *P. longirostris* and *P. roblini*, three statistically significant PCs were identified (broken-stick test): PC1 (55.43%), PC2 (13.52%), and PC3 (8.10%). Two clusters were identified without *a priori* classification of shells, with and without the inclusion of centroid size (based on BIC score using mclust; Supplementary Figure 7.11). The clusters identified by the best supported models (with and without centroid size) matched the complete separation of *P. maximus* from *P. longirostris* and *P. roblini*, with high confidence (Figure 7.6a). In contrast, *P. longirostris* and *P. roblini* could not be distinguished from each other (Figure 7.6a). Using PCA ordination, with specimens classified as taxonomic species, it was obvious that when separately compared, *P. maximus* does not overlap in morphospace with *P. longirostris* or *P. roblini*, although again the two latter species could not be distinguished based by 90% mean confidence ellipses (Figure 7.6b).

It is therefore unlikely that the two fossil species are closely related to *P. maximus*. *Penion longirostris* and *P. roblini* may be conspecific as they share geological localities, and the traits used to differentiate them focus on subtle features such as teleoconch whorl angles and shell sculpture (Ponder 1973), which are known to be highly variable features within most species of siphon whelk (Powell 1979).

Furthermore, in combination with the earlier analyses including all *Penion* and the closely related genera (Figure 7.3b, Supplementary Figures 7.4 and 7.5), it is probable that *P. longirostris* and *P. roblini* do not belong in *Penion* as they occur outside the range of morphospace occupied by the rest of the clade (extinct and extant), akin to *P. spatiosus*.

FIGURE 7.6a
Bayesian assignment probability of all sampled specimens classified as *P. maximus* (extant only), *P. roblini* and *P. longirostris*, to clusters estimated by the R model-based clustering package mclust 5.2. Specimens are coloured by assignment probability to clusters. Specimens are ordered by age and are labelled based on putative taxonomic classification and sampling region or geological site. We only show the EEE2 model, which was the best supported model using the statistically significant PC 1 – 3 with and without centroid size. The plot for the EEE2 model using centroid size as well as PC1 – 3 is not shown as it is identical. See Supplementary Figure 7.11 for a comparison for BIC values among clustering models.

FIGURE 7.6b
A principal component analysis ordination (PCs 1 and 2) of all sampled specimens classified as *P. maximus* (extant only), *P. roblini* and *P. longirostris*. Specimens are coloured according to putative taxonomic classification. Mean confidence ellipses (90%; same colouration) indicate that the group means of *P. roblini and P. longirostris* are likely to overlap with one another, but that the mean of *P. maximus* can be readily distinguished.



Penion sulcatus, Penion clifdenensis, Penion marwicki, Penion exoptatus

Penion sulcatus has traditionally been hypothesised as being related to the extinct fossil taxa including *P. clifdenensis*, *P. marwicki* and *P. exoptatus* (Powell 1947, Beu and Maxwell 1990). All of these fossil species exhibit shell sizes within the range of modern *P. sulcatus*, and like *P. sulcatus* they often have prominent axial ribs and short siphonal canals compared to other New Zealand species. *Penion marwicki* appears to be morphologically distinct, but the species overlapped in morphospace with extant specimens of *P. sulcatus* (Figures 7.3a and 7.3b, Supplementary Table 7.1). In contrast, *P. clifdenensis* and *P. exoptatus* appeared to be similar in shell shape and size to most species of New Zealand siphon whelks (Figures 7.3a and 7.3b). To determine whether these four taxa could represent a single evolutionary lineage we used all extant sampling from *P. sulcatus*, and fossil specimens from Te Piki and Wanganui (incorporating findings reported above, including fossils from Wanganui and Te Piki newly identified as *P. sulcatus*).

Three statistically significant PCs were identified (broken-stick test): PC1 (34.11%), PC2 (21.79%), and PC3 (14.08%). Two clusters were resolved when the significant PCs were analysed, with and without the inclusion of centroid size (based on BIC score using mclust; Supplementary Figure 7.12). With shape variation alone, one cluster contained the majority of *P. sulcatus*, all sampled specimens of *P. exoptatus* and *P. clifdenensis*, and some specimens of *P. marwicki* whereas the second cluster contained 75% of *P. marwicki* fossils with some extant *P. sulcatus* (Figure 7.7a). When centroid size was included, *P. exoptatus* and *P. clifdenensis* remained within a cluster dominated by *P. sulcatus*, but the discrimination of *P. sulcatus* and *P. marwicki* was less pronounced with many more individuals of *P. sulcatus* being assigned to a cluster containing almost all *P. marwicki* (Figure 7.7a).

Bayesian assignment and traditional taxonomic classification of species using PCA ordination (Figures 7.7b and 7.7c), show that *P. clifdenensis* and *P. exoptatus* are contained in the same clusters as *P. sulcatus*. The second cluster identified does contain most *P. marwicki*, but the cluster overlaps with the distribution of specimens classified as *P. sulcatus* (Figures 7.7b and 7.7c). Given these results, it is likely that *P. sulcatus* is related to *P. clifdenensis* and *P. exoptatus*, or that all three species are conspecific despite occurring over a 20 million year temporal range (which is compatible with the dated phylogeny estimated in Chapter 3). The situation with *P. marwicki* is less clear: naïve assignment using mclust does not distinguish *P. marwicki* and *P. sulcatus* (as

shell size is unhelpful, Figures 7.7b and 7.7c), but when identified by traditional taxonomic characters the specimens of both species can be readily distinguished (Supplementary Table 7.1). This result is less clear than the comparison of *P. maximus* and fossil taxa (Supplementary Figures 7.6a and 7.6b). The ability to separate these taxa is reminiscent of previous, similar results when attempting to distinguish closely related living species such as *P. c. jeakingsi* and *P. ormesi* (Chapter 6). We infer that two evolutionary hypotheses are possible, 1) an evolutionary lineage containing all four species, or 2) a lineage containing all species except *P. marwicki*.

FIGURE 7.7a
Bayesian assignment probability of all specimens classified as *P. sulcatus* (fossil and extant), *P. clifdenensis*, *P. marwicki*, and *P. exoptatus*, to clusters estimated by the R model-based clustering package mclust 5.2. Specimens are coloured by assignment probability to clusters. Specimens are ordered by age and are labelled based on putative taxonomic classification and sampling region or geological site. The EEE2 model (top) was the best supported model using only the statistically significant PCs 1 – 3, whereas the VVE2 model (bottom) received the highest BIC support when centroid size was also included. See Supplementary Figure 7.12 for a comparison for BIC values among clustering models.

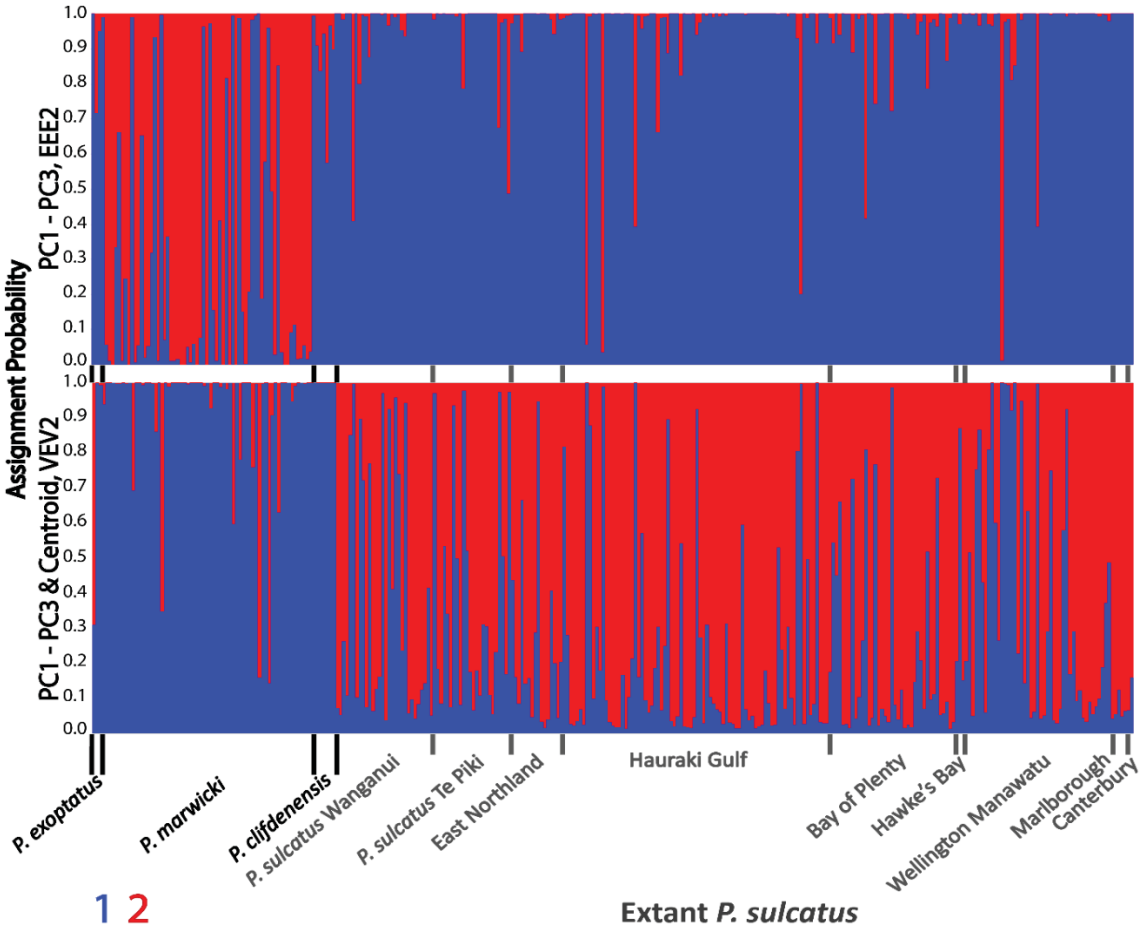


FIGURE 7.7b
A principal component analysis ordination (PCs 1 and 2) of all specimens classified as *P. sulcatus* (fossil and extant), *P. clifdenensis*, *P. marwicki*, and *P. exoptatus*. Colouration corresponds to clusters identified naïvely by the R model-based clustering package mclust 5.2. Mean confidence ellipses (90%; same colouration) indicate that the group means are unlikely to overlap. The clusters shown were identified by the VVE2 model using the statistically significant PCs 1 – 3 and centroid size.

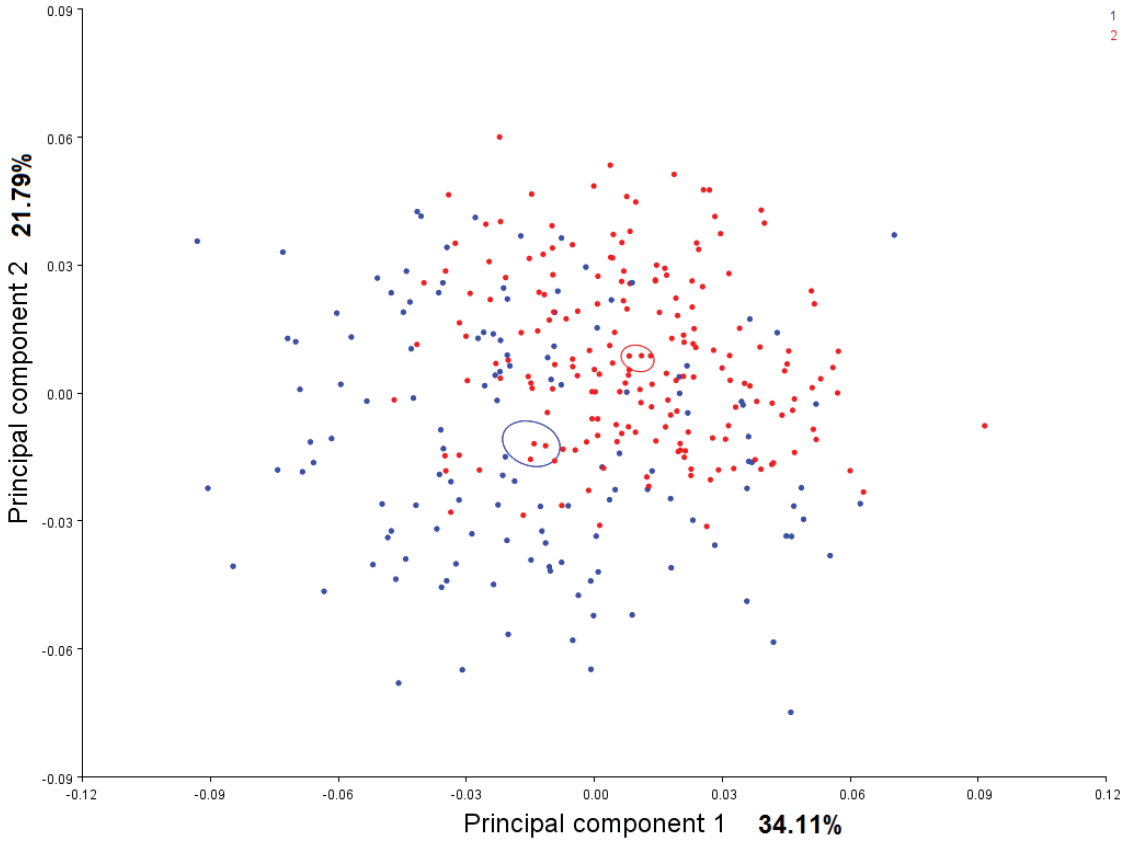
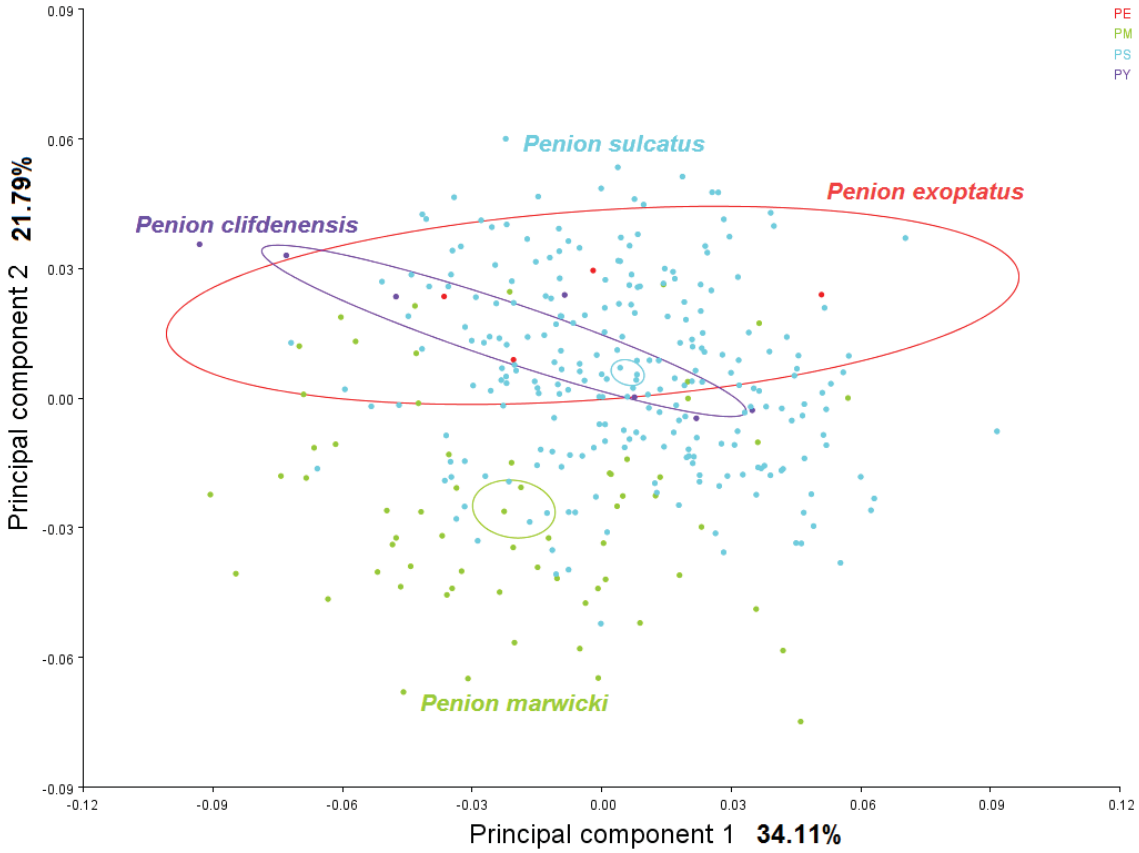


FIGURE 7.7c
A principal component analysis ordination (PCs 1 and 2) of all specimens classified as *P. sulcatus* (fossil and extant), *P. clifdenensis*, *P. marwicki*, and *P. exoptatus*. Specimens are coloured according to putative taxonomic classification. Mean confidence ellipses (90%; same colouration) indicate that the group means of *P. marwicki* and *P. sulcatus* are unlikely to overlap, however the group means of *P. clifdenensis* and *P. exopatus* (both with lower sampling) are likely to overlap with that of *P. sulcatus*.



We used the above data (lineage 1 containing all four species) to investigate how shell morphology has changed through time. Specimens were organised by geological locality, and because of small sample sizes from individual horizons, specimens from Wanganui were organised into composite time bins combining multiple adjacent strata. Based on marine oxygen isotope stage determinations for the Wanganui locality (Beu 2006, Beu 2011), the first time bin (20 fossils) spans 0.424 – 0.621 Ma (containing specimens from Shakespeare Cliff Sand, Pinnacle Sand, the Tainui Shellbed, and upper and lower Castlecliff Sand), and the second bin (11 fossils) specimens covers 0.712 – 0.970 Ma (containing specimens from Okehu Shell Grit, Kaimatira Pumice sand, Omapu shellbed, and the Kupe Formation; Fleming 1947, Fleming 1953, Naish *et al.* 2005).

The three statistically significant PCs and centroid size for all specimens were analysed using paleoTS. Based on AICc scores, all four traits were indicated to have undergone an unbiased random walk (URW) through evolutionary time (Figure 7.8a; Table 7.2a), suggesting that change in these variables over generations has been stochastic. Data for PC2 fitted models of stasis and URW almost equally well (Table 7.2a). For the second dataset including only sampling of *P. sulcatus*, *P. clifdenensis* and *P. exoptatus*, four PCs were identified as statistically significant (broken-stick test): PC1 (35.18%), PC2 (21.66%), PC3 (10.84%), and PC4 (5.74%). These significant PCs and centroid size for all specimens were analysed using paleoTS. Based on AICc scores, PC1, PC3 – PC4 and centroid size supported an URW as the best fitting model of evolutionary change (Figure 7.8b; Table 7.2b). Evolutionary stasis was best supported for PC2 however (Figure 7.8b; Table 7.2b), indicating that aspects of shell shape captured by this variable have not changed significantly with minimal, constrained drift for over 20 million years from *P. exoptatus* to modern populations of *P. sulcatus* (Figure 7.8b).

The actual shell shape variation captured by a PC can be difficult to identify. Although PCs are biologically relevant, a single PC can represent a complex combination of different aspects of shape variation – rather than a simple feature that is obvious from the examination of an object. However, PC2 for the second dataset primarily represents variation in the height of the teleoconch spire, as well as differences in the shape of the top of the aperture (Figure 7.8c). PC2 for the first dataset including *P. marwicki*, which was almost supported for evolutionary stasis as well, appeared to represent variation in the same traits. These seem like reasonable traits to

been subject to evolutionary stasis, as all four putative species considered are similar for these traits (Powell 1947, Beu and Maxwell 1990).

When viewed as a PCA ordination, the 90% mean confidence ellipses of modern populations and geological sites provide a potential map of shifting of shell shape through time. Using PC1 and PC2 from the second dataset (56.85% of variation), it was apparent that morphospace occupied by shells most localities overlap with one another (Figure 7.8d). All fossil sites exhibit overlapping shape distributions, despite the significant time difference between sets of samples (Figure 7.8d). In addition, most modern populations from the Cook Strait are centred on the same region of morphospace, but much of the modern sampling from locations on the north coast of the North Island – particularly from the Bay of Plenty, occurs within a range of morphospace for PC2 hardly occupied by fossil specimens (Figure 7.8d). This suggests that the lineage has undergone an expansion of morphological diversity within recent time, focussed within that geographic region.

FIGURE 7.8a
A diagram produced using paleoTS 0.4.4 (Hunt 2006, Hunt 2007), which plots change in a trait mean through time – in this case the statistically significant PCs 1 – 3 and centroid size – for sampling from *P. sulcatus*, *P. clifdenensis*, *P. marwicki* and *P. exoptatus*. An unbiased random-walk was the best-fitting model of evolutionary change for variation in each trait through time. Support for models is determined using the Aikaike information criterion with correction for finite sample sizes (AICc), utilising Aikaike weights.

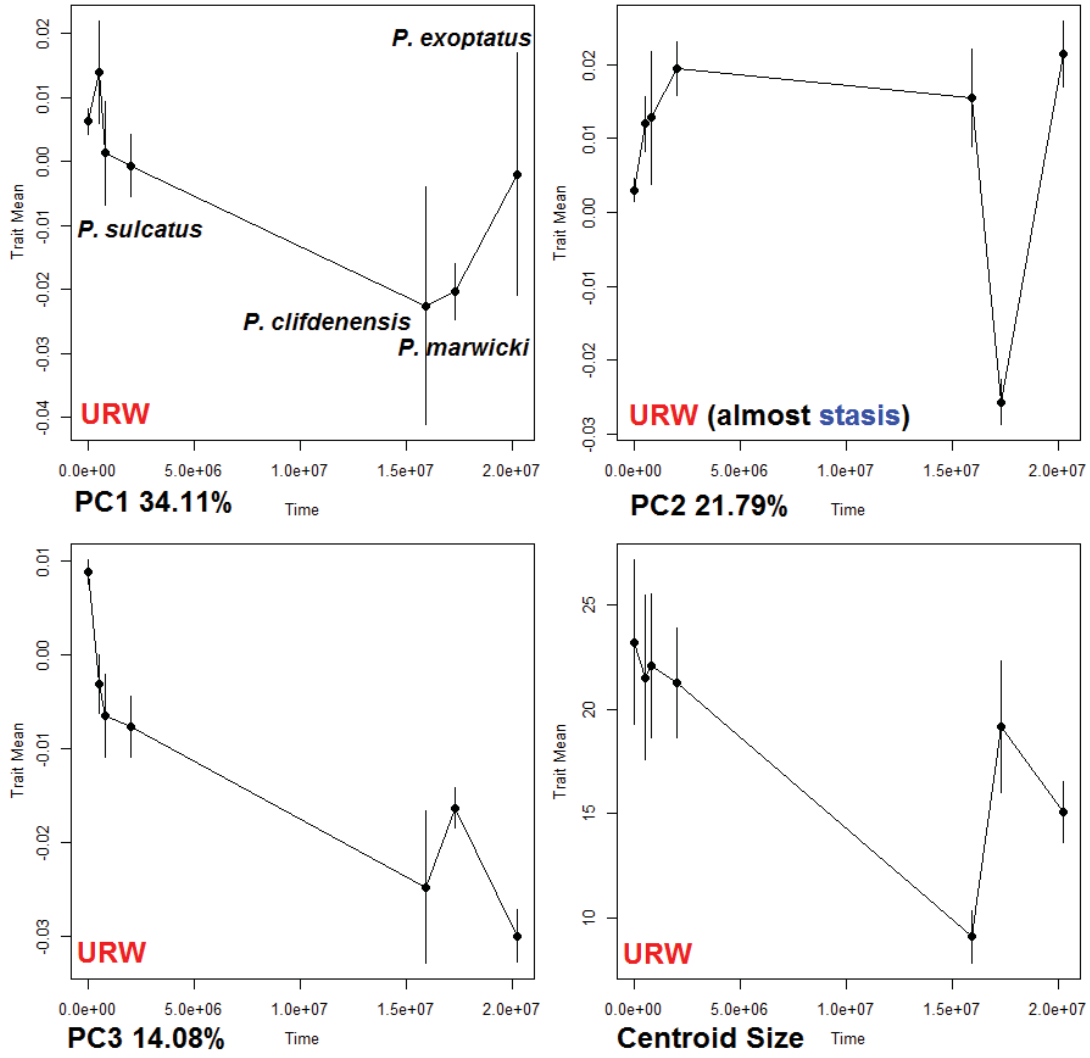


FIGURE 7.8b

A diagram produced using paleoTS 0.4.4 (Hunt 2006, Hunt 2007), which plots change in a trait mean through time – in this case the statistically significant PCs 1 – 4 and centroid size for sampling from *P. sulcatus, P. clifdenensi,* and *P. exoptatus*. An unbiased random-walk was the best-fitting model of evolutionary change for variation in PC1, PC3 and PC4 and centroid size through time. However, PC2 was fitted best by a model of evolutionary stasis. Support for models is determined using the Aikaike information criterion with correction for finite sample sizes (AICc), utilising Aikaike weights.

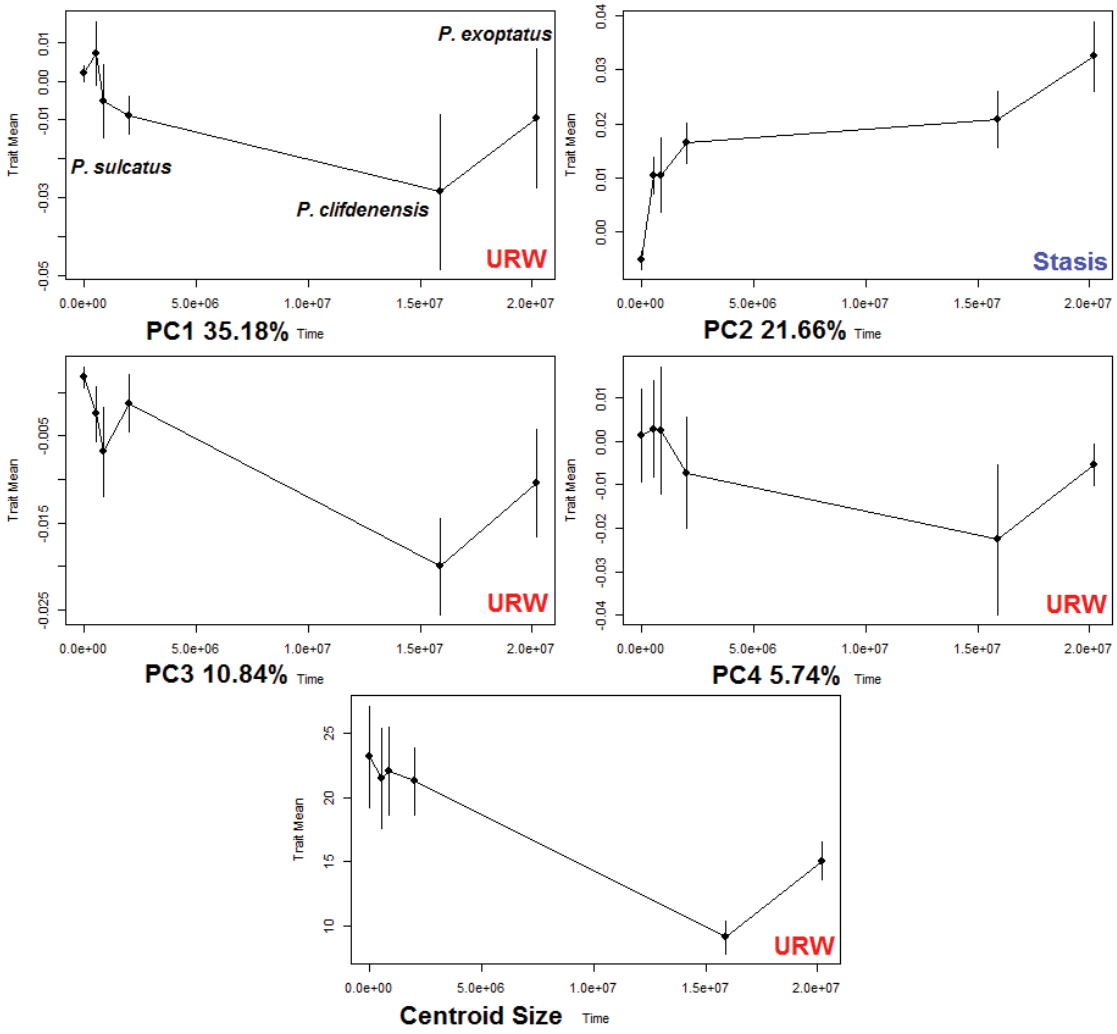


FIGURE 7.8c
Thin plate spline (TPS) diagrams with a transformation grid, showing the shape differences represented by PC1 – PC3 for the PCA of sampling from *P. sulcatus, P. clifdenensis* and *P. exoptatus*. PC1 and PC3 were indicated to have undergone an unbiased random-walk through evolutionary time via analysis using paleoTS 0.4.4 (Hunt 2006, Hunt 2007), but PC2 was estimated to have undergone evolutionary stasis. The shape variation represented by each axis is not as immediately obvious as in previous studies (see Chapter 6), but PC2 appears to primarily represent variation in the height of the teleoconch spire, as well as differences in the shape of the top of the aperture.

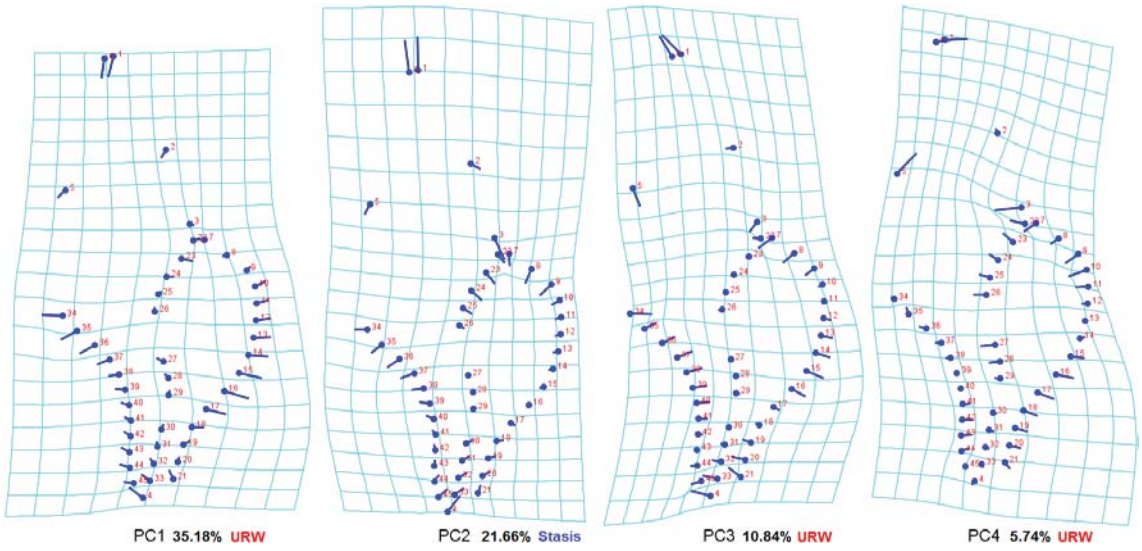


FIGURE 7.8d
A principal component analysis ordination (PCs 1 and 2) of all sampled specimens of *P. sulcatus* (fossil and extant), *P. clifdenensis* and *P. exoptatus*. Specimens are classified by geological locality (fossils) or geographic region (extant shells). Mean confidence ellipses (90%; same colouration) indicate that the group means of most sites are likely to overlap with at least one another location, although the mean of modern specimens from the Bay of Plenty is unlikely to overlap with any other group.

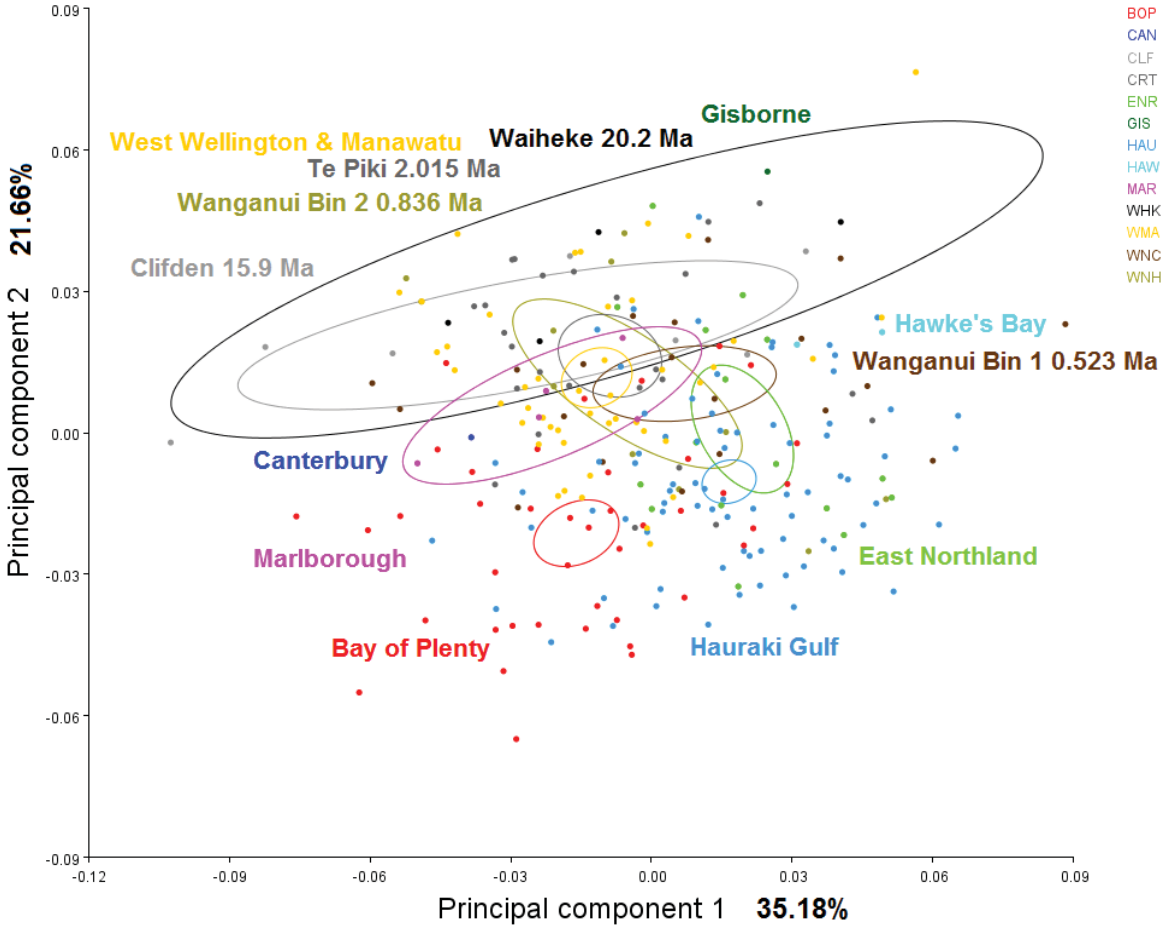


TABLE 7.2a
Support values (log likelihood, AICc and Akaike weight) for *Penion* shell morphological data fitted to different models of evolutionary change using PaleoTS 0.4.4. The data interpreted are the statistically significant PCs 1 – 3 and centroid size for the dataset including *P. sulcatus, P. clifdenensis, P. marwicki* and *P. exoptatus.* The model with the highest AICc support is marked with an asterisk for each trait.

	Model	logL	K	AICc	Akaike weight	
PC1 (34.11%)	GRW	18.96685	2	-29.93371	0.137	*
	URW	18.27712	1	-33.55425	0.837	
	Stasis	17.30628	2	-26.61256	0.026	
PC2 (21.79%)	GRW	13.6478	2	-19.2956	0.042	*
	URW	13.63373	1	-24.26747	0.504	
	Stasis	16.02981	2	-24.05963	0.454	
PC3 (14.08%)	GRW	19.25365	2	-30.5073	0.111	*
	URW	18.61463	1	-34.22925	0.715	
	Stasis	19.69785	2	-31.3957	0.173	
Centroid Size	GRW	-17.85106	2	43.70212	0.148	*
	URW	-18.81918	1	40.63835	0.685	
	Stasis	-17.72758	2	43.45517	0.167	

TABLE 7.2b
Support values (log likelihood, AICc and Akaike weight) for *Penion* shell morphological data fitted to different models of evolutionary change using PaleoTS 0.4.4. The data interpreted are the statistically significant PCs 1 – 3 and centroid size for the dataset including *P. sulcatus, P. clifdenensis* and *P. exoptatus.* The model with the highest AICc support is marked with an asterisk for each trait.

	Model	logL	K	AICc	Akaike weight	
PC1 (35.18%)	GRW	15.96398	2	-21.92796	0.064	
	URW	15.28431	1	-27.2353	0.906	*
	Stasis	15.22818	2	-20.45636	0.031	
PC2 (21.66%)	GRW	12.92622	2	-15.87245	0.003	
	URW	14.08669	1	-24.84005	0.302	
	Stasis	18.25243	2	-26.50486	0.694	*
PC3 (10.84%)	GRW	18.3085	2	-26.617	0.081	
	URW	17.32955	1	-31.32576	0.858	*
	Stasis	18.00701	2	-26.01403	0.060	
PC4 (5.74%)	GRW	-0.071022	2	10.14204	0.000	
	URW	14.576102	1	-25.81887	0.878	*
	Stasis	15.938338	2	-21.87668	0.122	
Centroid Size	GRW	-14.19752	2	38.39504	0.052	
	URW	-14.64974	1	32.63281	0.929	*
	Stasis	-15.19882	2	40.39763	0.019	

Conclusion

The results from this study suggest that geometric morphometric methods can yield new insight about even thoroughly collected and well-documented fossil records such as that of *Penion.* Geometric morphometric analysis of shells indicated that *P. longirostris, P. roblini* and *P. spatiosus* are readily distinguishable from living species, and that these fossil taxa are likely misclassified as they exhibit morphological differences beyond the range occupied by the related genera *Aeneator, Antarctonetpunea, Kelletia* and *Penion*. The second finding has profound impact upon the Australian fossil record of *Penion*, as the earliest possible occurrence of the genus from that region changes from 27.5 (*P. roblini*) to 4.3 Ma (*P. mandarinus*; Ponder 1973). This date suggests a relatively recent dispersal event of *Penion*, likely from the Zealandian continental shelf to Australia. Intriguingly, the earliest fossil occurrences of *P. mandarinus* and *P. maximus* are closer to the divergence date of 7.1 Ma estimated from genetic data with independent fossil calibrations (median 7.25 Ma; 95% HPD 9.86 – 5.05 Ma; Chapter 3). This overlap indicates that molecular data may support the exclusion of *P. longirostris, P. roblini* and *P. spatiosus* as relatives of the extant Australian fauna.

Geometric morphometric analysis of shells also assisted with the identification of specimens and interpretation of the fossil record of extant species. Almost all sampled fossils from Wanganui and adjacent geological localities, bear stronger resemblance to shells of *P. sulcatus*, rather than *P. ormesi, P. c. jeakingsi*, or extinct putative species such as *P. hiatulus*. This result is quite remarkable as the fossil record of *Penion* at these sites has been well collected (e.g. Powell 1947), indicating the benefit geometric morphometric analyses. Results of morphometric analysis suggested that many Te Piki fossils of *P. c. cuvierianus* to *P. sulcatus* should be reclassified, even though these specimens are challenging for molluscan taxonomists to separate confidently.

Morphometric variation in shell shape and size among extant and fossil specimens recognised as *P. sulcatus, P. clifdenensis, P. marwicki* and *P. exoptatus* indicated that two evolutionary lineages were possible (all species listed, or all species except *P. marwicki*), as naïve and *a priori* tests struggled to differentiate specimens that are classified as multiple species temporally distributed over 21.7 Ma. A molecular phylogeny of *Penion* using independent fossil calibrations estimated the median divergence date of *P. sulcatus* from a common ancestor with *Penion chathamensis* and

P. c. cuvierianus to be 40.62 Ma (95% HPD 50.04 – 32.53 Ma; Chapter 3), meaning that the fossil species considered occur within the expected time range of the lineage. This result emphasises that species are arbitrary segments of an evolutionary lineage, especially when the delimitation of a taxon is considered through time (Chapter 1: Vaux *et al.* 2016).

Exclusion of *P. marwicki* had a significant impact as the fit of each lineage to models of evolutionary change produced different results. With all four species treated as a single evolutionary lineage, variation in centroid size and all four aspects of shell shape variation fitted a random walk model (URW) through time. However, with the exclusion of *P. marwicki*, variation in PC2 fitted a model of morphological stasis (with all other variables continuing to support URW). This change stresses the importance of specimen identification when choosing time-slices for investigation of evolutionary models, and emphasises that distinguishing species by age is problematic (Chapter 1: Vaux *et al.* 2016).

Selecting specimens and time-slices inevitably affects results of temporal modelling, and mirrors the influence of taxonomic 'lumping and splitting', where species classification affects how morphological change and speciation in the fossil record is interpreted (Michaux 1989). Specifically, morphological change over time can be interpreted as being abrupt if fossils are treated as separate species, whereas if similar taxa are combined within a single evolutionary lineage, the same morphological change can be seen to be gradual (Michaux 1989).

Most genetically distinct lineages of *Penion* exhibit distinguishable differences in shell shape and size (Chapter 6). Without this knowledge, or without first investigating the relationship of *P. marwicki* to the other taxa using informed and *a priori* methods, it would have been difficult to hypothesise a meaningful lineage for paleoTS analysis. The need for a molecular framework behind potential lineages before attempting to interpret patterns of evolutionary change has been stressed (e.g. Aze *et al.* 2013), but our study demonstrates that statistical methods incorporated into packages such as mclust and paleoTS can be utilised to robustly investigate morphometric data, especially for the investigation of potential evolutionary lineages.

There are, however, limitations to our approach. Excluding *P. marwicki* based on identifiable differences in shell morphology, restricted our analysis to only morphologically similar populations through time – meaning that support for evolutionary stasis may have been favoured. There also remains the possibility that *P.*

sulcatus, *P. clifdenensis* and *P. exoptatus* are examples of evolutionary convergence, rather than representatives of a single evolutionary lineage. It is also possible that our analysis was biased by constraints of palaeontological sampling. For example, large shells are preserved more frequently than small specimens (Foote *et al.* 2015). This could cause under representation of smaller specimens and taxa through time, affecting our estimates that use centroid size. Shell sampling in the *Penion* fossil record is likely affected by less frequent preservation of bathyal and rocky-shore environments (Crampton *et al.* 2003), that could in the case of *Penion* influence estimates of shape change in *P. sulcatus* and related taxa through time, because features such as the length of the siphonal canal frequently appear to be affected by water depth (Ponder 1971). A further potential bias is that some fossil localities (and strata) sampled for PaleoTS analysis exhibit low sampling, and time slices are not evenly distributed over the total 21.7 Ma time range. This sampling may have caused a bias in our data towards the URW model, rather than directional change.

However, we believe that progress has been made to interpret the fossil record of *Penion* from an evolutionary perspective – and accepting these limitations only highlights the ongoing effort to extract more knowledge from the fossil record. Investigating morphological variation among fossils without *a priori* hypotheses, and testing the fit of evolutionary models to variation through time is a necessary step before more elaborate analyses can be conducted.

"We believe there is great value in simply determining the extent to which species diversification and phenotypic evolution are coupled in the natural world. Once we understand the generality of the pattern, we can begin the more challenging process of decomposing it into specific causal mechanisms." (Hunt and Rabosky 2014)

Future inclusion of *Penion* fossils from Antarctica, Argentina and Chile (e.g. Frassinetti 2000, Frassinetti 2001, Nielsen 2003, Beu 2009, Reichler 2010), and *Kelletia* worldwide (e.g. Anderson and Martin 1914, Ozaki 1954, Olsson 1964, Addicott 1970), using the same geometric morphometric method employed here would be beneficial. It would then be possible to compare the morphology of fossil and living taxa worldwide. It may also be of interest to digitise and analyse fossils from North America (e.g. Palmer and Bran 1965, Kollmann and Peel 1983, CoBabe and Allmon 1994), and Europe (e.g. Anderson 1973, Gilbert 1973, Moths and Albrecht 2010), which have been

variously as *Penion*, *Kelletia* or *Boreokelletia* Anderson, 1964 in order to assess the accuracy of their classification.

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Supplementary Data for Chapter Seven

Supplementary Tables

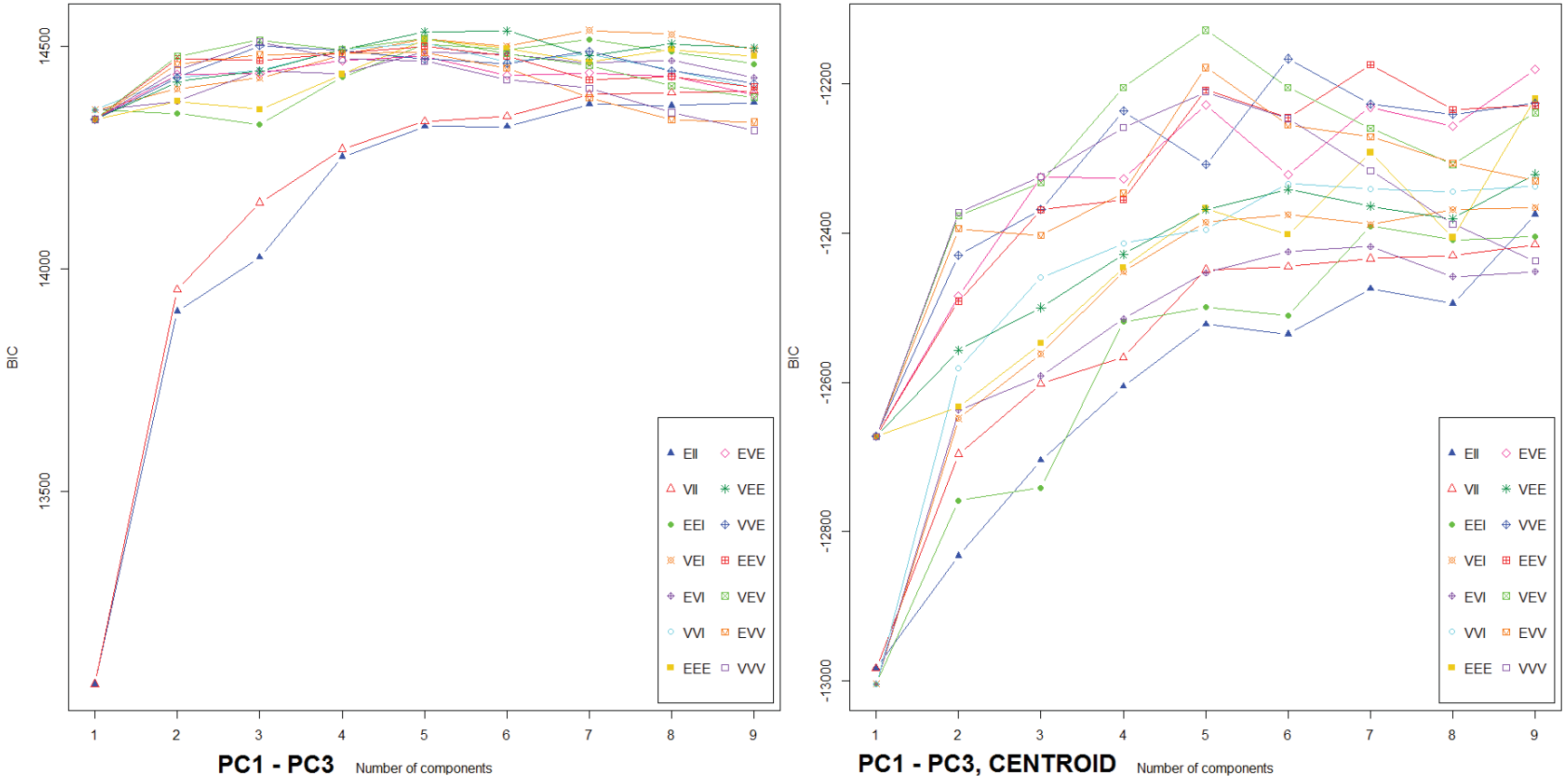
SUPPLEMENTARY TABLE 7.1
Cross-validation scores estimated for the discrimination of *P. marwicki* and extant *Penion* species with similar levels of sampling. Cross-validation scores were estimated via canonical variates analysis (CVA) implemented in the R package MASS 7.3-26 (Venables and Ripley 2002), using principal components generated from PCA in MorphoJ 1.06c (Klingenberg 2011). Colours reflect the frequency of individuals identified within each group (green = low, orange = intermediate, red = high).

	A. benthicola	*P. chathamensis*	*P. c. cuvierianus*	*P. mandarinus*	*P. marwicki*	*P. maximus*	*P. ormesi*	*P. sulcatus*
A. benthicola	58	0	1	0	0	0	0	1
P. chathamensis	0	165	0	0	0	0	8	0
P. c. cuvierianus	0	1	199	0	0	0	18	3
P. mandarinus	0	1	0	87	0	0	1	0
P. marwicki	0	0	0	0	62	0	0	2
P. maximus	0	0	0	0	0	114	0	0
P. ormesi	0	4	9	0	0	0	118	1
P. sulcatus	0	4	2	0	0	0	6	178

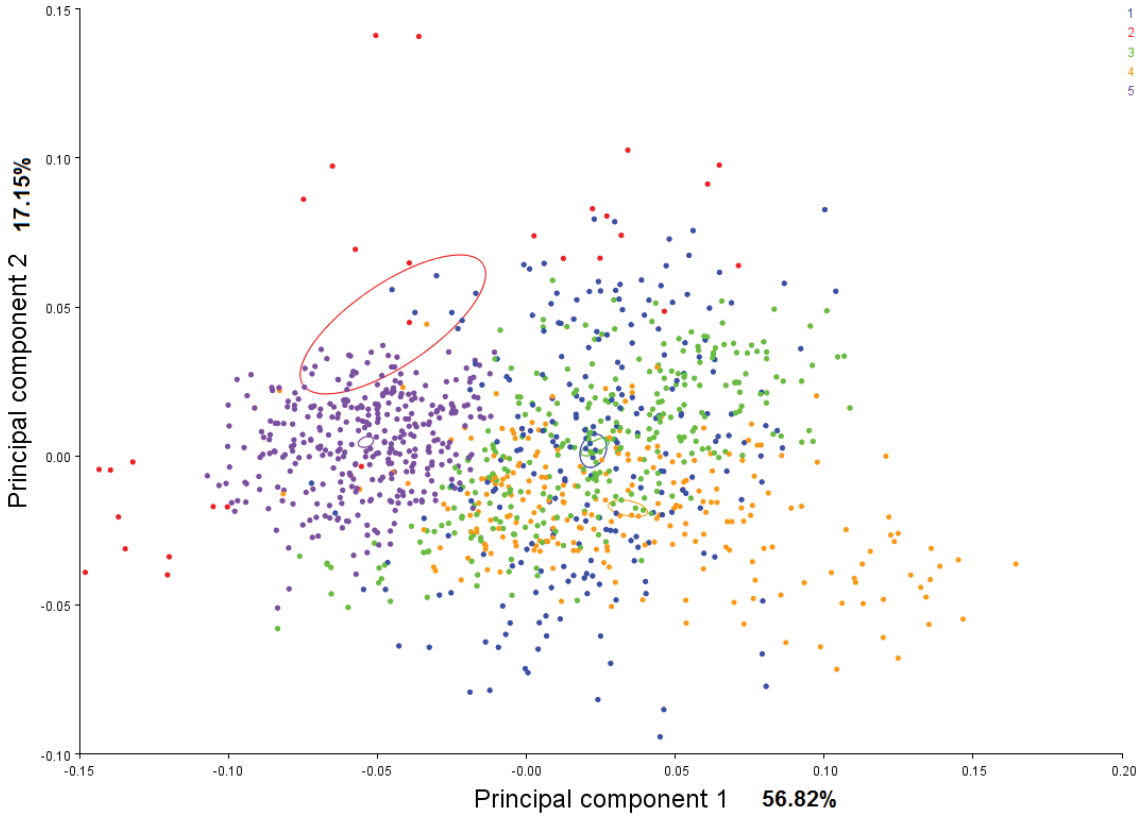
Supplementary Figures

SUPPLEMENTARY FIGURE 7.1

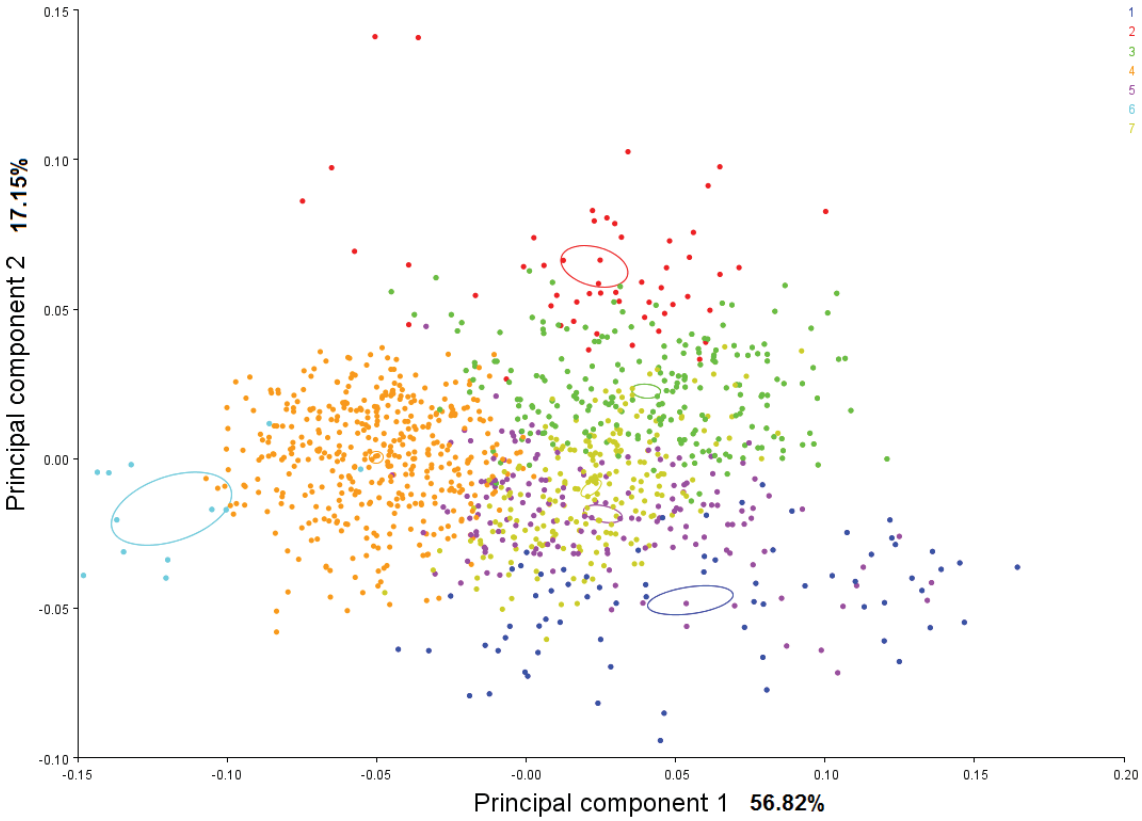
A plot of Bayesian information criterion (BIC) scores for fitting various clustering models to variation among all sampled fossils classified as extinct *Penion* species, with all extant sampling from *Penion*, *Kelletia* and *Antarctoneptunea* variation among all sampled shells classified as *Penion*, *Kelletia* and *Antarctonetpunea* using mclust 5.2. On the left, the BIC scores are shown using only the statistically significant PCs 1 – 3, and on the right the analysis is repeated when centroid size is also incorporated. See Fraley and Raftery (2012), for an explanation of the models listed.



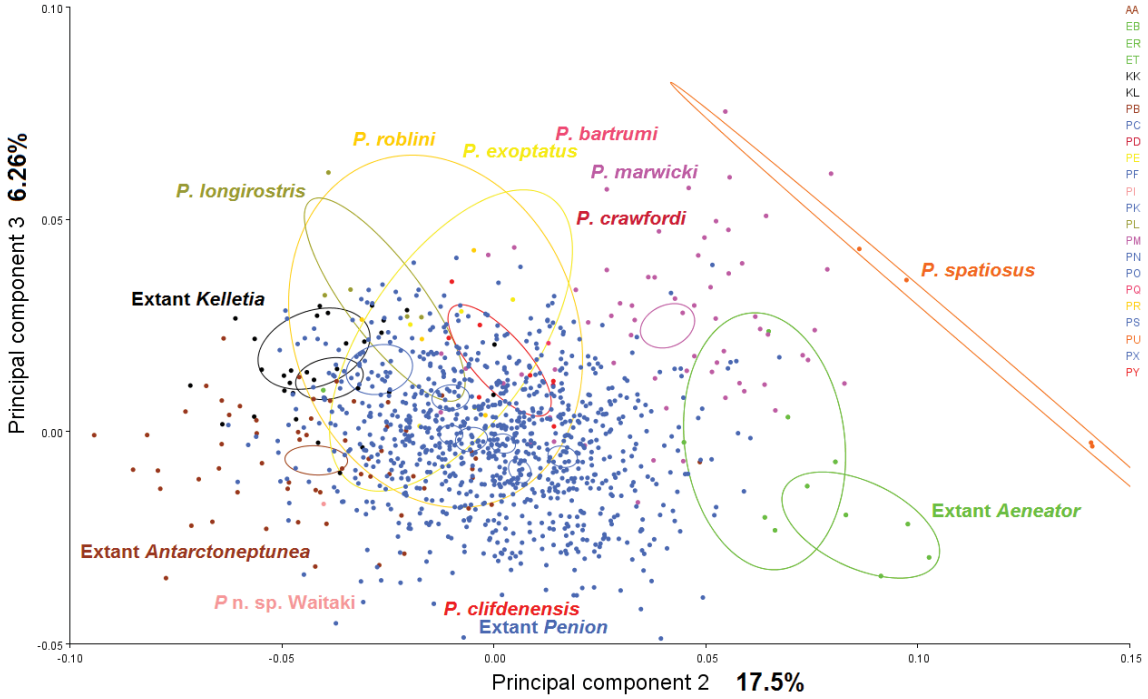
SUPPLEMENTARY FIGURE 7.2
A principal component analysis ordination (PCs 1 and 2) of all sampled fossils classified as extinct *Penion* species, with all extant sampling from *Penion*, *Kelletia* and *Antarctoneptunea*. Colouration corresponds to clusters identified naïvely by the R model-based clustering package mclust 5.2. Mean confidence ellipses (90%; same colouration) indicate that the groups means are unlikely to overlap. The clusters shown were identified by the VEV5 model using only the statistically significant PCs 1 – 3 and centroid size.



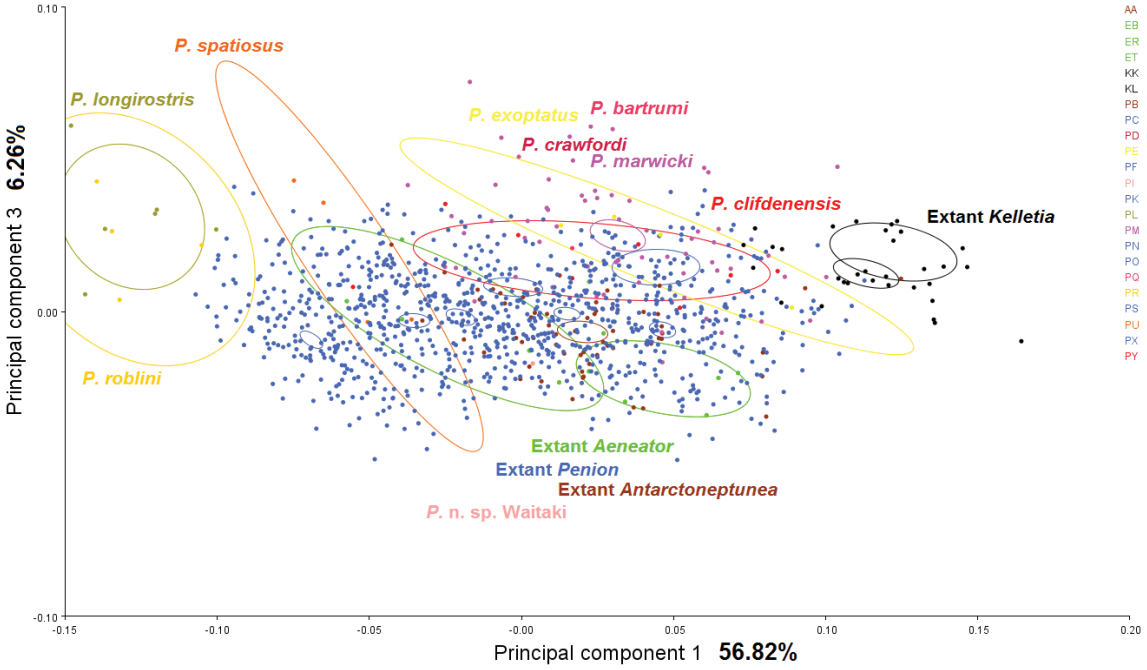
SUPPLEMENTARY FIGURE 7.3
A principal component analysis ordination (PCs 1 and 2) of all sampled fossils classified as extinct *Penion* species, with all extant sampling from *Penion*, *Kelletia* and *Antarctoneptunea*. Colouration corresponds to clusters identified naïvely by the R model-based clustering package mclust 5.2. Mean confidence ellipses (90%; same colouration) indicate that the groups means are unlikely to overlap. The clusters shown were identified by the EEV7 model using only the statistically significant PCs 1 – 3 and centroid size.



SUPPLEMENTARY FIGURE 7.4
A principal component analysis ordination (PCs 2 and 3) of all sampled fossils classified as extinct *Penion* species, with all extant sampling from *Penion*, *Kelletia* and *Antarctoneptunea*. Extant specimens are coloured by genera (blue for *Penion*, black for *Kelletia*, brown for *Antarctonetpunea* inclusive of *P. benthicolus* (see Chapter 3). Fossils are coloured according to putative taxonomic classification. Mean confidence ellipses (90%; same colouration) indicate that if the group means of species (fossil or extant) are likely to overlap. Specific extant species are not labelled for clarity, but there position can be readily estimated via comparison to previous PCA diagrams (see Chapter 6).

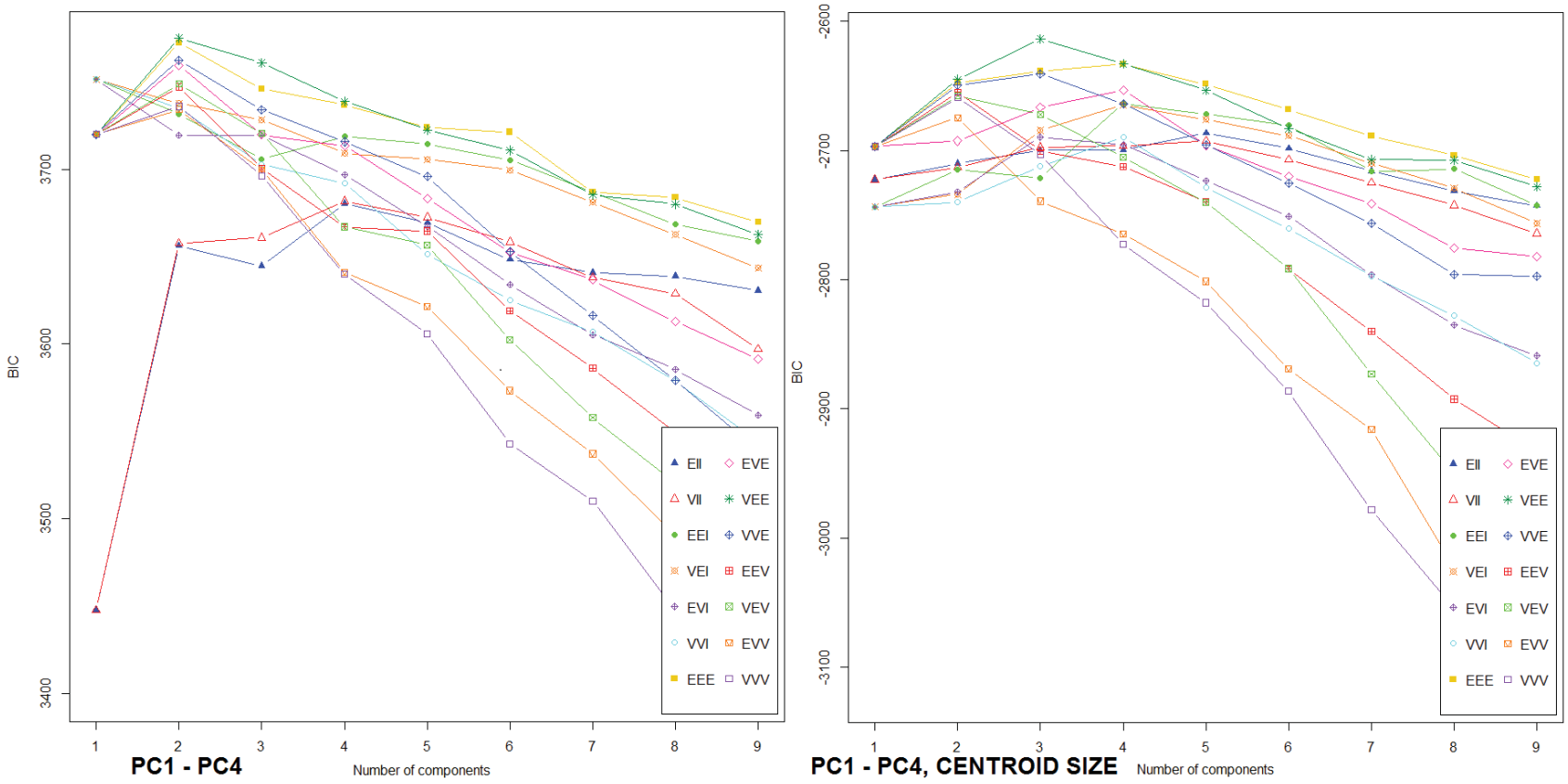


SUPPLEMENTARY FIGURE 7.5
A principal component analysis ordination (PCs 1 and 3) of all sampled fossils classified as extinct *Penion* species, with all extant sampling from *Penion*, *Kelletia* and *Antarctoneptunea*. Extant specimens are coloured by genera (blue for *Penion*, black for *Kelletia*, brown for *Antarctonetpunea* inclusive of *P. benthicolus* (see Chapter 3). Fossils are coloured according to putative taxonomic classification. Mean confidence ellipses (90%; same colouration) indicate that if the group means of species (fossil or extant) are likely to overlap. Specific extant species are not labelled for clarity, but there position can be readily estimated via comparison to previous PCA diagrams (see Chapter 6).



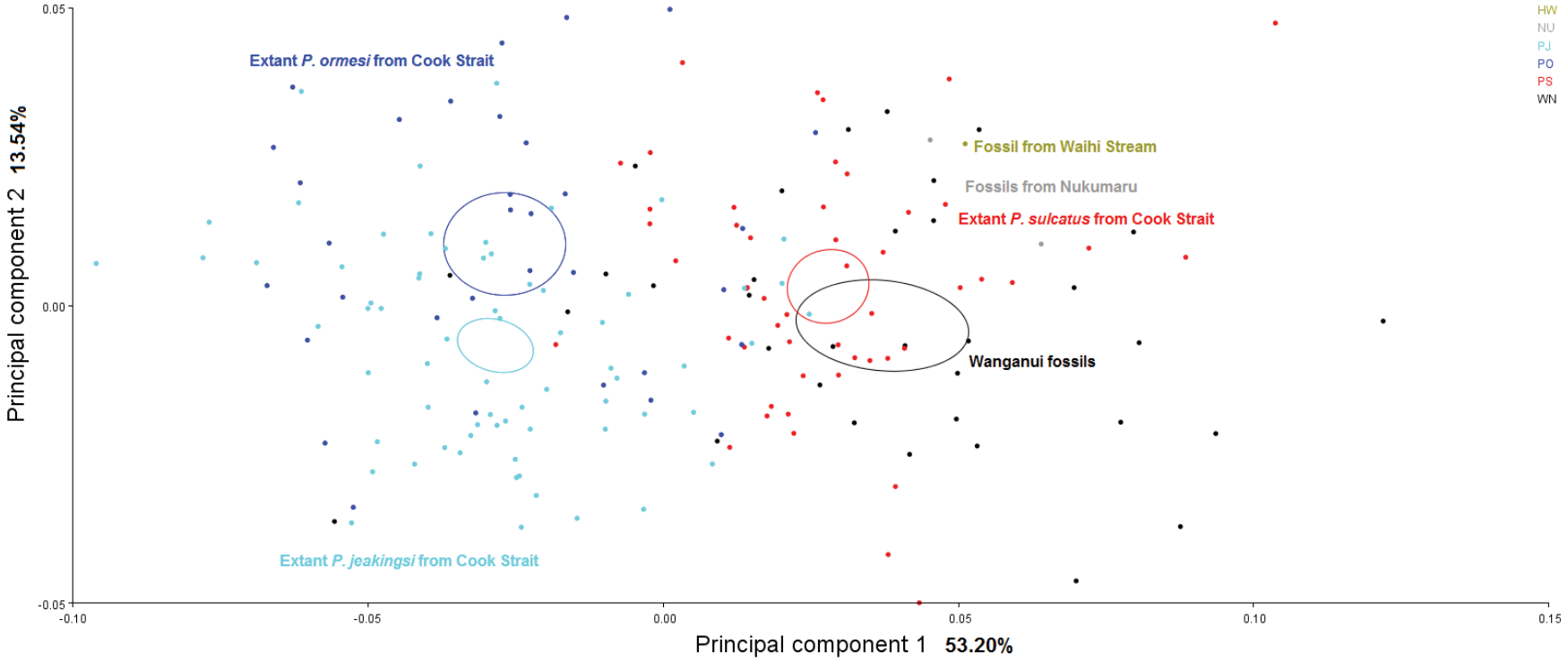
SUPPLEMENTARY FIGURE 7.6

A plot of Bayesian information criterion (BIC) scores for fitting various clustering models to variation among all sampled *Penion* fossils from Wanganui Beach and nearby geological localities, with all extant sampling from *P. sulcatus, P. ormesi,* and *P. c. jeakingsi* from nearby geographic regions, using mclust 5.2.On the left, the BIC scores are shown using only the statistically significant PCs 1 – 4, and on the right the analysis is repeated when centroid size is also incorporated. See Fraley and Raftery (2012), for an explanation of the models listed.



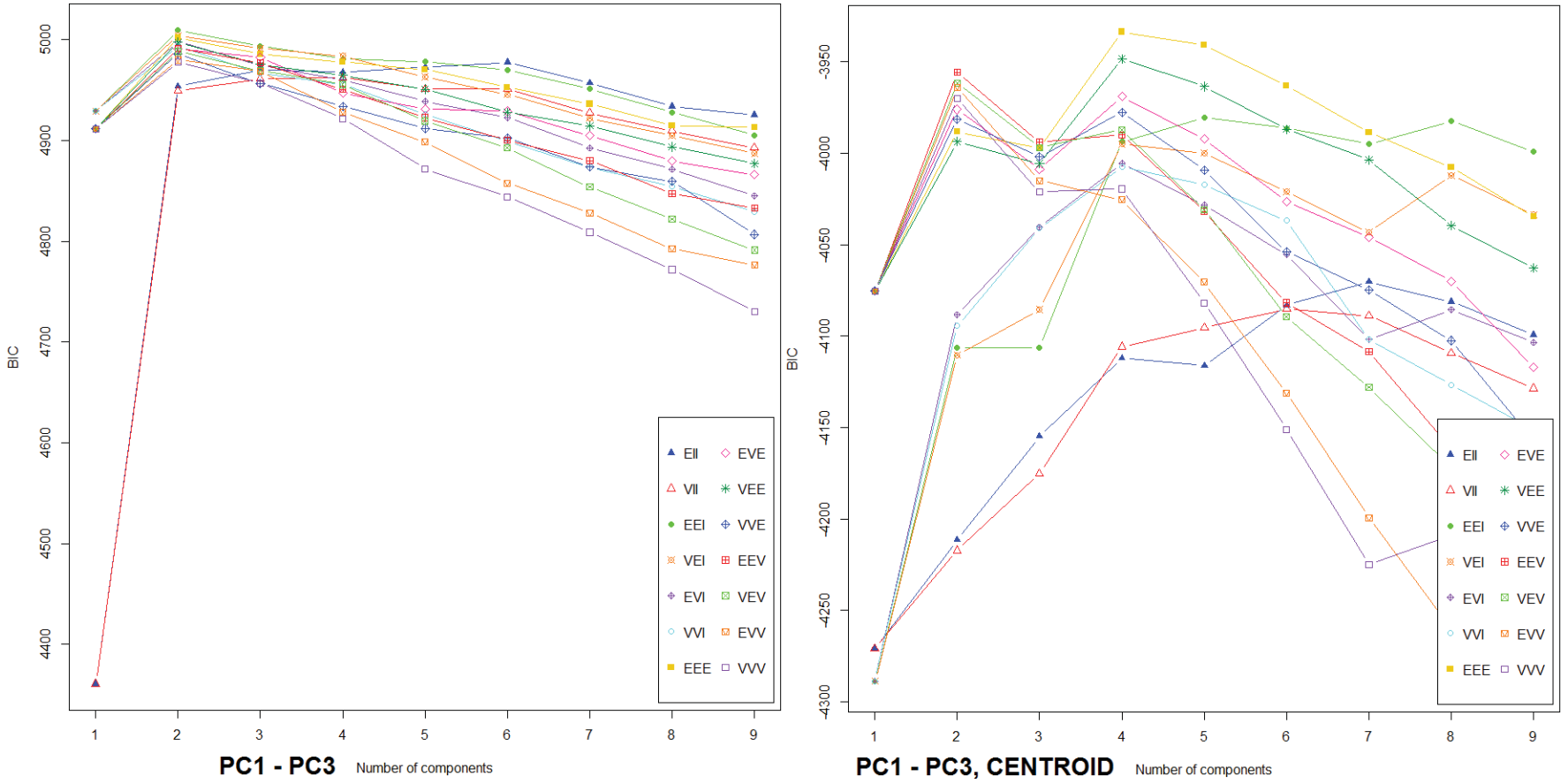
SUPPLEMENTARY FIGURE 7.7

A principal component analysis ordination (PCs 1 and 2) of all sampled *Penion* fossils from Wanganui Beach and nearby geological localities, with all extant sampling from *P. sulcatus*, *P. ormesi*, and *P. c. jeakingsi* from nearby geographic regions. Fossils are coloured according to geological locality (no *a priori* classification of specimens), and extant shells are coloured according to putative taxonomic classification. Mean confidence ellipses (90%; same colouration) indicate that the group mean of fossils from Wanganui is likely to overlap with modern *P. sulcatus* from nearby locations.

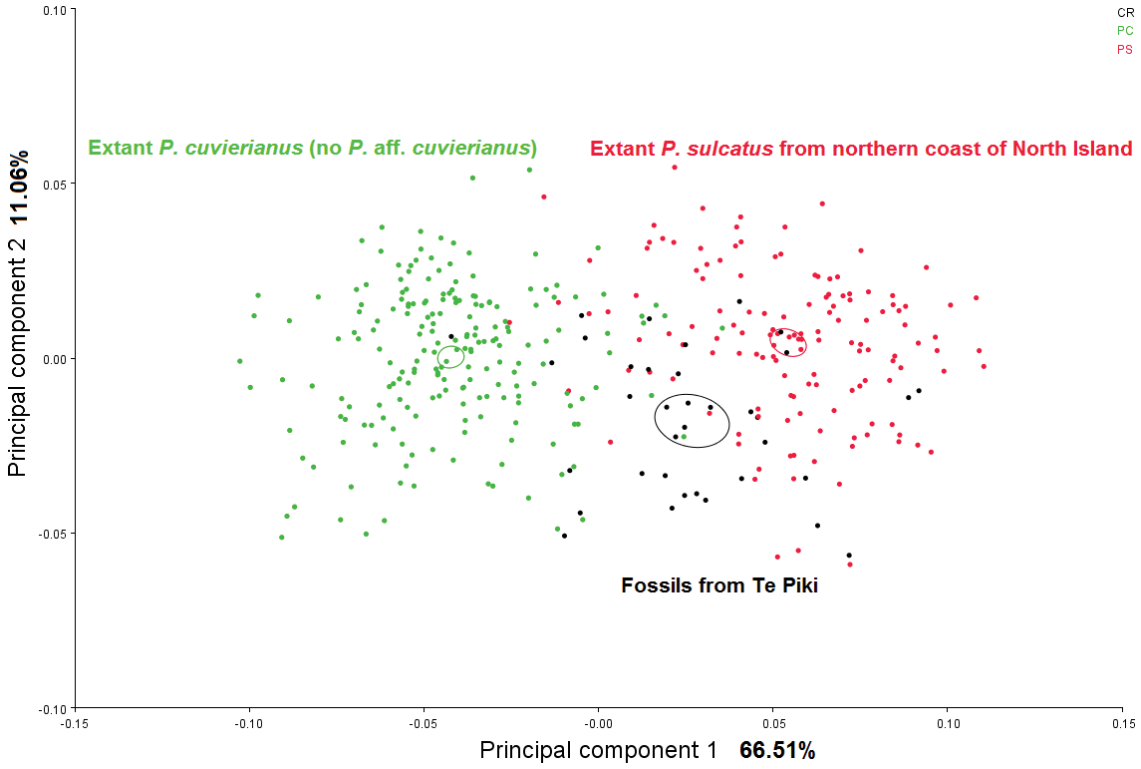


SUPPLEMENTARY FIGURE 7.8

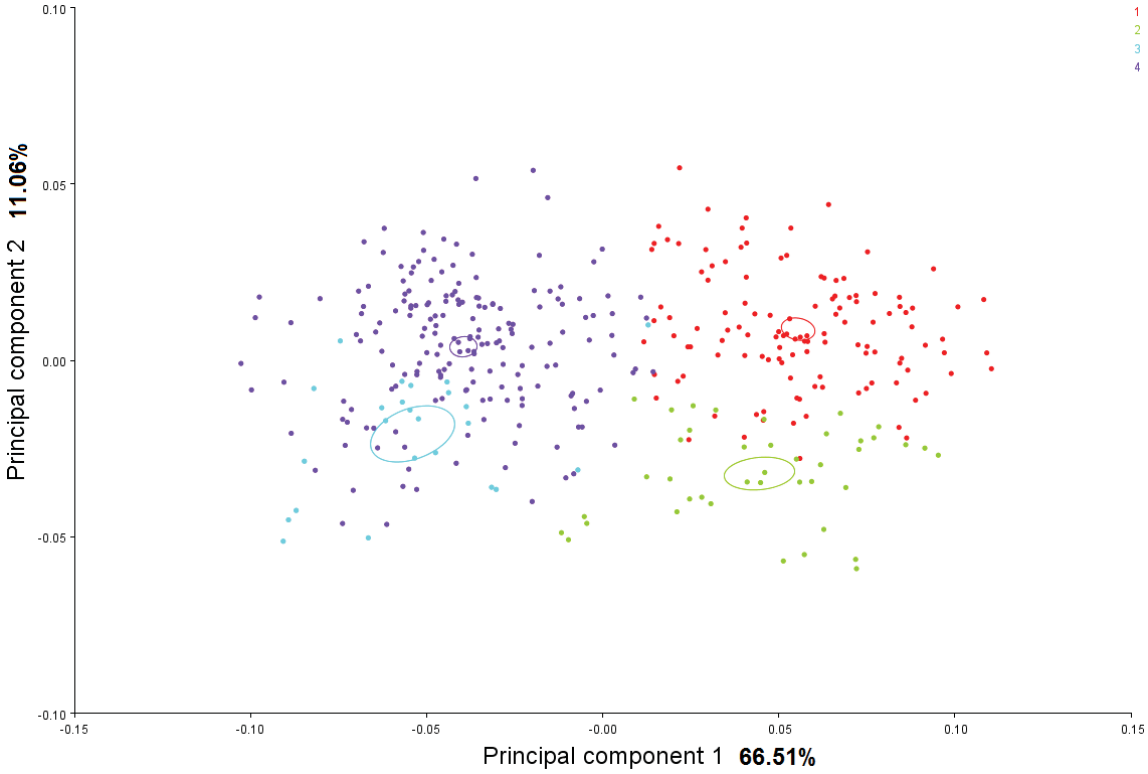
A plot of Bayesian information criterion (BIC) scores for fitting various clustering models to variation among of all sampled *Penion* fossils from Te Piki geological locality, and all extant sampling from *P. sulcatus* and *P. c. cuvierianus* from nearby geographic regions, using mclust 5.2. On the left, the BIC scores are shown using only the statistically significant PCs 1 – 3, and on the right the analysis is repeated when centroid size is also incorporated. See Fraley and Raftery (2012), for an explanation of the models listed.



SUPPLEMENTARY FIGURE 7.9
A principal component analysis (PCs 1 – 2) of all sampled *Penion* fossils from Te Piki geological locality, and all extant sampling from *P. sulcatus* and *P. c. cuvierianus* from nearby geographic regions. Fossils are coloured as a single geological locality (i.e. Te Piki fossils with no *a priori* classification of specimens), and extant shells are coloured according to putative taxonomic classification. Mean confidence ellipses (90%; same colouration) indicate that the mean of each group is unlikely to overlap with another, suggesting that all groups can be distinguished from one another.

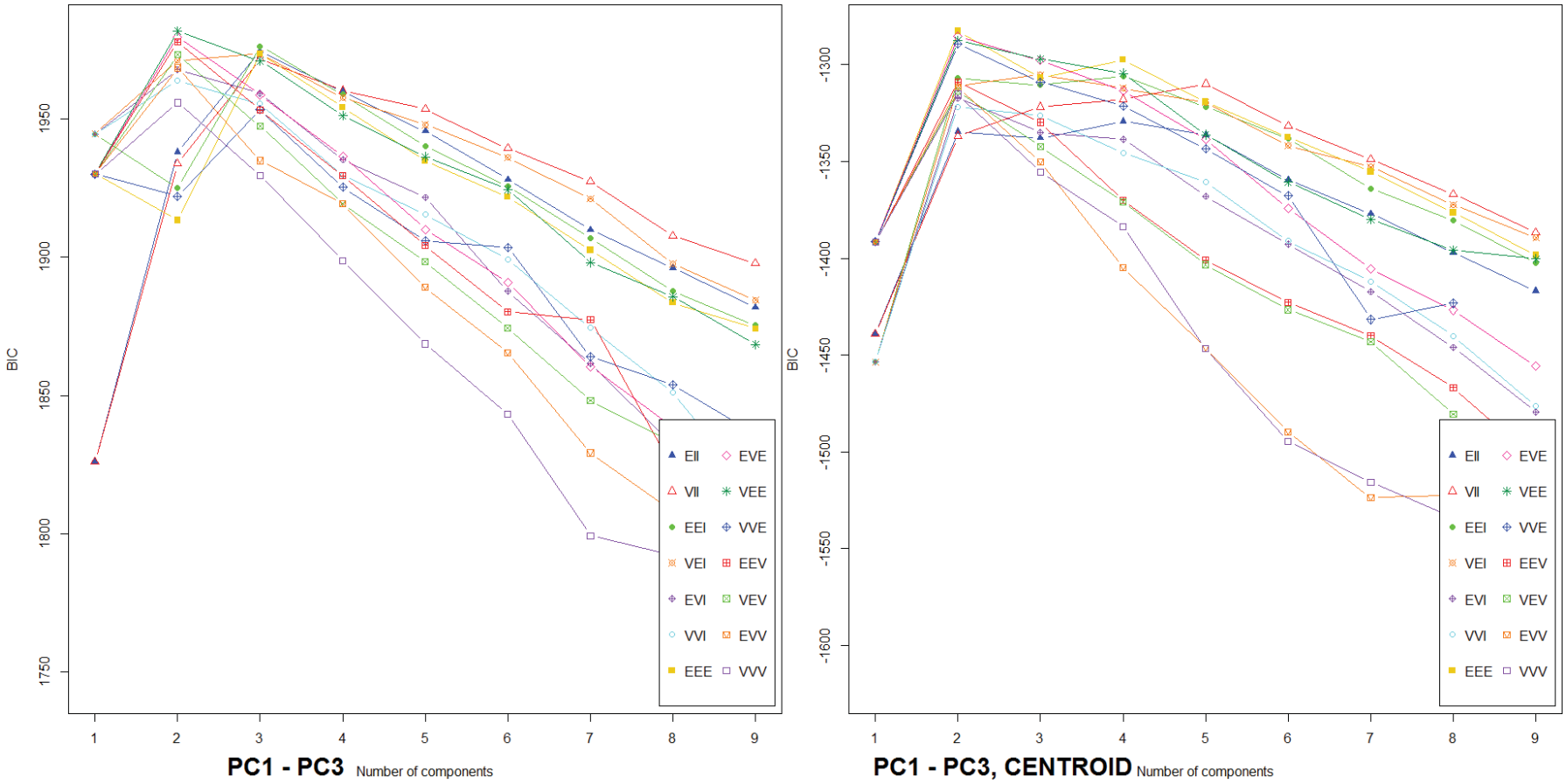


SUPPLEMENTARY FIGURE 7.10
A principal component analysis (PCs 1 – 2) of all sampled *Penion* fossils from Te Piki geological locality, and all extant sampling from *P. sulcatus* and *P. c. cuvierianus* from nearby geographic regions. Colouration corresponds to clusters identified naïvely by the R model-based clustering package mclust 5.2. Mean confidence ellipses (90%; same colouration) of group means indicate that the taxa are unlikely to overlap. The clusters shown were identified by the EEE4 model using only the statistically significant PCs 1 – 3 and centroid size.



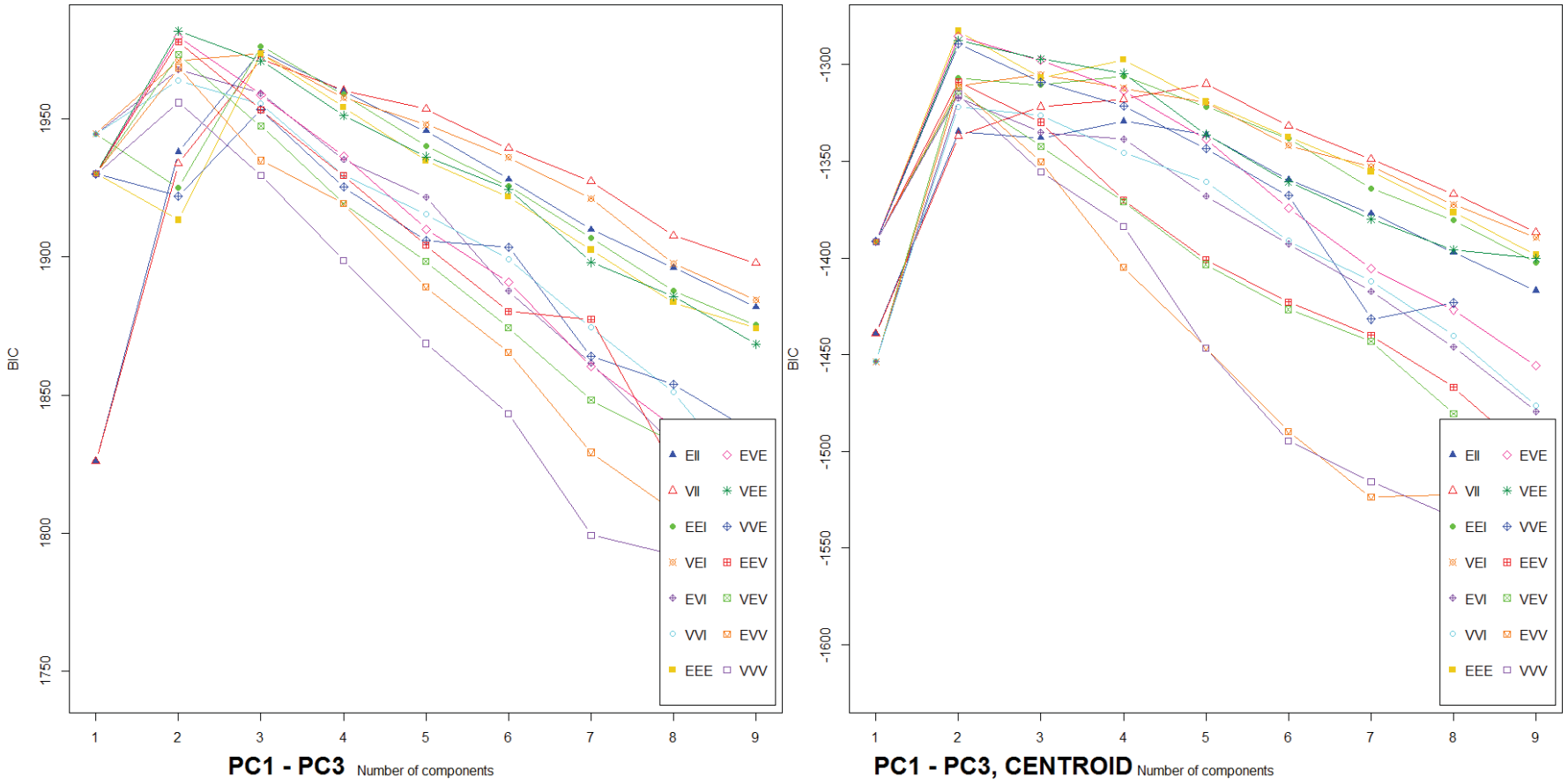
SUPPLEMENTARY FIGURE 7.11

A plot of Bayesian information criterion (BIC) scores for fitting various clustering models to variation among of all sampled specimens classified as *P. maximus* (extant only), *P. roblini* and *P. longirostris*, using mclust 5.2. On the left, the BIC scores are shown using only the statistically significant PCs 1 – 3, and on the right the analysis is repeated when centroid size is also incorporated. See Fraley and Raftery (2012), for an explanation of the models listed.



SUPPLEMENTARY FIGURE 7.12

A plot of Bayesian information criterion (BIC) scores for fitting various clustering models to variation among of all sampled specimens of *P. sulcatus* (fossil and extant), *P. clifdenensis P. marwicki*, and *P. exoptatus*, using mclust 5.2. On the left, the BIC scores are shown using only the statistically significant PCs 1 – 3, and on the right the analysis is repeated when centroid size is also incorporated. See Fraley and Raftery (2012), for an explanation of the models listed.



Chapter Eight

A review of the extant and fossil taxonomy of *Antarctoneptunea, Kelletia* and *Penion*

Left to right, fossils originally classified as:
P. c. cuvierianus (2.40 – 1.63 Ma),
P. haweraensis† (3.7 Ma) and *P. finlayi*† (21.7 Ma).

Introduction

The taxonomy of *Antarctoneptunea* Dell, 1972, *Kelletia* Bayle, 1884 and *Penion* Fischer, 1884 is challenging to interpret as there is no single source that attempts to consolidate all worldwide extant and fossil information for the clade. Important sources also exist in four languages and not all documents are currently available digitally. Although the research conducted in this thesis should be comprehensible as an evolutionary study, we believe that the taxonomy and known geological history of the clade should be easy to reference. Here we outline the current taxonomy of *Penion, Kelletia* and *Antarctoneptunea* and then suggest revisions based on the two sources of evidence described below. These changes are the operational taxonomic units (OTUs) used in this thesis.

In our methods for Chapters 3 – 7, we consolidated a number of fossil species and extant subspecies of *Penion* as they were argued to be misclassified. Two new fossil species of *Penion* were also recorded and were used in some analyses. We provide below our evidence for making these decisions. The majority of taxa synonymised were fossils, which appear to have been affected by the perennial problem of over splitting in taxonomy. All sampled specimens were identified by experienced molluscan taxonomists: Bruce A. Marshall (Collection Manager Sciences, Museum of New Zealand Te Papa Tongarewa) and Alan G. Beu (Palaeontologist, GNS). These changes were made independent of the molecular phylogenetic and geometric morphometric analyses in Chapters 3 – 7.

Our results from Chapters 3 – 7, based on molecular phylogenetics and the geometric morphometric analysis of shell shape, suggested further taxonomic changes. These changes included the discovery of a new extant species of *Penion*, and the misclassification of a lineage of *Antarctoneptunea* as *Penion*. We also include these revisions below. We hope this clarifies how the taxonomy of the clade may be affected overall by the work of this thesis. We list clearly when changes are based on the traditional examination or the molecular phylogenetic and geometric morphometric results of previous chapters. We also illustrate the combined geological history of the clade worldwide.

Taxonomy of *Antarctoneptunea, Kelletia* and *Penion*

Siphon whelk taxonomy has been reviewed in five key publications covering extant New Zealand (Powell 1979), fossil New Zealand (Beu and Maxwell 1990),

extant and fossil Australian (Ponder 1973), fossil Chilean and Argentinian taxa (Nielsen 2003), and fossil Antarctica taxa (Beu 2009). No previous work has combined information from all five sources however, and an abundance of synonyms generate further confusion for those trying to study siphon whelks (e.g. Ponder 1975). Further species have also subsequently been described (e.g. Reichler 2010).

Here we list the current taxonomy of *Antarctoneptunea, Kelletia* and *Penion* based on published research, followed by suggested revisions. Species marked with a single asterisk (*) were sampled and analysed within Chapters 3 – 7. Sampling includes DNA sequencing, digitisation of shells for geometric morphometrics, and photography of specimens that were too poorly preserved for morphometric analysis but often adequate for traditional taxonomic assessment. Revisions have not been suggested for taxa outside of Australasia as sampling was limited. We only list taxonomic synonyms implied by the results of this thesis, we do not include all synonyms.

In this review we exclude fossils that have been putatively classified as *Penion* from the south-eastern USA (e.g. Palmer and Bran 1965, CoBabe and Allmon 1994), Greenland (Kollmann and Peel 1983), and France (Pacaud *et al.* 2000), as well as fossils classified as either *Kelletia* or *Boreokelletia* Anderson, 1964 from Northern Europe (e.g. Anderson 1973, Gilbert 1973, Moths and Albrecht 2010). These fossil occurrences are excluded as their taxonomic classification is often noted to be dubious (e.g. Kollmann and Peel 1983), as recent reviews have suggested alternative classifications for many of these species (e.g. Snyder 2003), and because there has been no comprehensive comparison of many of these fossils with type specimens from the widely accepted ranges of *Penion* and *Kelletia.* However, although many of these fossils are likely to be misclassified examples of evolutionary convergence, we do suggest a future analysis of these shells given the long distance dispersal that has occurred in the evolution of the *Antarctoneptunea, Kelletia* and *Penion* clade (Chapter 3).

The geographic location of taxa is also listed (ANT = Antarctica, ARG = Argentina, AUS = Australia, CHL = Chile, ECU = Ecuador, JPN = Japan, KOR = Republic of Korea, MEX = Mexico, NZ = New Zealand, USA = United States of America).

Current taxonomy of *Antarctoneptunea*, *Kelletia* and *Penion* organised by region:

BUCCINOIDEA
BUCCINIDAE [true whelks] OR BUCCINULIDAE [Southern Hemisphere true whelks]

Antarctoneptunea Dell, 1972		
Antarctoneptunea aurora (Hedley, 1916)	ANT	*
Kelletia Bayle, 1884 = Kellet's whelks		
Kelletia ecuadoriana† Olsson, 1964	ECU	
Kelletia rugosa† Olsson, 1964	ECU	
Kelletia lischkei Kuroda, 1938	JAP, KOR	*
Kelletia brevis† Ozaki, 1954	JAP	
Kelletia kanakoffi† Hertlein, 1970	USA	
Kelletia kelletii (Forbes, 1850)	USA, MEX	*
Kelletia kettlemanensis† (Arnold, 1910)	USA	
Kelletia lorata† (Addicott, 1970)	USA	
Kelletia posoensis† (Anderson & Martin, 1914)	USA	
Kelletia vladimiri† Kanakoff, 1954	USA	
Penion Fischer, 1884 = Siphon whelks		
Penion australocapax† Stillwell & Zinsmeister, 1992	ANT	
Penion longirostrsis† (Tate, 1888)	AUS	*
Penion mandarinus (Duclos, 1831)	AUS	*
Penion maximus (Tryon, 1881)	AUS	*
Penion roblini roblini† (Tenison Woods, 1876)	AUS	*
Penion roblini simulans† (Tenison Woods, 1876)	AUS	*
Penion spatiosus† (Tate, 1888)	AUS	*
Penion crassus† Frassinetti, 2000	CHL	
Penion darwinianus (Philipphi, 1887)	CHL	
Penion diversum† Frassinetti, 2000	CHL	
Penion domeykoanus† (Philippi, 1887)	CHL, ARG	*
Penion macsporrani† (Philippi, 1887)	CHL	
Penion oncodes† (Philippi, 1887)	CHL	
Penion patagonensis† Reichler, 2010	ARG	
Penion petitianus† (d'Orbigny, 1842)	CHL	
Penion subrectus† (Ihering, 1899)	CHL, ARG	
Penion subreflexus† (Sowerby, 1846)	CHL	
Penion subregularis† (d'Orbigny, 1852)	CHL	
Penion affixus† (Finlay, 1930)	NZ	
Penion asper† (Marwick, 1928)	NZ	*
Penion bartrumi† (Laws, 1941)	NZ	*
Penion brazieri† (Fleming, 1955)	NZ	*
Penion benthicolus Dell, 1956	NZ	*
Penion chathamensis (Powell, 1938)	NZ	*
Penion clifdenensis† (Finlay, 1930)	NZ	*
Penion crawfordi† (Hutton, 1873)	NZ	*
Penion cuvierianus cuvierianus (Powell, 1927)	NZ	*
Penion cuvierianus jeakingsi (Powell, 1947)	NZ	*

Penion exoptatus† (Powell & Batrum, 1929)	NZ	*
Penion fairfieldae (Powell, 1947)	NZ	*
Penion finlayi† (Laws, 1930)	NZ	*
Penion gauli† (Marwick, 1928)	NZ	*
Penion haweraensis† (Powell, 1931)	NZ	*
Penion huttoni† (King, 1934)	NZ	*
Penion imperfectus† (Powell, 1947)	NZ	*
Penion interjunctus† (Finlay, 1930)	NZ	*
Penion koruahinensis† (Powell & Bartrum, 1928)	NZ	*
Penion marwicki† (Finlay, 1930)	NZ	*
Penion ormesi (Powell, 1927)	NZ	*
Penion parans† (Finlay, 1930)	NZ	*
Penion proavitus† (Finlay & Marwick, 1937)	NZ	*
Penion sulcatus (Lamarck, 1816)	NZ	*
Penion winthropi† (Marwick, 1965)	NZ	*

Revised taxonomy of *Antarctoneptunea*, *Kelletia* and *Penion* organised by region:

BUCCINOIDEA
BUCCINIDAE [true whelks]

Antarctoneptunea Dell, 1972

Antarctoneptunea aurora (Hedley, 1916)	ANT	*
Antarctoneptunea benthicola (Dell, 1956)	NZ	*

Kelletia Bayle, 1884 = Kellet's whelks

Kelletia ecuadoriana† Olsson, 1964	ECU	
Kelletia rugosa† Olsson, 1964	ECU	
Kelletia lischkei Kuroda, 1938	JAP, KOR	*
Kelletia brevis† Ozaki, 1954	JAP	
Kelletia kanakoffi† Hertlein, 1970	USA	
Kelletia kelletii (Forbes, 1850)	USA, MEX	*
Kelletia kettlemanensis† (Arnold, 1910)	USA	
Kelletia lorata† (Addicott, 1970)	USA	
Kelletia posoensis† (Anderson & Martin, 1914)	USA	
Kelletia vladimiri† Kanakoff, 1954	USA	

Penion Fischer, 1884 = Siphon whelks

Penion australocapax† Stillwell & Zinsmeister, 1992	ANT	
Penion mandarinus (Duclos, 1832)	AUS	*
Penion maximus (Tryon, 1881)	AUS	*
Penion crassus† Frassinetti, 2000	CHL	
Penion darwinianus (Philipphi, 1887)	CHL	
Penion diversum† Frassinetti, 2000	CHL	
Penion domeykoanus† (Philippi, 1887)	CHL, ARG	*
Penion macsporrani† (Philippi, 1887)	CHL	
Penion oncodes† (Philippi, 1887)	CHL	
Penion patagonensis† Reichler, 2010	ARG	
‡*Penion petitianus*† (d'Orbigny, 1842)	CHL	
Penion subrectus† (Ihering, 1899)	CHL, ARG	

Penion subreflexus† (Sowerby, 1846)	CHL	
Penion subregularis† (d'Orbigny, 1852)	CHL	
Penion asper† (Marwick, 1928)	NZ	*
Penion bartrumi† (Laws, 1941)	NZ	*
Penion chathamensis (Powell, 1938)	NZ	*
Penion clifdenensis† (Finlay, 1930)	NZ	*
Penion crawfordi† (Hutton, 1873)	NZ	*
Penion cuvierianus (Powell, 1927)	NZ	*
Penion aff. *cuvierianus*	NZ	*
Penion exoptatus† (Powell & Batrum, 1929)	NZ	*
Penion imperfectus† (Powell, 1947)	NZ	*
Penion jeakingsi (Powell, 1947)**	NZ	*
Penion marwicki† (Finlay, 1930)	NZ	*
Penion ormesi (Powell, 1927)**	NZ	*
Penion proavitus† (Finlay & Marwick, 1937)	NZ	*
Penion sulcatus (Lamarck, 1816)	NZ	*
Penion n. sp. Three Kings Islands	NZ	*
Penion n. sp. Waimumu†	NZ	*
Penion n. sp. Waitaki†	NZ	*
Penion n. sp. West Coast**	NZ	*

* Taxa sampled within this thesis.

** *Penion ormesi*, *P. jeakingsi* and *P.* n. sp. West Coast appear to form a species complex ('*P. ormesi* species complex') and all taxa may be conspecific. Further molecular investigation is required.

† Extinct taxa.

‡ The classification of *P. petitanus* is not confident because the only known specimen is highly fragmented.

Distributions of *Antarctoneptunea, Kelletia* and *Penion*

Figure 8.1
Revised distribution of *Antarctoneptunea*, *Kelletia* and *Penion*
The *P. ormesi* complex includes *P. ormesi*, *P. jeakingsi* and *P.* n. sp. West Coast. Compare to Figure 3.7 for distribution of *Antarctoneptunea*, *Kelletia* and *Penion* under previous taxonomy prior to the molecular and morphometric results of Chapters 3 and 4, 6.

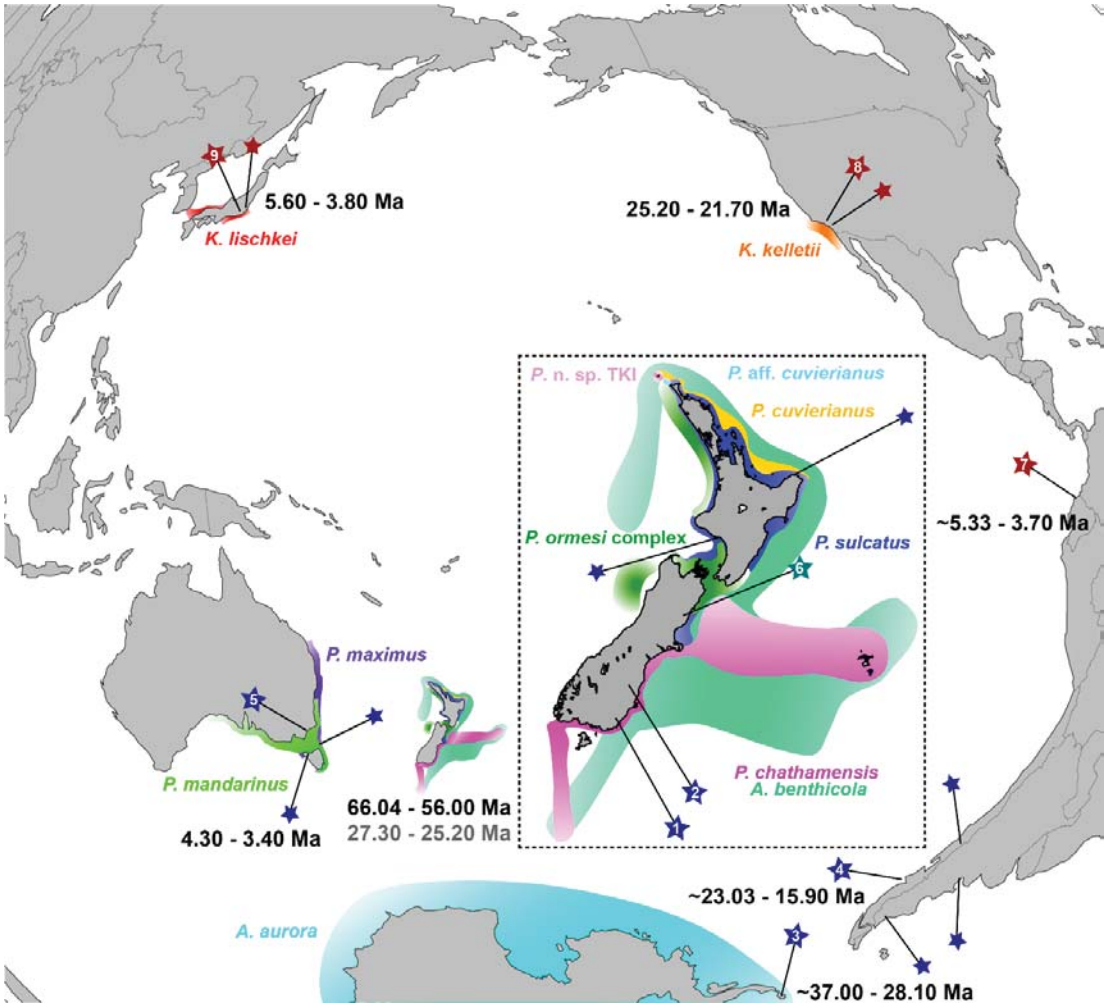
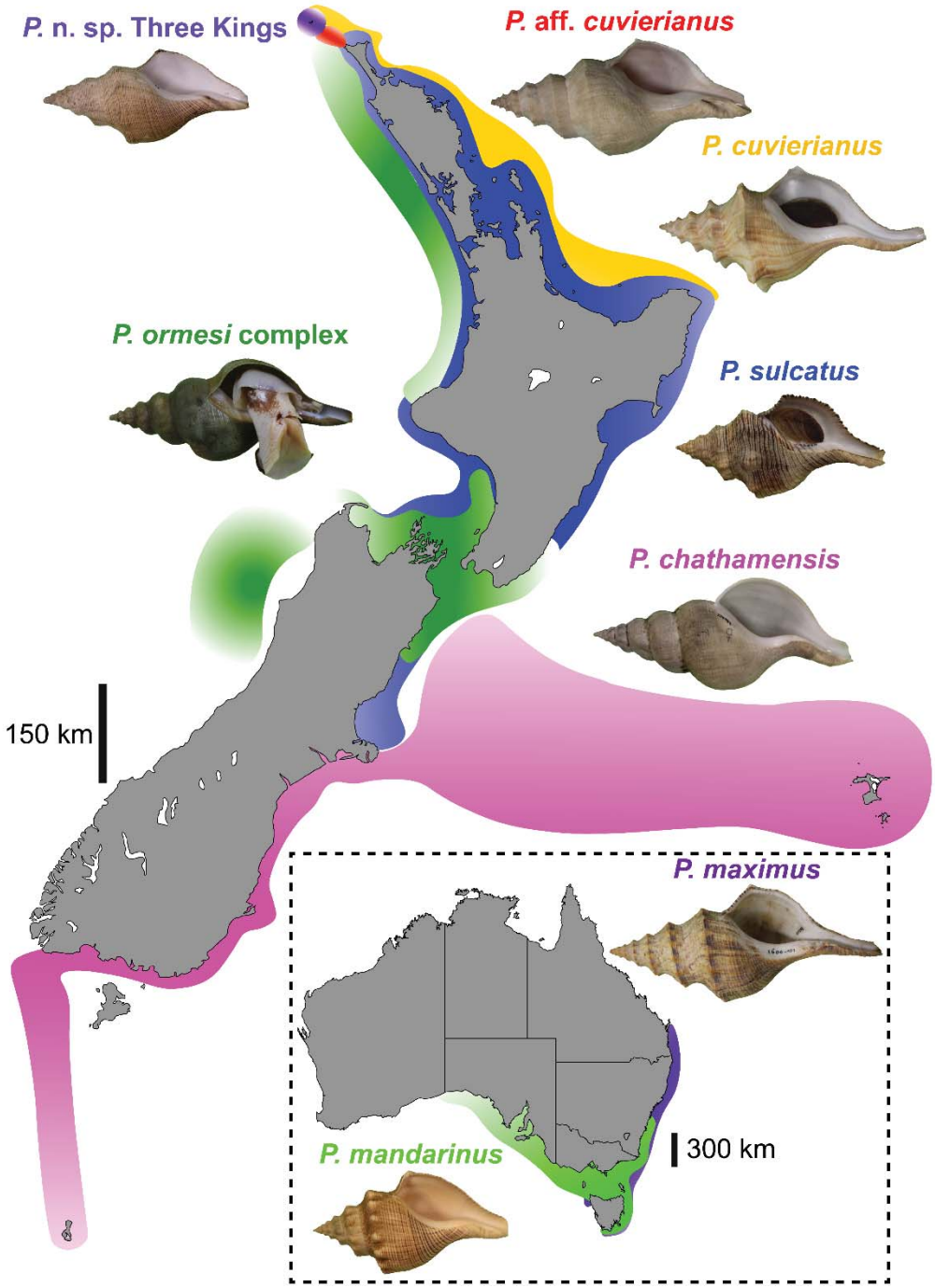


Figure 8.2
Revised distribution of extant monophyletic *Penion*
The *P. ormesi* complex includes *P. ormesi*, *P. jeakingsi* and *P.* n. sp. West Coast. Compare to Figure 4.1 for distribution of monophyletic New Zealand *Penion* under previous taxonomy.



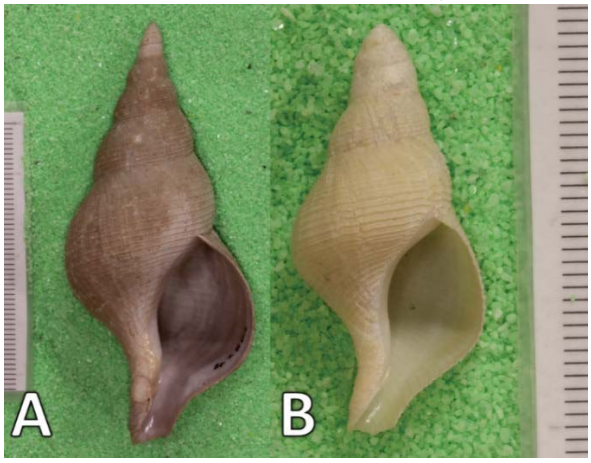
Taxonomic catalogue of *Antarctoneptunea, Kelletia* and *Penion*

Species are listed in alphabetical order and organised into genera. The museum identification number is provided for figured specimens, as are host museums using abbreviations listed below:

AM	Auckland War Memorial Museum
AU	Auckland University
AUS	Australian Museum
GNS	GNS Science
LACMIP	Natural History Museum of Los Angeles County
MNZ	Museum of New Zealand Te Papa Tongarewa
MU	Massey University
NMNS	National Museum of Nature and Science, Japan
USNM	National Museum of Natural History, USA
VIC	Museum Victoria, Melbourne
VU	Victoria University of Wellington

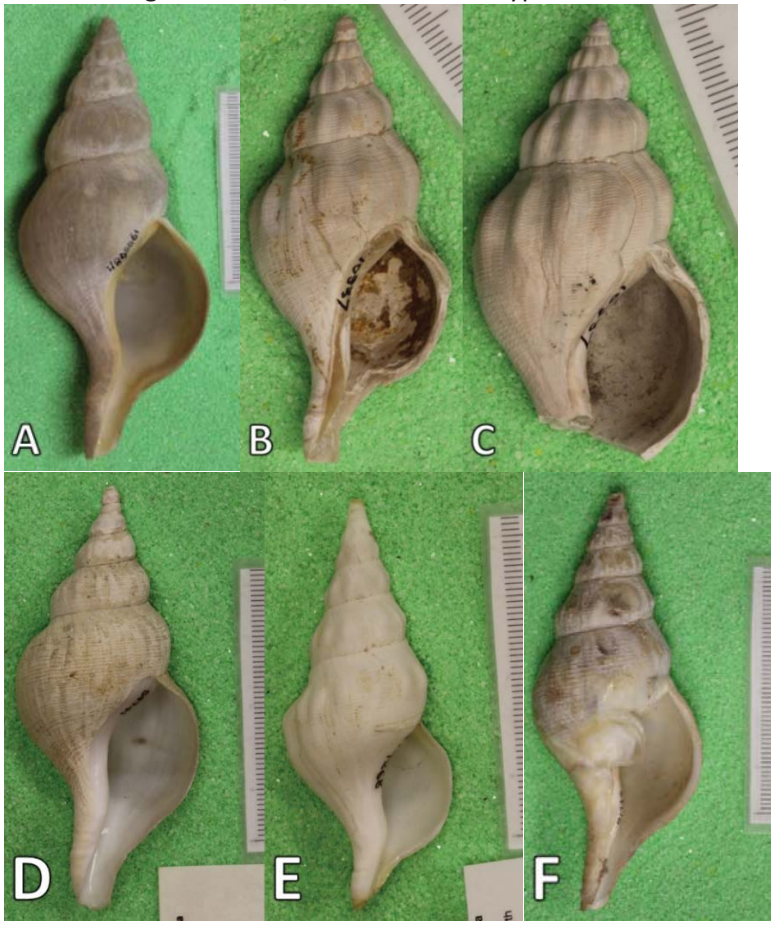
Antarctoneptunea

Antarctoneptunea aurora (Hedley, 1916)
Figure 8.3
A: M.242882 [MNZ] from the Ross Sea; B: M.260977 [MNZ] juvenile specimen from the Ross Sea.



Evidence used for change	Molecular phylogenetics, traditional examination of shell traits, geometric morphometric analysis of shells.
Comments	Molecular phylogenetic results indicate that *Antarctoneptunea aurora* is a sister lineage to *P. benthicolus* (Chapter 3). Traditional morphological examination of shells also indicates strong similarity between the species (Chapter 3). Morphometric analysis of shells with limited sampling of *A. aurora* also indicated similar morphology to *P. benthicolus* (Chapter 6). *Antarctoneptunea aurora* exhibits less prominent axial ribs (although this trait is variable in *P. benthicolus*), and may exhibit a shorter siphonal canal. Both species also appear restricted to deep water and potentially exhibit overlapping distributions south of New Zealand. The species do however differ in radulae morphology (Dell 1972).
Extant references	Hedley 1916 (taxonomic description) Dell 1972
Geological range	Present.
Fossil localities	None yet recognised.
Sampled depth range	132 – 400 m, deep.
Protoconch	3 – 4 whorls, large and 'beehive-shaped' with first protoconch whorl forming a cap.

Antarctoneptunea benthicola (Dell, 1956)
Figure 8.4
A: M.190068/1 [MNZ] from Chatham Rise, tissue sequenced in Chapter 3; B – C: GS10337 [GNS] fossil specimens from Oaro, figured in Beu 1979; D: M.274268 [MNZ] from off Cape Kidnappers, tissue sequenced in Chapter 4; E: M.059741 [MNZ] from Hikurangi Trench; F: MA71333 [AM] from off Poor Knights Islands, classified as holotype of *P. benthicolus delli* Powell, 1971.

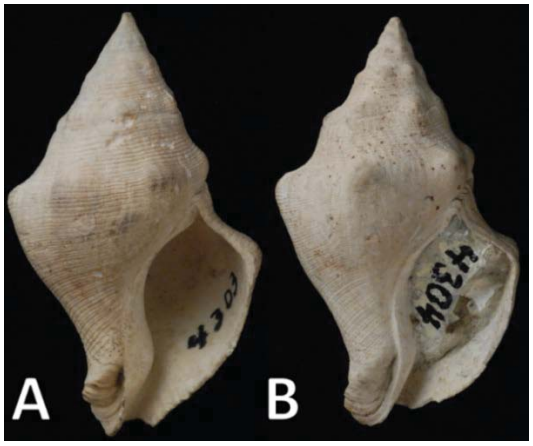


Synonyms	*Penion benthicolus* (Dell, 1956)
Evidence used for change	Molecular phylogenetics, traditional examination of shell traits, geometric morphometric analysis of shells.
Comments	Molecular phylogenetic results indicate that *P. benthicolus* is is paraphyletic to other *Penion*, with the species instead forming a clade with *Antarctoneptunea aurora* (Chapter 3). Geometric morphometric analyses find *P. benthicolus* to be easily distinguishable from other *Penion* (Chapter 6). Traditional examination of shell morphology indicates that *P. benthicolus* and *A. aurora* are similar, although *P. benthicolus* possibly exhibits a longer siphonal canal and more prominent axial ribs. These results contrast with previous suggestions that *P. benthicolus* most closely resembles *P. chathamensis* (Dell 1956, Powell 1979). Both *A. aurora* and *P. benthicolus* exhibit similar depth distributions and possibly overlap in their geographic distributions south of New Zealand. We suggest that *P. benthicolus* is reclassified within the genus *Antarctoneptunea*, representing a sister lineage to *A. aurora*.
Extant references	Dell 1956 (taxonomic description) Powell 1979 (taxonomic description)

	Dell 1995 (synonymy of *P. benthicolus delli*) Spencer *et al.* 2009 Spencer *et al.* 2017
Geological range	Present, Nukumaruan (2.40 – 1.63 Ma).
Fossil localities	Oaro, Canterbury (2.40 – 1.63 Ma). Cheviot, Canterbury (2.40 – 1.63 Ma).
Geological references	Beu 1979 Beu and Maxwell 1990
Sampled depth range	350 – 1723 m, deep.
Protoconch	3 – 4 whorls, large and 'beehive-shaped' with first protoconch whorl forming a cap.
Mean teleoconch length	9.41 cm (n = 52, SD = 1.16 cm).
Mean aperture length	5.35 cm (n = 52, SD = 0.71 cm).
Mean base protoconch width	3.32 mm (n = 52, SD = 0.45 mm).

Kelletia (Kellet's whelks)

Kelletia brevis† Ozaki, 1954
Figure 8.5
A: 4304a [NMNS] fossil holotype from Cape Inuwaka; B: 4303a [NMNS] fossil paratype from Cape Inuwaka.



Geological range	Very Late Miocene to Early Pliocene (5.60 – 3.80 Ma).
Fossil localities	Cape Inuwaka, Chōshi, Chiba Prefecture (5.60 – 3.80 Ma). Ochiai Formation, Kanagawa Prefecture (5.60 – 3.80 Ma). Naarai Formation, Chiba Prefecture (5.57 – 4.37 Ma). Osozawa Sandstone, Iitomi Formation, Yamanashi Prefecture (5.33 Ma).
Geological references	Matsushima *et al.* 2003 Ogasawara 2002 Okumura *et al.* 2011 Ozaki 1954 (taxonomic description) Shiba *et al.* 2014 Shiba *et al.* 2012 (for age estimation) Wade *et al.* 2011 (for age estimation)

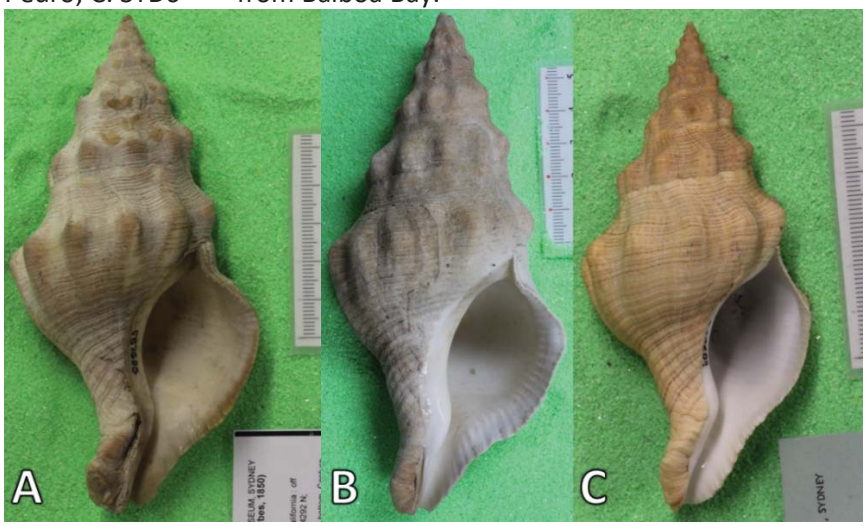
Kelletia ecuadoriana† Olsson, 1964
Figure 8.6
A: 644206 [USNM] fossil holotype from Quebrada, image courtesy of Daniel Levin (National Museum of Natural History, USA).

Comments	Shell morphology is similar to *K. kanakoffi* from California, USA.
Geological range	Miocene or Pliocene (limited stratigraphy, approximately 5.33 – 3.7 Ma).
Fossil localities	Esmeraldas Formation, Quebrada, Camarones, Ecuador (limited stratigraphy, approximately 5.33 – 3.7 Ma).
Geological references	Olsson 1964 (taxonomic description)

Kelletia kanakoffi† Hertlein, 1970
Figure 8.7
A: 22456 [LACMIP] fossil holotype from Lomita Marl, figured in Hertlein 1970.

Comments	Shell morphology is similar to *K. ecuadoriana* from Camarones, Ecuador.
Geological range	Pleistocene (0.57 – 0.40 Ma).
Fossil localities	Lomita Marl, California (0.57 – 0.40 Ma).
Geological references	Hertlein 1970 (taxonomic description)

Kelletia kelletii (Forbes, 1850)
Figure 8.8
A: C.87505 [AUS] from Redondo Beach, figured in Ponder 1973; B: M.217824 [MNZ] from off San Pedro; C: SYD6 [AUS] from Balboa Bay.

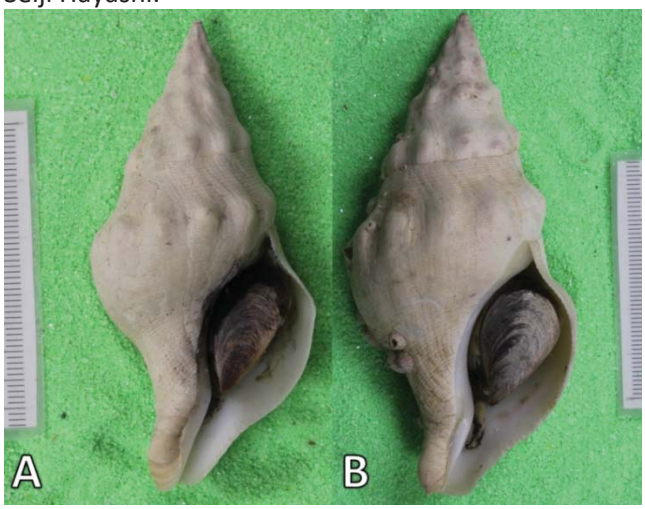


Geological range	Present (0.18 onwards), Late Pliocene (2.58 – 3.60 Ma).
Fossil localities	San Nicolas Island, California (0.18 – 0.08 Ma). Pico Formation, California (3.60 – 2.58 Ma).
Geological references	Powell and Stevens 2000 Schenck 1945 Vedder and Norris 1963 Zacherl *et al.* 2003
Sampled depth range	36 – 55, shallow.
Protoconch	1½ whorls, tiny; development confirmed as facultative planktotrophic by *in vitro* rearing (Vendetti 2009).
Mean teleoconch length	11.12 cm (n = 24, SD = 2.01 cm).
Mean aperture length	6.54 cm (n = 24, SD = 1.09 cm).
Mean base protoconch width	2.57 mm (n = 24, SD = 1.05 mm).

Kelletia kettlemanensis† (Arnold, 1910)

Geological range	Lower middle Pliocene (approximately 4.0 – 3.0 Ma).
Fossil localities	Basal Etchegoin formation, Kettleman Hills district, California (approximately 4.0 – 3.0 Ma).
Geological references	Arnold 1910 (taxonomic description)

Kelletia lischkei Kuroda, 1938
Figure 8.9
A – B: KL3 [MU] and KL2 [MU] from off Nayaura and Hoza-ura fisheries respectively, collected by Seiji Hayashi.



Geological range	Present (0.13 onwards).
Fossil localities	Katori Formation, Chiba Prefecture (0.13 Ma).
Geological references	Ogasawara 2002
Sampled depth range	5 – 15, shallow.
Protoconch	1.5 whorls, tiny.
Mean teleoconch length	9.49 cm (n = 8, SD = 1.49 cm).
Mean aperture length	5.55 cm (n = 8, SD = 0.65 cm).
Mean base protoconch width	2.03 mm (n = 8, SD = 0.70 mm).

Kelletia lorata† (Addicott, 1970)

Geological range	Middle Miocene (limited stratigraphy).
Fossil localities	Upper Olcese Sand, UCLA locality AC-2-34, east of Oil Center quadrangle, California.
Geological references	Addicott 1970 (taxonomic description)

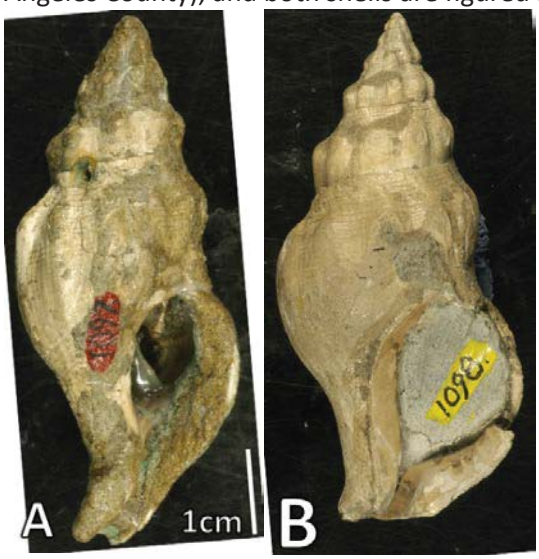
Kelletia posoensis† (Anderson & Martin, 1914)
Figure 8.10
A: MO650152 [USNM] fossil hypotype from Kern County, figured in Addicott 1970, image courtesy of Daniel Levin (National Museum of Natural History, USA).

Geological range	Early Miocene (approximately 25.2 – 21.7 Ma).
Fossil localities	Temblor Formation, CAS126, small creek near centre of section 34, San Luis Obispo County, California (approximately 25.2 – 21.7 Ma).
Geological references	Anderson and Martin 1914 (taxonomic description) Addicott 1970

Kelletia rugosa† (Olsson, 1964)
Figure 8.11
A: 644023 [USNM] fossil holotype from Punta Gorda, image courtesy of Daniel Levin (National Museum of Natural History, USA).

Geological range	Miocene or Pliocene (limited stratigraphy, approximately 5.33 – 3.7 Ma).
Fossil localities	Esmeraldas Formation, Quebrada, Camarones, Ecuador (limited stratigraphy, approximately 5.33 – 3.7 Ma).
Geological references	Olsson 1964 (taxonomic description)

Kelletia vladimiri† Kanakoff, 1954
Figure 8.12
A – B: 1097 [LACMIP] fossil holotype and 1098 [LACMIP] fossil paratype, both from section 27, Humprey quad. Both photos are courtesy of Lindsey T. Groves (Natural History Museum of Los Angeles County), and both shells are figured in Kanakoff 1954.



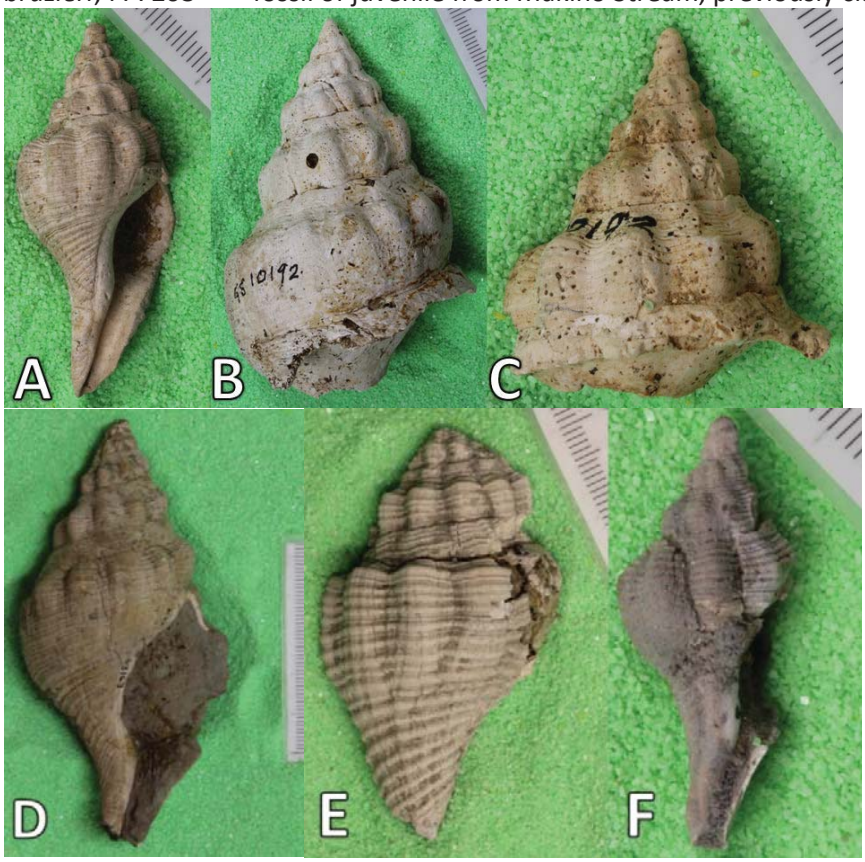
Geological range	Late Pliocene (3.60 – 2.58 Ma).
Fossil localities	Pico Formation, California (3.60 – 2.58 Ma). [Includes Humprey quad, LA County, California.]
Geological references	Kanakoff 1954 (taxonomic description)

Penion (siphon whelks)

Penion asper† (Marwick, 1928)

Figure 8.13

A – C: GNSE [GNS] fossil, and two GS10192 [GNS] fossils from Whenuataru Peninsula, D: M.43163 [MNZ] fossil from Marohau Point; E: 5170 [AU] fossil from Point Craig, classified as holotype of *P. brazieri*; F: 7203 [GNS] fossil of juvenile from Makino Stream, previously classified as *P. brazieri*.



Synonyms	*P. brazieri*† (Fleming, 1955)
Evidence used for change	Traditional examination of shell traits.
Comments	We struggle to distinguish *P. brazieri* from *P. asper.* The species resembles *P. chathamensis* (through comparison to synonym *P. fairfieldae;* Powell 1947), but specimens are smaller with more prominent axial ribs. No complete specimens are preserved.
Geological range	Kapitean to Mangapanian (7.20 – 2.40 Ma)
Fossil localities	Whenuataru Peninsula, Pitt Island, Chatham Islands (3.7 – 2.4 Ma). Flower Pot Harbour, Pitt Island, Chatham Islands (5.33 – 3.70 Ma). Maruhou Point, east of Te Araroa, Gisborne (7.20 – 5.33 Ma). Point Craig, Te Waewae Bay, Southland (7.20 – 5.33 Ma). Makino Stream, Huiroa, Taranaki (7.20 – 5.33 Ma)
Geological references	Beu and Maxwell 1990
Protoconch	2 ½ whorls, large.

Penion australocapax† Stilwell & Zinsmeister, 1992

Comments	It has been suggested that *P. australocapax* is a misclassified species of *Antarctoneptunea* (Beu 2009), although the overall shell morphology is most similar to species such as *P. sulcatus.* A number of similar fossils from Antarctic geological localities, previously also called *Penion*, have since been reclassified as other genera (Beu 2009).
Geological range	Upper Eocene to Lower Oligocene (limited stratigraphy, approximately 37.0 – 28.1 Ma)
Fossil localities	La Mesta Formation, Seymour Island, Antarctica Peninsula.
Geological references	Stilwell and Zinsmeister 1992 (taxonomic description) Beu 2009

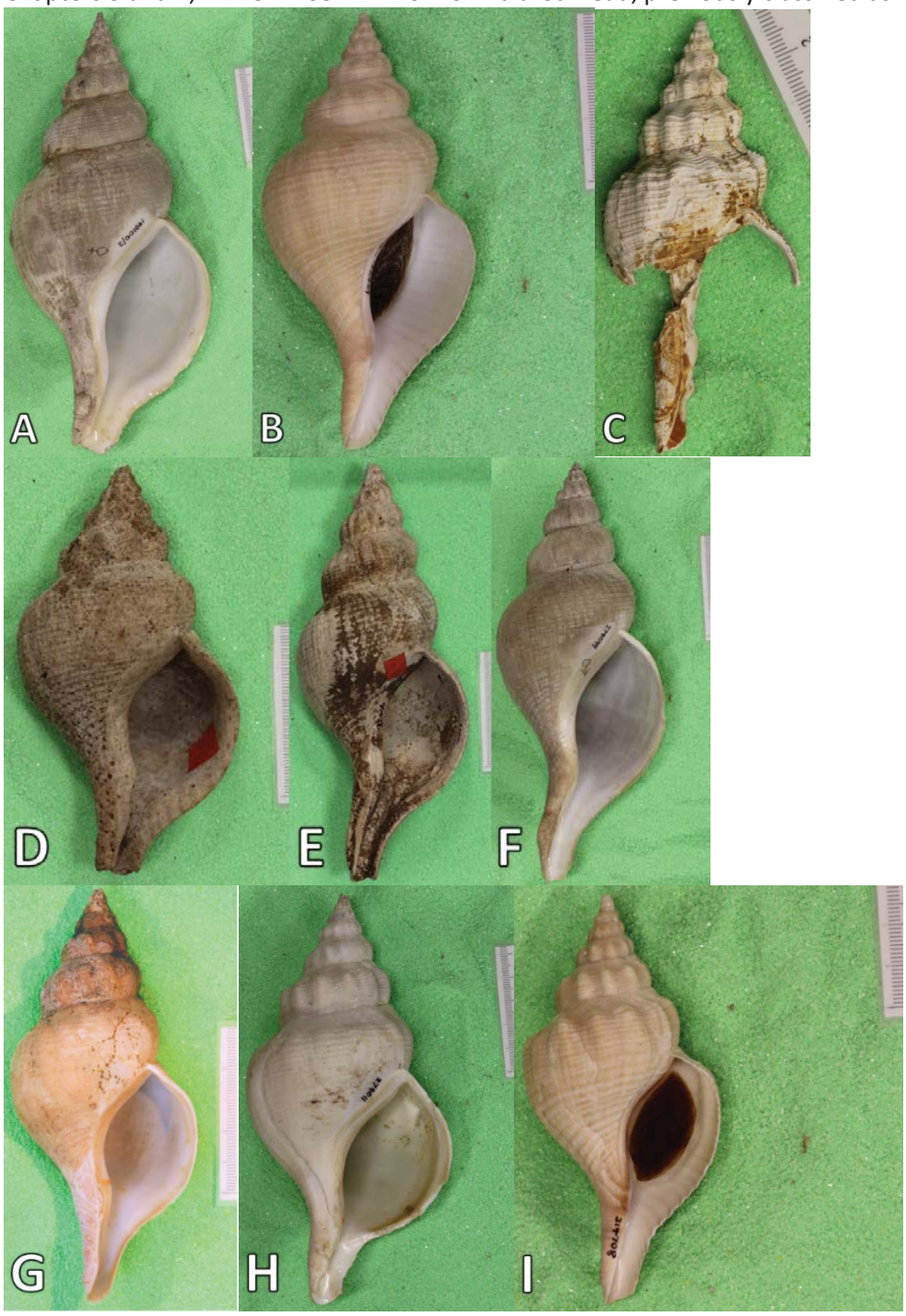
Penion bartrumi† (Laws, 1941)

Figure 8.14

A: TM1288 [GNS] fossil from Kaipara Harbour, figured in Beu and Raine 2009.

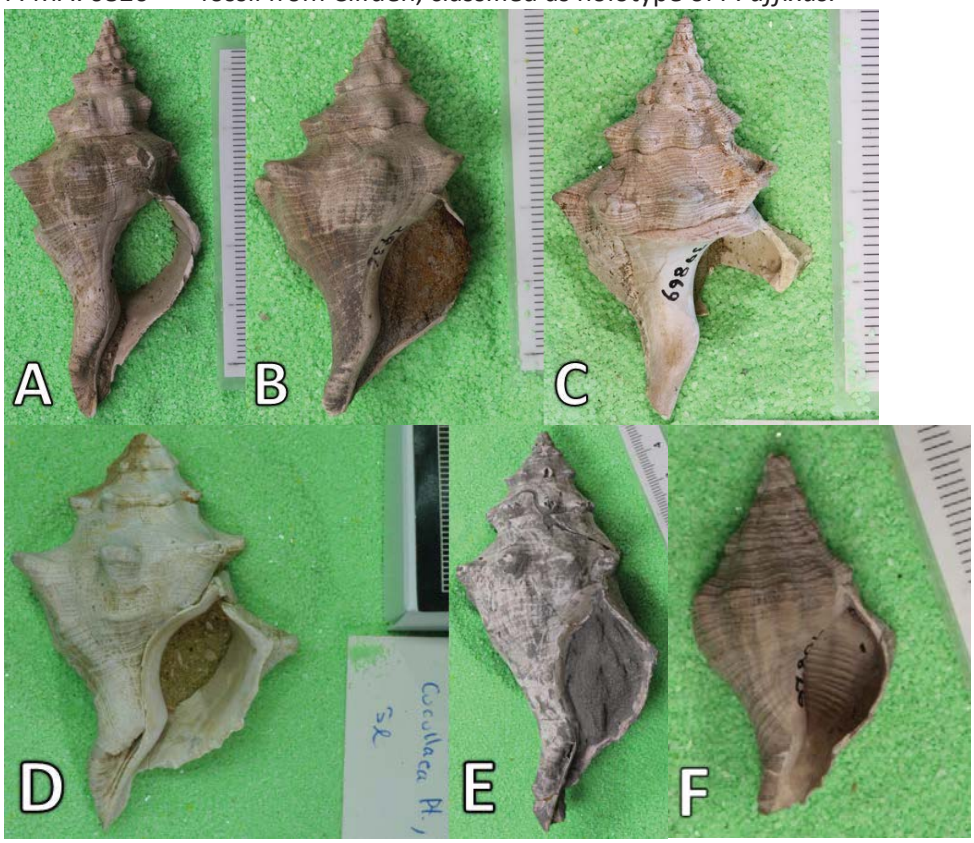
Geological range	Altonian (18.7 – 15.9 Ma).
Fossil localities	Pakurangi Formation, Kaipara Harbour, Auckland (18.7 – 15.9 Ma).
Geological references	Beu and Raine 2009 (taxonomic description)
Protoconch	1½ whorls, tiny.

Penion chathamensis (Powell, 1938)
Figure 8.15
A: M.190100 [MNZ] from Mernoo Bank, tissue sequenced in Chapter 3; B: M.132409 [MNZ] from Otago Penisula; C: GS10337 [GNS] from Oaro, formerly classified as *P. fairfieldae* fossil, figured in Beu 1979; D: MA71145 [AM] from off Otago, classified as holotype of *P. fairfieldae*; E: MA7077 [AM] from Kaingaora Beach, holotype of *P. chathamensis*; F: M.274099 [MNZ] from Mernoo Bank; G: Phoenix1 [MU] from off Otago Peninsula, previously classified as *P. fairfieldae*, tissue sequenced in Chapter 4; H: M.274011 [MNZ] from east of Auckland Islands, tissue sequenced in Chapters 3 and 4; I: M.314708 [MNZ] from off Taiaroa Head, previously classified as *P. fairfieldae.*



Synonyms	*P. fairfieldae* (Powell, 1947)
Evidence used for change	Molecular phylogenetics, traditional examination of shell traits, geometric morphometric analysis of shells.
Comments	Based on recent re-examination of shells, *P. fairfieldae* is similar to *P. chathamensis* in shell morphology, although shells of *P. fairfieldae* are often smaller and exhibit shorter siphonal canals. The two species were believed to have distinct but adjacent geographic ranges, with *P. fairfieldae* restricted to coastal waters off Otago and Canterbury. Molecular phylogenetics and geometric morphometric analysis of shell shape indicates that the two species are indistinguishable and likely to be conspecific (Chapters 4, 6). Previous taxonomic assessments suggested similarities between *P. chathamensis* and *P. ormesi* (Powell 1947, 1979). The length of the siphonal canal likely does exhibit a phylogenetic signal among buccinids, but it can also be highly variable due to ecology. A likely mechanism is that individuals in shallow-water populations are more exposed to wave action, preventing the mantle from extending far enough to grow a long siphonal canal (Ponder 1971). Many individuals of all species also appear to have grown and subsequently broken longer siphonal canals, giving arise to broader apertures that end abruptly. Some extant and fossils specimens exhibit more prominent axial ribs (e.g. C, H – I; across both putative *P. chathamensis* and *P. fairfieldae*), which raises the possibility that *P. asper* and *P. imperfectus* may actually be conspecific to *P. chathamensis* (see Powell 1979).
Extant references	Powell 1947 Powell 1979 Spencer *et al.* 2009 Spencer *et al.* 2017
Geological range	Present, Nukumaruan (2.40 – 1.63 Ma).
Fossil localities	Oaro, Canterbury (2.40 – 1.63 Ma).
Geological references	Beu 1979
Sampled depth range	27 – 620 m, predominantly deep.
Protoconch	3 – 3½ whorls, large.
Mean teleoconch length	17.22 cm (n = 84, SD = 2.89 cm).
Mean aperture length	10.41 cm (n = 84, SD = 1.33 cm).
Mean base protoconch width	2.97 mm (n = 84, SD = 0.44 mm).

Penion clifdenensis† (Finlay, 1930)
Figure 8.16
A – C: all fossils from Clifden, 10365 [GNS] previously classified as *P. parans* topotype, 2936 [GNS] topotype, MA36591 [AM]; D: V587 [VU] fossil from Clifden; E: GNSD [GNS] fossil from Weka Creek; F: MA70826 [AM] fossil from Clifden, classified as holotype of *P. affixus.*



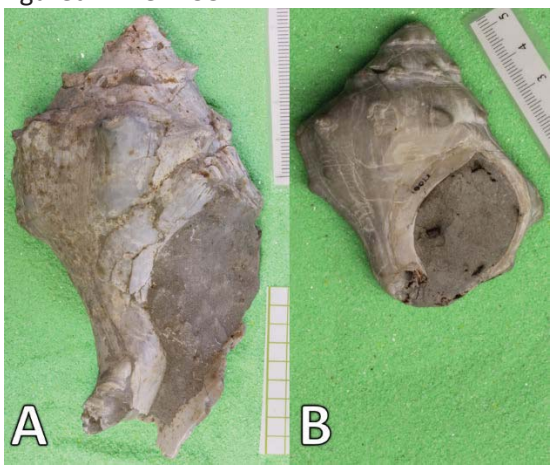
Synonyms	*P. affixus*† (P. Fischer, 1884), *P. parans*† (Finlay, 1930)
Evidence used for change	Traditional examination of shell traits.
Comments	*Penion affixus* and *P. parans* are restricted to the Clifden locality, which also hosts the majority of *P. clifdenensis* specimens. We argue that all three taxa are conspecific as there is no obvious feature to distinguish them. Shells of all species are small, have prominent axial ribs, and protoconchs and siphonal canals of similar dimensions. The holotype of *P. affixus* (F) is likely a juvenile due to its small size and low number of teleconch whorls, and although it is smoother than most specimens of *P. clifdenensis*, the fourth teleoconch whorl is beginning to show axial rib growth that resembles typical specimens of *P. clifdenensis* and *P. parans*. Small specimens classified as juveniles of *P. clifdenensis* and *P. parans* also resemble this holotype specimen.
Protoconch	2 ½ to 3 whorls, large.
Geological range	Altonian to Lilburnian (18.70 – 13.05 Ma).
Fossil localities	Te Awaite, Wairarapa, Wellington (11.04 Ma). Clifden, Waiau River, Southland (15.9 – 13.05 Ma). Weka Creek, Canterbury (18.7 – 15.9 Ma).
Geological references	Beu and Maxwell 1990
Protoconch	2½ – 3 whorls, medium to large.
Mean teleoconch length	5.32 cm (n = 6, SD = 2.89 cm).

Mean aperture length	3.20 cm (n = 6, SD = 0.46 cm).
Mean base protoconch width	1.64 mm (n = 6, SD = 0.27 mm).

Penion crassus† Frassinetti 2000

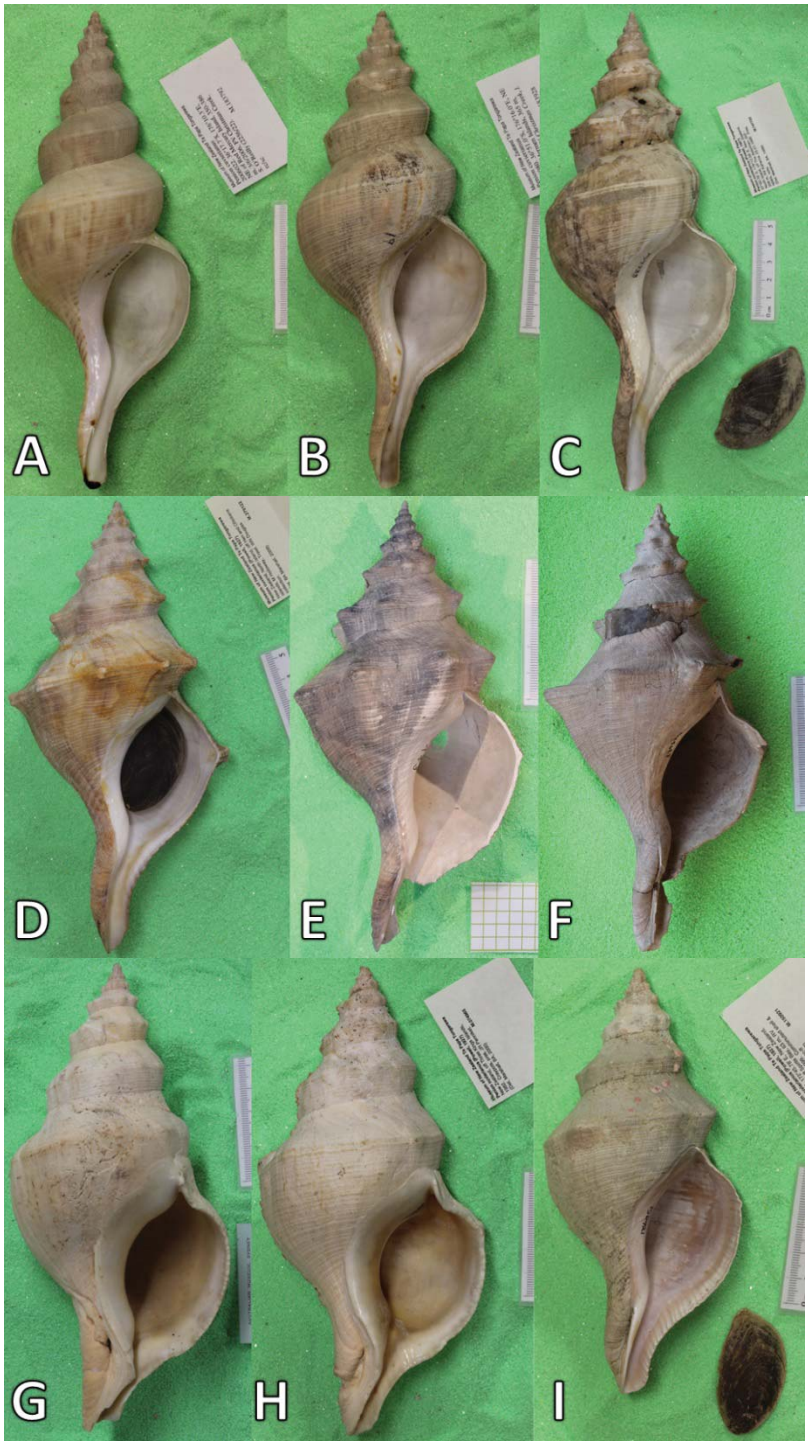
Geological range	Early Pliocene (limited stratigraphy, approximately 5.0 Ma).
Fossil localities	Guafo Island (approximately 5.0 Ma).
Geological references	Frassinetti 2000 (taxonomic description)

Penion crawfordi† (Hutton, 1873)
Figure 8.17
A: MA36586 [AM] fossil from Hurupi Stream; B: GNSM [GNS] fossil holotype from Te Awaiti, figured in Dell 1952.



Geological range	Lilburnian to Opoitian (15.1 – 3.7 Ma).
Fossil localities	Mangatoro Reserve, Dannevirke, Manawatu-Wanganui (5.33 – 3.70 Ma). Hurupi Stream, Cape Palliser, Wairarapa, Wellington (11.04 – 7.20 Ma). South of Takapau, Hawke's Bay (11.04 – 7.20 Ma). Tutamoe formation, Takapau, Hawke's Bay (13.05 – 7.20 Ma). Burnt Hill, Oxford, Canterbury (13.05 – 11.04 Ma).
Geological references	Dell 1952 Beu and Raine 2009
Protoconch	None preserved.

Penion cuvierianus (Powell, 1927)
Figure 8.18
A: M.183792 [MNZ] from off Red Mercury Island, tissue sequenced in Chapters 3 and 4; B: M.183928 [MNZ] from tissue from off Alderman Islands, tissue sequenced in Chapters 3 and 4; C: M.147733 [MNZ] from off Alderman Islands; D: M.279122 [MNZ] from off Hen and Chickens Islands; E: GS15443 [GNS] fossil from Te Piki; F: AU5627 [AU] fossil from Ohiwa Harbour; G – H: C90358 [AUS] and M.074965 [MNZ] from off Three Kings Islands, classified as *P.* aff. *cuvierianus*; I: M.150921 [MNZ] from Spirits Bay, classified as *P.* aff. *cuvierianus*.



Common names	Flaring *Penion*
Synonyms	*P. cuvierianus cuvierianus* (Powell, 1927)*; P.* aff. *cuvierianus cuvierianus*
Evidence used for change	Molecular phylogenetics, traditional examination of shell traits, geometric morphometric analysis of shells.
Comments	*Penion cuvierianus* is one of the most morphologically variable species of *Penion.* Specimens off far northern Northland (e.g. G – I), referred to as ***P.* aff. *c. cuvierianus***, were investigated as being a distinct species in Chapters 4 and 6. Based on the traditional examination of shell traits these specimens from far north Northland most closely resemble *P. c. cuvierianus*, but shells are thicker and heavier, with short siphonal canals. Geometric morphometric analysis of shells indicates that the two taxa can be readily distinguished (Chapter 6). However, mtDNA and rDNA sequence from a single individual of *P.* aff. *c. cuvierianus* indicates that there is little genetic difference from individuals of *P. c. cuvierianus* (Chapter 4). Since only one individual was available for sequencing, and because no faster evolving nuclear markers were sequenced, it is premature to deny the possibility that this population represents a very recent event of speciation, hybridisation or genetic isolation by distance. Nonetheless, current evidence merely suggests that *P. cuvierianus* is a single species with significant intraspecific morphological variation. We suggest referring to the taxon as *P. cuvierianus* as the former subspecies *P. c. jeakingsi* is not closely related and is morphometrically distinguishable (Chapters 4, 6).
Extant references	Powell 1927 (taxonomic description) Ponder 1975 Powell 1979 Spencer *et al.* 2009 Spencer *et al.* 2017
P. cuvierianus (sensu stricto)	
Geological range	Present, Nukumaruan to Castlecliffian (2.40 – 0.34 Ma).
Fossil localities	Ohiwa Harbour, Bay of Plenty (1.63 – 0.34 Ma). Devil's Elbow, Hawke's Bay (2.40 – 1.63 Ma). Te Piki, Cape Runaway, Bay of Plenty (2.40 – 1.63 Ma).
Geological references	Beu and Maxwell 1990
Sampled depth range	3 – 503 m, shallow to deep.
Protoconch	3 – 4 whorls, large.
Mean teleoconch length	15.64 cm (n = 64, SD = 3.37 cm).
Mean aperture length	9.61 cm (n = 64, SD = 1.92 cm).
Mean base protoconch width	3.56 mm (n = 64, SD = 0.64 mm).
P. aff. *cuvierianus* only	
Geological range	Present.
Fossil localities	None yet recognised.
Sampled depth range	63 – 110 m, deep.
Protoconch	2 to 2½ whorls, large.
Mean teleoconch length	17.94 cm (n = 7, SD = 2.27 cm).
Mean aperture length	10.89 cm (n = 7, SD = 1.28 cm).

Mean base protoconch width	3.98 mm (n = 7, SD = 0.50 mm).

Penion darwinianus† (Philippi, 1887)

Geological range	Early Miocene (limited stratigraphy, 23.03 – 15.9 Ma?)
Fossil localities	Stokes Island (limited stratigraphy, 23.03 – 15.9 Ma?)
Geological references	Frassinetti 2001

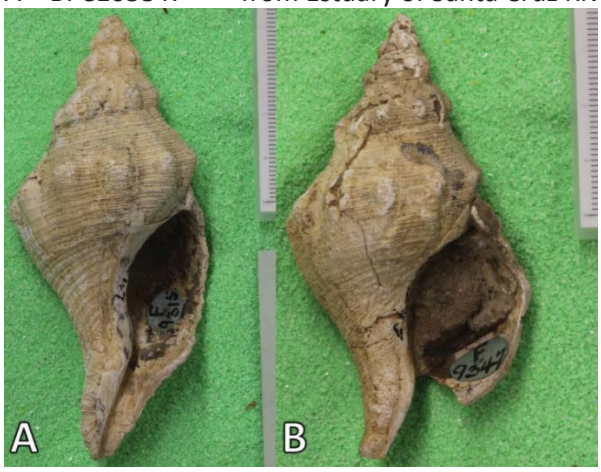
Penion diversum† Frassinetti, 2000

Geological range	Early Pliocene (limited stratigraphy, approximately 5.0 Ma).
Fossil localities	Guafo Island (approximately 5.0 Ma).
Geological references	Frassinetti 2000 (taxonomic description)

Penion domeykoanus† (Philippi, 1887)

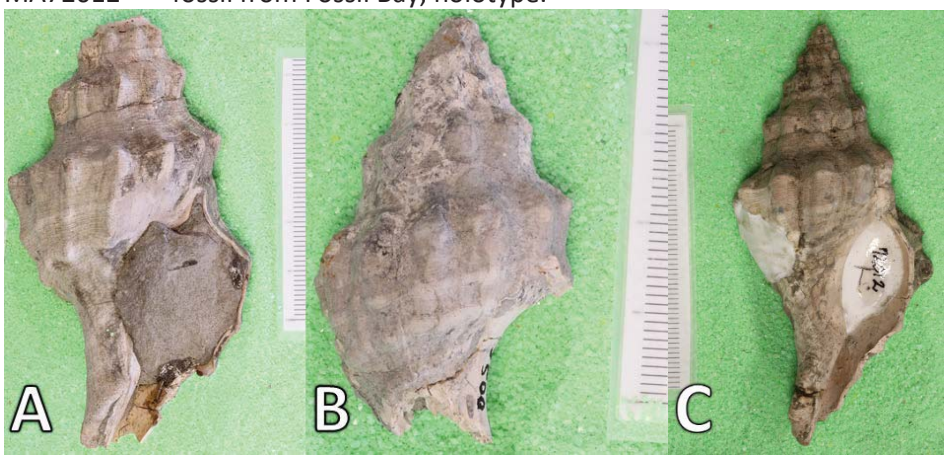
Figure 8.19

A – B: C163847 [AUS] from Estuary of Santa Cruz River, Argentina.



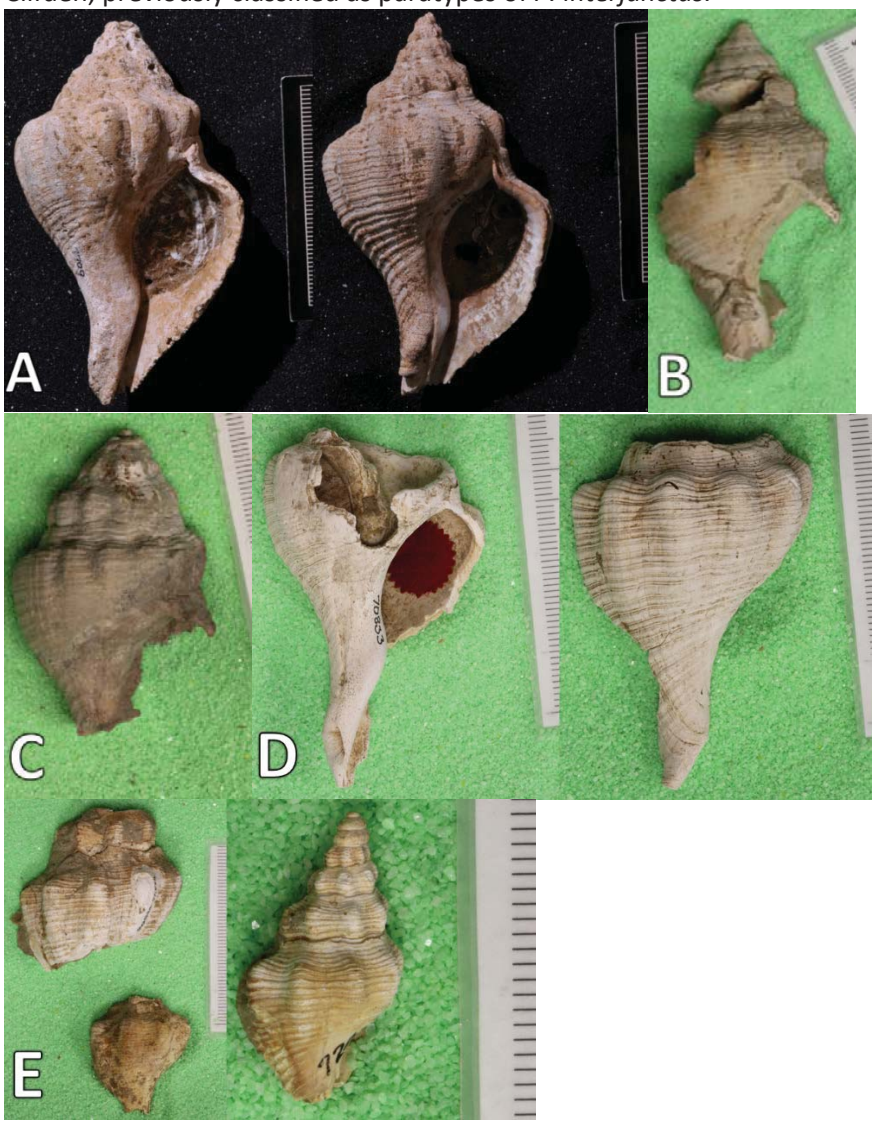
Synonyms	*P. domeykoana* (Philippi, 1887)
Geological range	Early Miocene (limited stratigraphy, 23.03 – 15.9 Ma?)
Fossil localities	Santa Cruz, Chile (limited stratigraphy, 23.03 – 15.9 Ma?). Estuary of Río Santa Cruz, Patagonia, Argentina (limited stratigraphy, 23.03 Ma?)
Geological references	Ponder 1973 Nielsen 2003 (taxonomic description)

Penion exoptatus† (Powell & Bartrum, 1929)
Figure 8.20
A: MA36579 [AM] fossil from Squadron Bay; B: MA36564 [AM] fossil from Little Oneroa; C: MA72012 [AM] fossil from Fossil Bay, holotype.



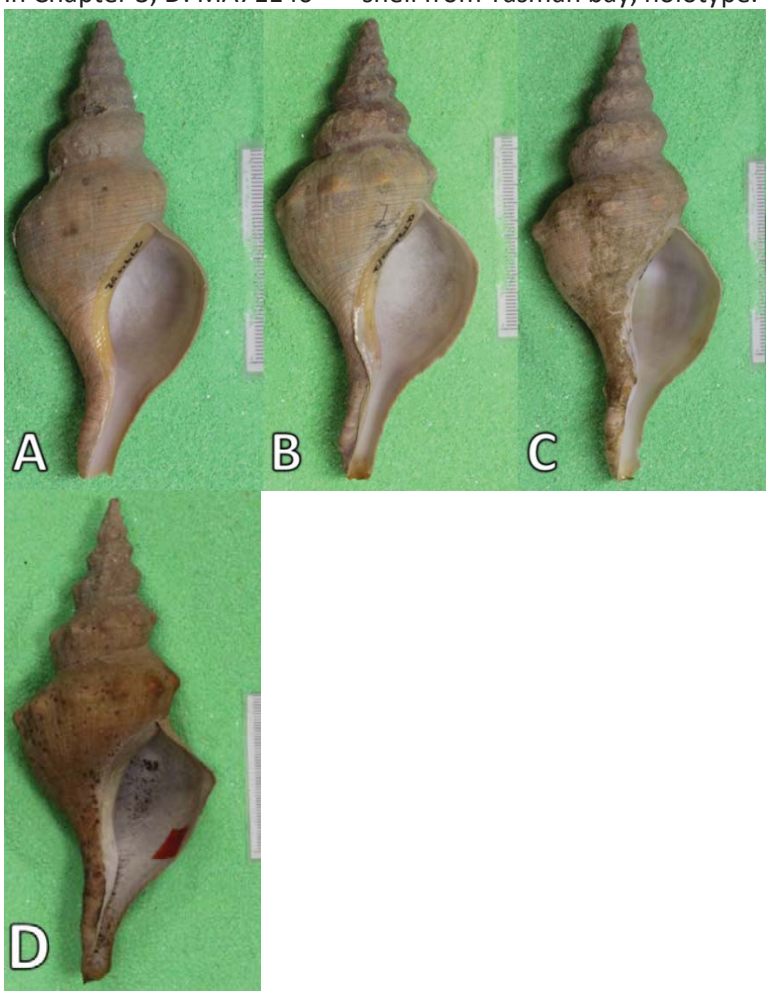
Geological range	Otaian (21.7 – 18.7 Ma).
Fossil localities	Fossil Bay, Waiheke Island (21.7 – 18.7 Ma). Little Oneroa, Waiheke Island (21.7 – 18.7 Ma). Squadron Bay, Waiheke Island (21.7 – 18.7 Ma).
Geological references	Beu and Maxwell 1990
Protoconch	None preserved.

Penion imperfectus† (Marwick, 1928)
Figure 8.21
A: GS7709 [GNS] two fossils from Clifden, previously classified as *P. interjunctus*; B: GNSO [GNS] fossil from Awamoa Beach, holotype; C: GNSP [GNS] fossil from Pareora, paratype; D: MA70833 [AM] fossil from Clifden, classified as holotype of *P. interjunctus*; E: MA72476 [AM] fossils from Clifden, previously classified as paratypes of *P. interjunctus.*



Synonyms	*P. interjunctus*† (Finlay, 1930)
Evidence used for change	Traditional examination of shell traits.
Comments	Likely shares common ancestry with *P. asper* and *P. chathamensis* (Powell 1979). We argue that *P. interjunctus* is conspecific with *P. imperfectus* as fossils are similar in size with smooth axial ribs and originate from localities somewhat close in geological time.
Geological range	Lilburnian to Waiauan (15.10 – 11.04 Ma), Altonian (18.7 – 15.9 Ma).
Fossil localities	Clifden, Southland (15.10 – 11.04 Ma). Awamoa Beach, Oamaru, Otago (18.7 – 15.9 Ma). Pareora, Canterbury (18.7 – 15.9 Ma).
Geological references	Powell 1947 (taxonomic description) Beu and Maxwell 1990
Protoconch	2½ whorls, large.

Penion jeakingsi (Powell, 1947)
Figure 8.22
A – C: M.279432-1, -2, -3 [MNZ] extant shells from Tasman Bay, tissue from all three sequenced in Chapter 3; D: MA71146 [AM] shell from Tasman bay, holotype.



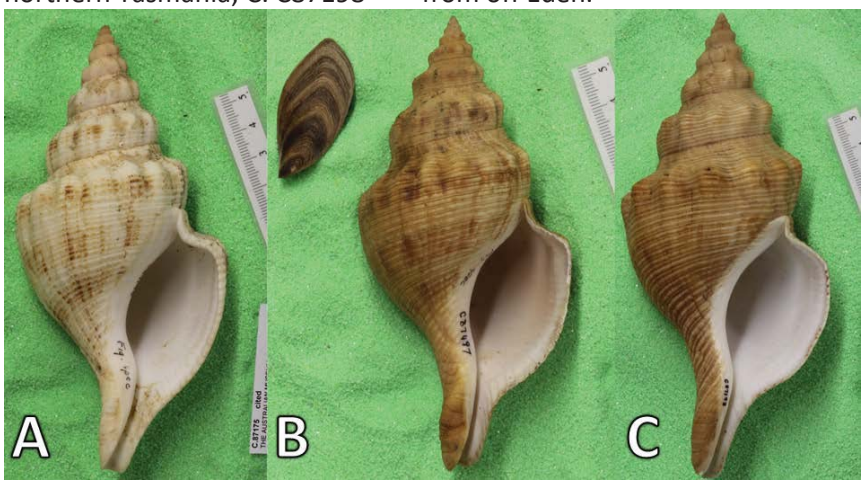
Synonyms	*P. cuvierianus jeakingsi* (Powell, 1947)
Evidence used for change	Molecular phylogenetics, traditional examination of shell traits, geometric morphometric analysis of shells.
Comments	Formerly classified as *P. cuvierianus jeakingsi*, we suggest that this taxon is reclassified as a distinct species based on molecular data (Chapters 4). *Penion jeakingsi* resembles *P. cuvierianus* using traditional shell traits, however *P. jeakingsi* has blunted axial ribs (or none) past the first three teleoconch whorls and is often smaller. *Penion cuvierianus* is also restricted to the northern coast of the North Island, whereas *P. jeakingsi* is restricted to the Cook Strait and the western coast of the North Island. The species only potentially overlaps within proximity to the Three Kings Islands. Some specimens of *P. jeakingsi* resemble *P. ormesi*, but *P. jeakingsi* has a shorter teleoconch spire and more prominent axial ribs. Based on mtDNA and nuclear rDNA sequence data, *P. ormesi* is closely related to *P. jeakingsi* and *P.* n. sp. West Coast (Chapter 4). Short length mtDNA sequence data from further individuals of each species indicated that there are multiple genetic lineages within *P. jeakingsi,* and nuclear SNP analysis was able

	to differentiate *P. ormesi* and *P. jeakingsi* (Chapter 4). Morphometric results to distinguish *P. ormesi* and *P. jeakingsi* were mixed (Chapter 6). Further genetic sampling is needed to investigate the situation within the *P. ormesi* species complex overall.
Extant references	Powell 1947 (taxonomic description) Powell 1979 Spencer *et al.* 2009 Spencer *et al.* 2017
Geological range	Present.
Fossil localities	None yet recognised.
Sampled depth range	Shallow to deep, 18 – 587 m.
Protoconch	2½ – 4 whorls, medium to large.
Mean teleoconch length	13.66 cm (n = 63, SD = 1.91 cm).
Mean aperture length	8.39 cm (n = 63, SD = 1.07 cm).
Mean base protoconch width	3.70 mm (n = 63, SD = 0.91 mm).

Penion macsporrani† (Philippi, 1887)

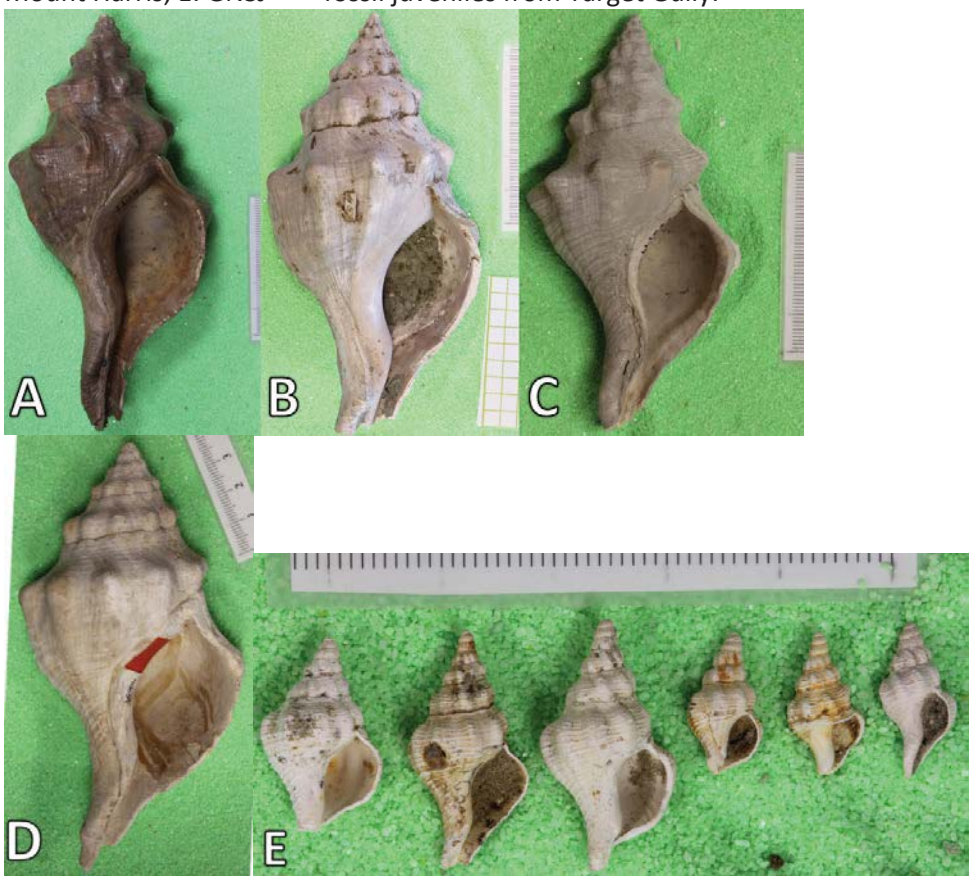
Geological range	Early Miocene (limited stratigraphy, 23.03 – 15.9 Ma?)
Fossil localities	Santa Cruz, Chile (limited stratigraphy, 23.03 – 15.9 Ma?). Isla Crosslet, Chile (limited stratigraphy, 23.03 – 15.9 Ma?). Isla Hereford, Chile (limited stratigraphy, 23.03 – 15.9 Ma?).
Geological references	Covacevich and Frassinetti 1986 Nielsen 2003 (taxonomic description)

Penion mandarinus (Duclos, 1832)
Figure 8.23
All figured in Ponder 1973. A: C.87175 [AUS] from Port Macdonell; B: C.87497 [AUS] from off northern Tasmania; C: C87198 [AUS] from off Eden.



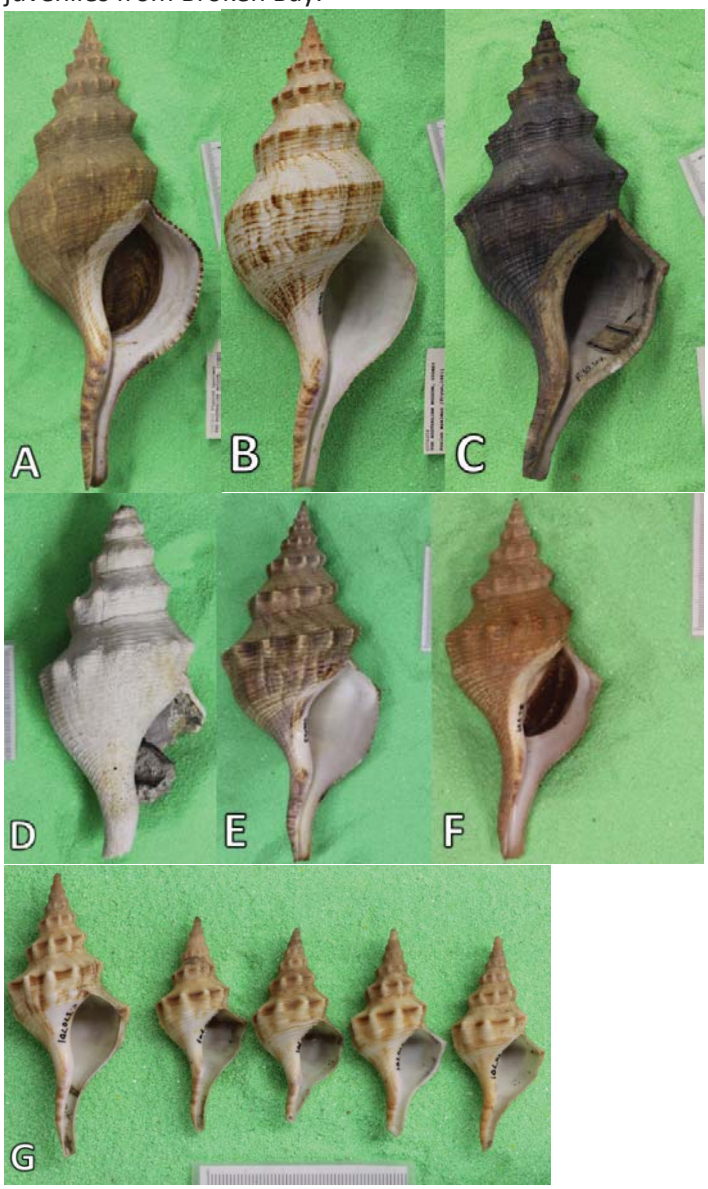
Common names	Mandarin *Penion*; southern siphon whelk; Waite's *Buccinum* whelk
Geological range	Present, Werrikooian (1.806 – 1.000 Ma), Kalimnan to Yatalan (4.3 – 2.5 Ma).
Fossil localities	Strathdowne, Victoria (1.806 – 1.000 Ma). Cameron Inlet Formation, Flinders Island, Tasmania (3.5 – 2.5 Ma). Grange Burn Formation, Orange Burn, Victoria (4.3 – 3.4 Ma).
Geological references	Ponder 1973 (taxonomic description)
Sampled depth range	7 – 549 m, shallow to deep.
Protoconch	1½ – 2 whorls, tiny. Speculated to have planktonic larvae (Ponder 1973).
Mean teleoconch length	14.30 cm (n = 50, SD = 2.55 cm).
Mean aperture length	8.66 cm (n = 50, SD = 1.48 cm).
Mean base protoconch width	2.26 mm (n = 50, SD = 0.54 mm).

Penion marwicki† (Finlay 1930)
Figure 8.24
A: GNSB [GNS] fossil from Mount Harris; B: MA36600 [AM] fossil from Pareora, formerly classified as *P. finlayi*; C: M.288199 [MNZ] from Awamoa Beach; D: MA70834 [AM] fossil holotype from Mount Harris; E: GNSJ [GNS] fossil juveniles from Target Gully.



Synonyms	*P. finlayi*† (Laws, 1930)
Evidence used for change	Traditional examination of shell traits.
Comments	We treat *P. finlayi* as conspecific to *P. marwicki* as there is no clear trait in shell morphology to separate them, and because specimens originate from the same geological sites and strata.
Geological range	Altonian (18.7 – 15.9 Ma).
Fossil localities	Mount Harris, Canterbury (18.7 – 15.9 Ma). Timaru, Canterbury (18.7 – 15.9 Ma). Pareora, Canterbuty (18.7 – 15.9 Ma). Awamoa Beach, Ardgowan shellbeds, Oamaru, Otago (18.7 – 15.9 Ma). Target Gully, Otago (18.7 – 15.9 Ma).
Geological references	Beu and Raine 2009 (taxonomic description)
Protoconch	2½ – 3½ whorls, medium.

Penion maximus (Tryon, 1881)
Figure 8.25
A: C.87203 [AUS] from off Cape Moreton, figured in Ponder 1973; B: C076058 [AUS] from Shell Harbour; C: C370952 [AUS] fossil from Stockton; D: MF.262364 trawled off Eden; E: P316911 [VIC] fossil from Cameron Inlet; F: MF.34889 [MNZ] from D'Entrecasteaux Channel; G: C370701 [AUS] juveniles from Broken Bay.



Common names	Giant whelk; great whelk
Geological range	Present (0.34 Ma onwards), Kalimnan to Yatalan (3.5 – 2.5 Ma).
Fossil localities	Stockton, Newcastle, New South Wales (approximately 0.34 Ma). Cameron Inlet Formation, Flinders Island, Tasmania (3.5 – 2.5 Ma).
Geological references	Ponder 1973 (taxonomic description)
Sampled depth range	Predominantly deep, 36 – 220 m.
Protoconch	1½ – 3 whorls, tiny to small.
Mean teleoconch length	20.05 cm (n = 50, SD = 3.32 cm).
Mean aperture length	12.33 cm (n = 50, SD = 2.13 cm).
Mean base protoconch width	2.40 mm (n = 50, SD = 0.44 mm).

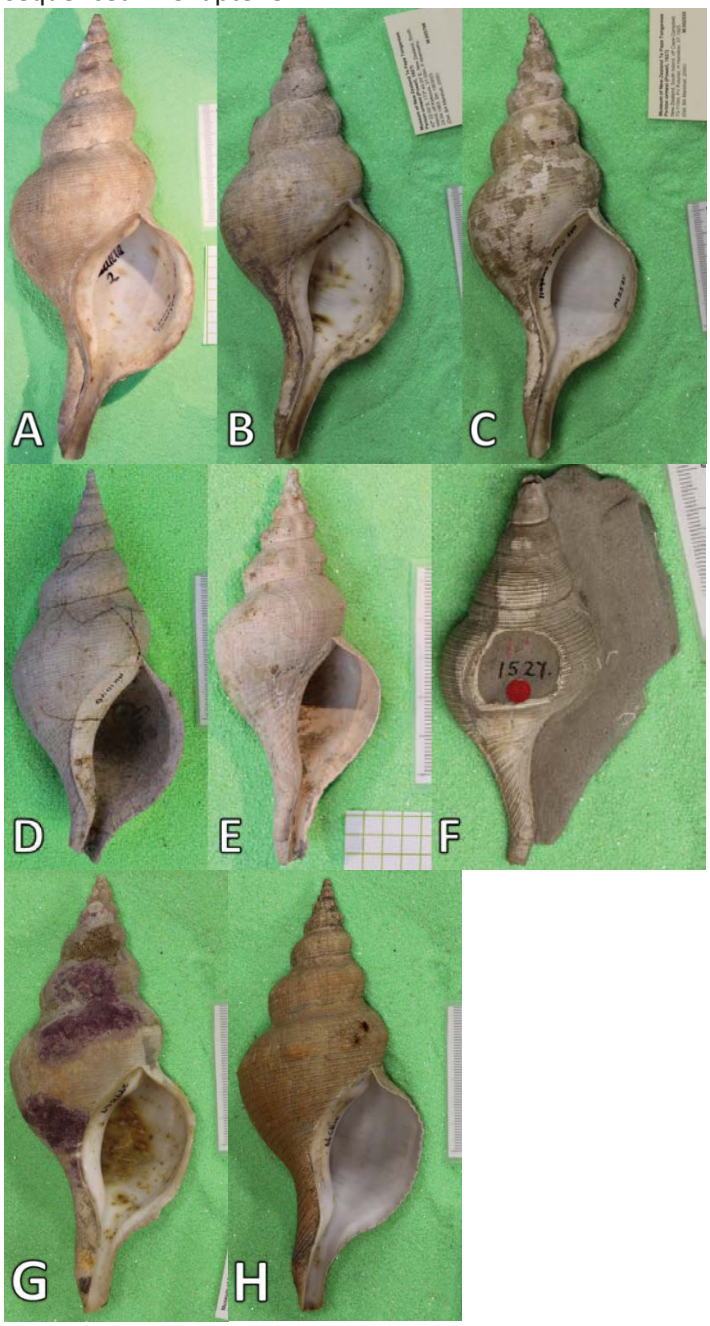
Penion oncodes† (Philippi, 1887)

Geological range	Early Miocene (limited stratigraphy, roughly 23.03 – 15.9 Ma).
Fossil localities	Santa Cruz, Chile (limited stratigraphy, roughly 23.03 – 15.9 Ma).
Geological references	Nielsen 2003 (taxonomic description)

Penion ormesi (Powell, 1927)

Figure 8.26

A: 257 [GNS] paratype from off Cape Campbell; B: M.005768 [MNZ] from off Kaikoura; C: M.002535 [MNZ] from off Cape Campbell; D: AU1046 [AU] fossil from Wanganui; E: 4082 [GNS] fossil from Castlecliff; F: GS1527 [GNS] fossil formerly classified as *P. winthropi* holotype; G: M.299869 [MNZ] from Cloudy Bay, tissue sequenced in Chapter 3; H: M.318599 [MNZ] from Pelorus Sound, tissue sequenced in Chapter 3.



Synonyms	*P. winthropi*† (Marwick, 1965), *Penion adustus worthyae* (Powell, 1947)
Evidence used for change	Molecular phylogenetics, traditional examination of shell traits, geometric morphometric analysis of shells.
Comments	Molecular phylogenetic and geometric morphometric results indicate that that some extant specimens of *P. sulcatus* from Tasman Bay (previously classified as *P. adustus worthyae* and then *P. sulcatus*) are actually *P. ormesi* (data in Chapters 4, 5 but not discussed). Fossils from Wanganui often exhibit short siphonal canals but otherwise match the shell morphology of extant populations (tall teleoconch spire, axial ribs only prominent on first few teleoconch whorls, large protoconch). The holotype specimen of *P. winthropi* is poorly preserved, but the tall spire resembles *P. ormesi.* Based on mtDNA and nuclear rDNA sequence data, *P. ormesi* is closely related to *P. jeakingsi* and *P.* n. sp. West Coast (Chapter 4). Short length mtDNA sequence data from further individuals of each species indicated that there are multiple genetic lineages within *P. jeakingsi,* and nuclear SNP analysis was able to differentiate *P. ormesi* and *P. jeakingsi* (Chapter 4). Morphometric results to distinguish *P. ormesi* and *P. jeakingsi* were mixed (Chapter 6). Further genetic sampling is needed to investigate the situation within the *P. ormesi* species complex overall.
Extant references	Powell 1927 (taxonomic description) Powell 1979 Spencer *et al.* 2009 Spencer *et al.* 2017
Geological range	Present, Castlecliffian (1.63 – 0.34 Ma), Waipipian (3.7 – 2.4 Ma).
Fossil localities	Castlecliff and nearby streams, Manawatu-Wanganui (1.63 – 0.34 Ma). Nukumaru, Manawatu-Wanganui (2.40 – 1.63 Ma). Waihi Beach, Hawera, Taranaki (3.7 – 3.0 Ma). Makaretu Stream, Levin, Manawatu-Wanganui (3.7 – 3.0 Ma).
Geological references	Beu and Maxwell 1990
Sampled depth range	37 – 695 m, predominantly deep.
Protoconch	3 – 4 whorls, large.
Mean teleoconch length	16.55 cm (n = 45, SD = 2.62 cm).
Mean aperture length	9.89 cm (n = 45, SD = 1.47 cm).
Mean base protoconch width	3.34 mm (n = 45, SD = 0.73 mm).

Penion patagonensis† Reichler, 2010

Geological range	Lower Miocene (limited stratigraphy, approx. 20.43 – 15.97 Ma)
Fossil localities	Gran Bajo del Gualicho Formation, Saladar member, Argentina (20.43 – 15.97 Ma).
Geological references	Reichler 2010 (taxonomic description)

Penion petitianus† (d'Orbigny, 1842)

Comments	Nielsen only cautiously classifies this species as it is based on a single small, but distinct teleoconch spire fragment. Since we have not sampled any fossils from South America we do not suggest a change, but we follow Nielsen's policy of a 'soft' classification.
Geological range	Early Miocene (limited stratigraphy, approximately 23.03 – 15.9 Ma).
Fossil localities	Santa Cruz, Chile (limited stratigraphy, approximately 23.03 – 15.9 Ma).
Geological references	Nielsen 2003 (taxonomic description)

Penion proavitus† (Finlay & Marwick, 1937)

Figure 8.27

A: MA73353 [AM] fossil juvenile from Boulder Hill, holotype; B: GNSC [GNS] fossil from Mitchell Rocks; C: GS10195 [GNS] fossil juvenile from Boulder Hill, paratype.

Comments	The holotype and paratype specimens of *P. proavitus* appear to be juveniles, and resemble those of other *Penion* species. The only apparent adult specimen of *P. proavitus* is bisected and too damaged for detailed comparison, and it may be misclassified specimen of another buccinid such as *Aeneator* Finlay, 1926.
Geological range	Teurian (66.04 – 55.80 Ma).
Fossil localities	Wangaloa Formation, Mitchell Rocks, Wangaloa, Otago (66.04 – 55.80 Ma). Boulder Hill, Dunedin, Otago (66.0 – 56.0 Ma).
Geological references	Finlay and Marwick 1937 Powell 1947 Beu and Maxwell 1990
Protoconch	None preserved.

Penion subrectus† (Ihering, 1899)

Geological range	Late Oligocene-Early Miocene (limited stratigraphy, approximately 23.03 – 15.97 Ma)
Fossil localities	Estuary of Río Santa Cruz, Argentina (approximately 23.03 Ma?) Estancia Busnadiego, Argentina (approximately 23.03 – 15.97 Ma?).
Geological references	Ponder 1973 Beu *et al.* 1997 del Rio 2004 Parras and Griffin 2009

Penion subreflexus† (Sowerby, 1846)

Synonyms	*Penion subreflexa* (Sowerby, 1846)
Geological range	Early Miocene (limited stratigraphy, approximately 23.03 – 15.9 Ma)
Fossil localities	Santa Cruz, Chile (limited stratigraphy, 23.03 – 15.9 Ma?). Isla Ipún, Chile (limited stratigraphy, 23.03 – 15.9 Ma?)
Geological references	Ponder 1973 Covacevich and Frassinetti 1986 Nielsen 2003 (taxonomic description)

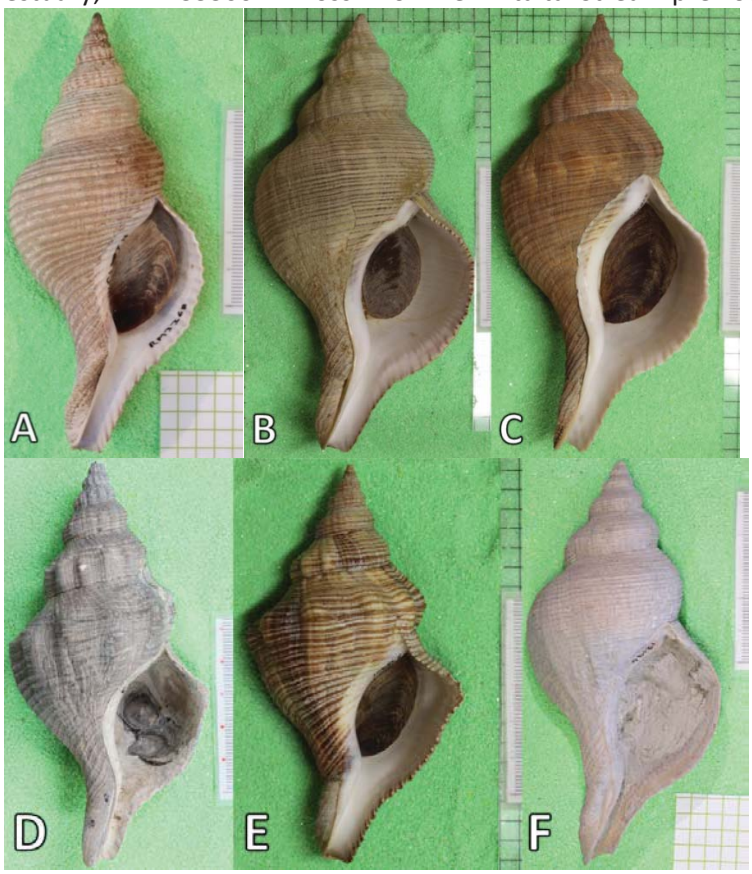
Penion subregularis† (d'Orbigny, 1852)

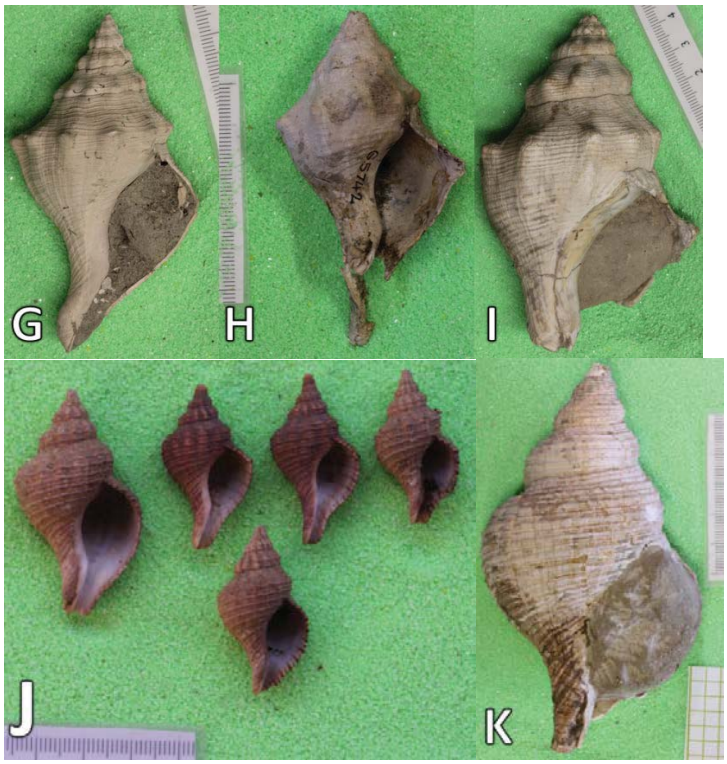
Geological range	Early Miocene (limited stratigraphy, approximately 23.03 – 15.9 Ma)
Fossil localities	Santa Cruz, Chile (limited stratigraphy, approximately 23.03 – 15.9 Ma).
Geological references	Nielsen 2003 (taxonomic description)

Penion sulcatus (Lamarck, 1816)

Figure 8.28

A: RM3368 [GNS] from Wellington harbour, from same collection lot as figured in Beu and Maxwell 1990; B: M.278791 [MNZ] from off Slipper Island; C: M.278792 [MNZ] from off Great Barrier Island; D: GS15443 fossil from Te Piki; E: M.278801 [MNZ] from Manakau Harbour; F: MA36574 [AM] fossil from Castlecliff; G: GS3538 [GNS] fossil previously classified as *P. gauli* paratype, from Otahuhu brewery well; H: G5742 [AU] fossil previously classified as *P. koruahinensis* holotype, from Kaawa Stream; I: GNSN [GNS] fossil previously classified as *P. huttoni* holotype, from Black Birch Creek; J: RX014 [AU] juvenile of *P. sulcatus* from off Waiau estuary; K: MA33360 [AM] fossil from Te Ahitaitai Stream previously classified as *P. haweraensis*.

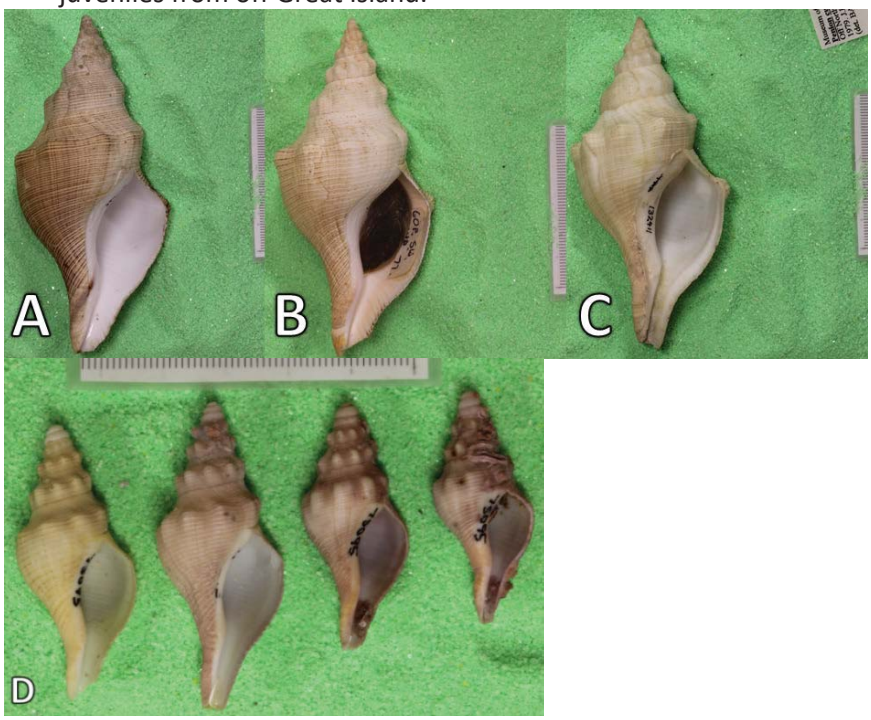




Common names	Northern siphon whelk; kākara nui
Synonyms	*P. gauli*† (Marwick, 1948)*, Penion haweraensis*† (Powell, 1931)*, P. hiatulus*† (Powell, 1947), *P. huttoni*† (King, 1934), *P. koruahinensis*† (Bartrum & Powell, 1928)
Evidence used for change	Traditional examination of shell traits, geometric morphometric analysis of shells.
Comments	We suggest that five fossil species are conspecific with *P. sulcatus*. This is determined using the traditional examination of shell traits, as all shells of all taxa overlap with the shape and size variation exhibited by modern populations of *P. sulcatus.* Extant *P. sulcatus* is highly variable in shell morphology, but molecular data indicates that this is phenotypic plasticity (Chapter 4). Each of the type specimens shown above are difficult to distinguish from forms observed in extant populations (G – I to E), and from fossils currently classified as *P. sulcatus* (G – I to D). The protoconch size of *P. sulcatus* is also particularly variable, however size seems to be strongly, positively associated with depth. Large fossils from Wanganui with well-rounded teleoconch whorls (e.g. F), can be confused with extant *P. ormesi*, however the teleoconch spire is shorter and the shell is thicker, corresponding to extant intraspecific variation in *P. sulcatus* (e.g. B). Similarly, some fossils of *P. hiatulus* bear some resemblance to *P. jeakingsi,* mainly the suddenly enlarged body-whorl described by Powell (1979), but geometric morphometric analysis of shell shape and size (Chapter 7), strongly suggests that these shells are in fact *P. sulcatus.*
Extant references	Powell 1927 (taxonomic description) Powell 1947 (taxonomic description) Ponder 1975 Powell 1979 (taxonomic description)

	Spencer *et al.* 2009 Spencer *et al.* 2017
Geological range	Present, Opoitian to Castlecliffian (5.33 – 0.34 Ma).
Fossil localities	Castlecliff, Kai Iwi, Okehu Stream, Manawatu-Wanganui (1.63 – 0.34 Ma). Manawatu Gorge, Manawatu-Wanganui (2.40 – 1.63 Ma). Maraekakaho and Kereru Road, Hawke's Bay (2.40 – 1.63 Ma). Petane Mudstone, Napier, Hawke's Bay (2.40 – 1.63 Ma). Te Ahitaitai Stream, Wairarapa, Wellington (2.40 – 1.63 Ma). Te Piki, Cape Runaway, Bay of Plenty (2.40 – 1.63 Ma). Hawera, Taranaki (3.00 – 2.40 Ma). Mangahoa River, Wairarapa, Wellington (3.70 – 3.00 Ma). Waverley, Taranaki (3.70 – 3.00 Ma). Otahuhu Brewery Well, Auckland (3.70 – 3.00 Ma). Oweka River, Gisborne (5.33 – 3.70 Ma). Estuary of Kaawa Stream, Waikaretu, Waikato (5.33 – 3.70 Ma). Black Birch Creek, Canterbury (stratigraphy unknown).
Geological references	Beu and Maxwell 1990
Sampled depth range	2 – 165 m, predominantly shallow.
Protoconch	2 to 3, medium to large.
Mean teleoconch length	12.10 cm (n = 55, SD = 2.70 cm).
Mean aperture length	7.62 cm (n = 55, SD = 1.71 cm).
Mean base protoconch width	2.60 mm (n = 55, SD = 0.64 mm).

Penion n. sp. Three Kings Islands (TBA)
Figure 8.29
A – B: 80439 [MNZ] from Three Kings Islands; C: M.132411 [MNZ] from North Cape; D: M.75095 [MNZ] juveniles from off Great Island.

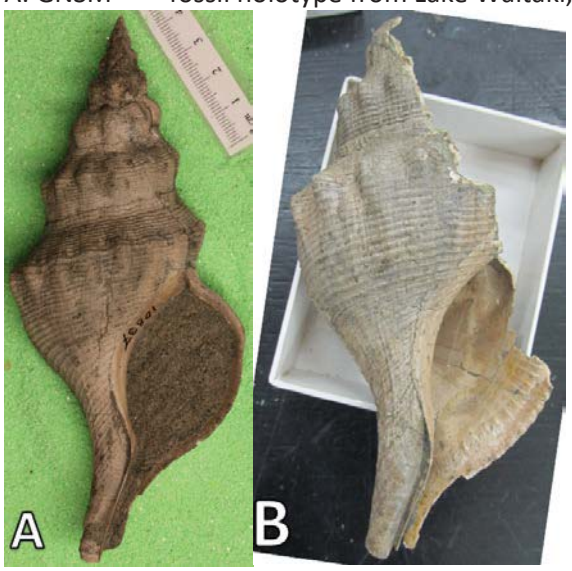


Evidence used for change	Molecular phylogenetics, traditional examination of shell traits, geometric morphometric analysis of shells.
Comments	Mitochondrial and nuclear rDNA sequence data was only available from a single individual, but results indicate that there is a distinct lineage of *Penion* restricted to the Three Kings Islands regions (Chapter 4). Geometric morphometric analysis of shells indicates that the species is readily distinguishable from other siphon whelk species (Chapter 6). Numerous traditional shell traits also distinguish the species, as shells are medium in size and patterned with fine, regular banding. Shells have smooth axial ribs that are often not prominent and the siphonal canal is short. The protoconch is large and especially wide.
Geological range	Present.
Fossil localities	None yet recognised.
Sampled depth range	90 – 503 m, deep.
Protoconch	3 whorls, large and slightly flattened dorsoventrally so that it appears especially wide.
Mean teleoconch length	14.86 cm (n = 26, SD = 2.72 cm).
Mean aperture length	8.78 cm (n = 26, SD = 1.45 cm).
Mean base protoconch width	4.18 mm (n = 26, SD = 0.58 mm).

Penion n. sp. Waimumu† (TBA)
Figure 8.30
A: GS15692 [GNS] fossil holotype from Cosy Dell Farm, Waimumu.

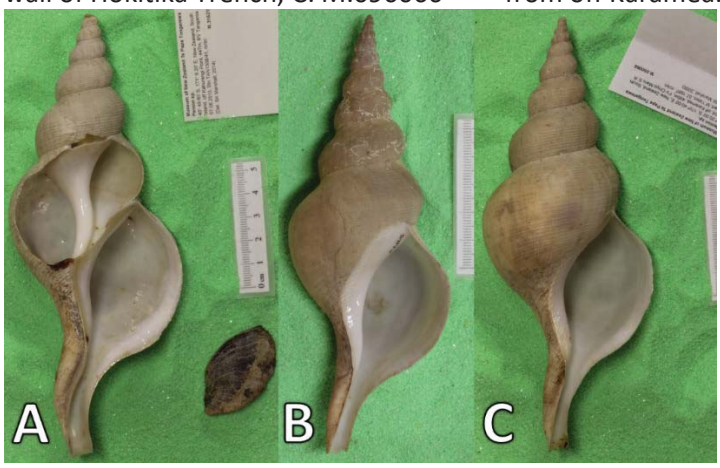
Evidence used for change	Traditional examination of shell traits.
Comments	The only specimen is too fragmented for geometric morphometric analysis or reliable morphological measurements, however the shell is clearly distinct in morphology from other *Penion* of similar geological age. The specimen exhibits a small aperture with a short siphonal canal and rounded axial ribs. Striations are prominent on the shell. The specimen possibly bears some resemblance to *P. exoptatus* based on the size and axial rib morphology, but does not appear to be similar to the contemporaneous fossil from Lake Waitaki.
Geological range	Duntroonian (27.3 – 25.2 Ma).
Fossil localities	Cosy Dell Farm, Waimumu, Southland (27.3 – 25.2 Ma).
Protoconch	Not preserved.

Penion n. sp. Waitaki† (TBA)
Figure 8.31
A: GNSM [GNS] fossil holotype from Lake Waitaki; B: GS10837 [GNS] fossil from Lake Waitaki.



Evidence used for change	Traditional examination of shell traits.
Comments	The species is classified based on two specimens from Lake Waitaki. The overall shell morphology is somewhat similar to that of *P. mandarinus* or *P. sulcatus*. Like *P. mandarinus*, the spire is slightly elongated relative to the rest of the teleoconch, the axial ribs are very regular (in contrast to e.g. *P. sulcatus*), and the axial ribs are not very prominent. The final protoconch whorl is partially preserved and indicates a small protoconch, as observed in *P. mandarinus* and *P. maximus*. However, shells of *P.* n. sp. Waitaki are large (similar in size to *P. cuvierianus*) and the species is obviously found in New Zealand rather than Australian fossil record.
Geological range	Duntroonian (27.3 – 25.2 Ma).
Fossil localities	Wharekune greensand, Lake Waitaki, Canterbury (27.3 – 25.2 Ma).
Protoconch	Small, likely only 2 whorls at most but not fully preserved.
Mean teleoconch length	15.51 cm (n = 1).
Mean aperture length	8.77 cm (n = 1).
Mean base protoconch width	2.10 mm (n = 1).

Penion n. sp. West Coast (TBA)
Figure 8.32
A: M.316215 [MNZ] from off Karamea, tissue sequenced in Chapter 3; B: M.059193 [MNZ] from wall of Hokitika Trench; C: M.090066 [MNZ] from off Karamea.



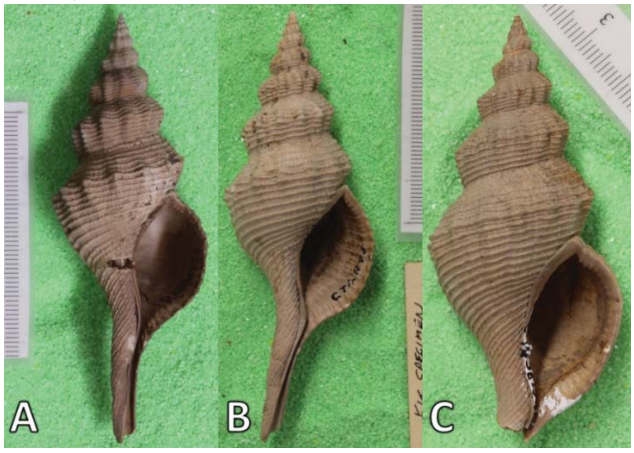
Evidence used for change	Molecular phylogenetics, traditional examination of shell traits, geometric morphometric analysis of shells.
Comments	The taxon includes specimens collected from waters off of the West Coast region of the South Island. All specimens are collected from deep water and exhibit large, thin shells that appear somewhat elongated compared to specimens of *P. jeakingsi* and *P. ormesi.* Phylogenetic analysis of mtDNA and nuclear rDNA from a single specimen (A; see Chapter 4), indicated that *P.* n. sp. West Coast is identical to some specimens of *P. jeakingsi*, however *P. jeakingsi* itself appeared to contain multiple genetic lineages (Chapter 3). Uninformed morphometric analysis could not differentiate *P.* n. sp. West Coast from *P. jeakingsi* or *P. ormesi*, however tests with *a priori* classification suggested that *P.* n. sp. West Coast may be morphologically distinct (Chapter 6). However, sampling was very limited (n = 4). Further genetic and morphological sampling is needed to investigate the situation within the *P. ormesi* species complex overall.
Geological range	Present.
Fossil localities	None yet recognised.
Sampled depth range	300 – 587 m, deep.
Protoconch	2½ – 4 whorls, medium to large.

Taxa that are likely misclassified

Penion longirostris† (Tate, 1888)

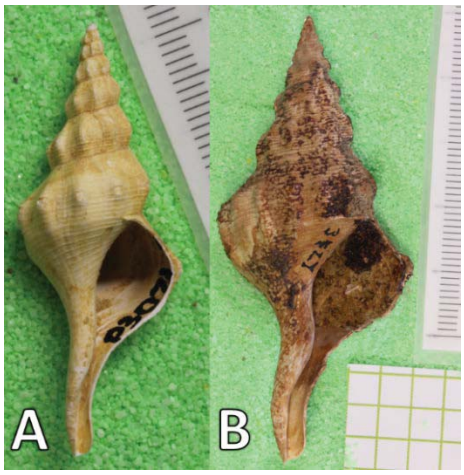
Figure 8.33

A – B: P316771 [VIC] and C74895 [AUS], fossils from Balcombe Bay; C: C163843 [AUS] from Muddy Creek, Hamilton.



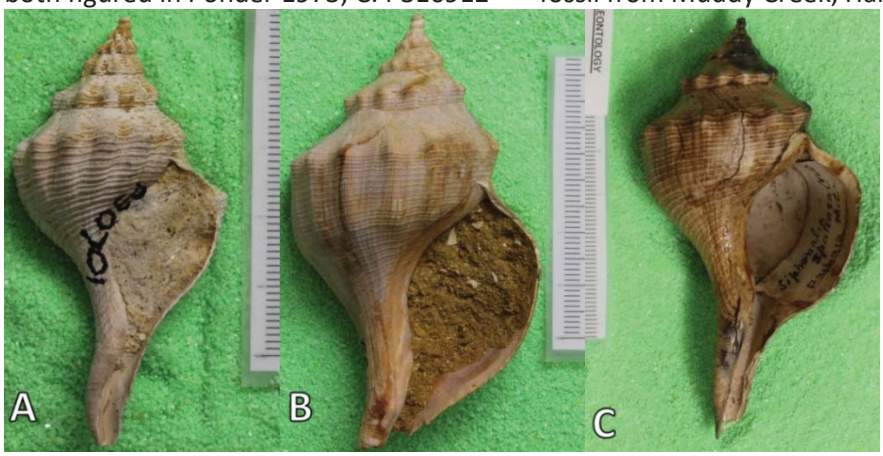
Evidence used for change	Traditional examination of shell traits, geometric morphometric analysis of shells.
Comments	*Penion longirostris* is an unusual looking species of *Penion* because of the strong shell texture and small size, however fossils do bear a resemblance to small and juvenile specimens of *P. maximus* also from Australia (e.g. *P. maximus* G). The species is possibly a misclassified Fasciolariid, possibly *Pleuroploca cristata* or *Pleuroploca rugata* (pers. com. Thomas Darragh 2015). However, *P. longirostris* lacks a collumellar fold (pers. comm. Alison Miller 2015), which is a frequent trait exhibited by Fasciolariidae. Based on geometric morphometric analysis of shell shape and size, *P. longirostris* is indistinguishable from *P. roblini* and is not similar to other taxa classified as *Penion* (Chapter 7).
Geological range	Cheltenhamian (5.0 – 4.3 Ma), Balcombian to Bairnsdalian (15.5 – 10.5 Ma).
Fossil localities	Spring Creek, Minhamite, Victoria (5.0 – 4.3 Ma). Cliffs south of Manyung Rocks, Mornington, Victoria (15.0 – 10.5 Ma) Barwon River, Murgheboluc, Victoria (15.0 – 10.5 Ma). Inverleigh, Victoria (15.0 – 10.5 Ma). Schanpper Point, Port Phillip, Victoria (15.5 – 15.0 Ma). Mornington, Balcombe Bay, Victoria (15.5 – 15.0 Ma). Muddy Creek, Hamilton, Victoria (15.5 – 15.0 Ma). Gellibrand Marl, Gibson Beach, Princetown, Victoria (15.5 – 15.0 Ma). Fossil Beach, Balcombe Bay, Mornington, Victoria (15.5 – 15.0 Ma).
Geological references	Ponder 1973 (taxonomic description)
Protoconch	1½ - 2 whorls, tiny.
Mean teleoconch length	5.80 cm (n = 6, SD = 3.01 cm).
Mean aperture length	3.44 cm (n = 6, SD = 1.98 cm).
Mean base protoconch width	1.86 mm (n = 6, SD = 0.39 mm).

Penion roblini† (Tate, 1888)
Figure 8.34
A: P30742 [VIC] fossil from Murray River Cliffs, previously classified as lectotype of *P. roblini simulans*, figured in Ponder 1973; B: MA28738 [AM] fossil from Table Cape previously classified as *P. roblini roblini*.



Synonyms	*P. roblini roblini*†, *P. roblini simulans*†
Evidence used for change	Traditional examination of shell traits, geometric morphometric analysis of shells.
Comments	*Penion roblini simulans* is an unusual in appearance: shells are small, the protoconch is tiny, axial ribs are acute/sharp, and the siphonal canal is very straight. However, these traits match with small specimens of *P. maximus* (*P. maximus* F and G (juveniles)). We suggest that *P. roblini simulans* and *P. roblini roblini* are conspecific as specimens are difficult to distinguish, which was noted in their initial description (Ponder 1973). However, as noted in Ponder 1973, some specimens of *P. roblini roblini* may be misclassified Fasciolariidae, namely *Fusinus johnstoni*. In addition, we did not manage to take new photographs of the holotype of *P. roblini simulans*, but based on the figures shown in Ponder 1973, we suggest that this specimen is actually a misclassified fossil of *P. maximus.* Finally, based on geometric morphometric analysis of shell shape and size, *P. roblini* is indistinguishable from *P. longirostris* and is not similar to other taxa classified as *Penion* (Chapter 7).
Geological range	Longfordian to Bairnsdalian (27.5 – 10.5 Ma).
Fossil localities	Cliffs south of Manyung Rocks, Mornington, Victoria (15.0 – 10.5 Ma) Western Gellibrand River estuary, Victoria (15.0 – 10.5 Ma) Murray River Cliffs, Morgan, South Australia (15.5 – 15.0 Ma). Muddy Creek, Hamilton, Victoria (16.5 – 15.5 Ma) Fossil Bluff, Table Cape, Tasmania (27.5 – 16.5 Ma).
Geological references	Ponder 1973 (taxonomic description)
Protoconch	1 – 2 whorls, tiny.
Mean teleoconch length	6.84 cm (n = 4, SD = 2.43 cm).
Mean aperture length	4.10 cm (n = 4, SD = 1.54 cm).
Mean base protoconch width	1.25 mm (n = 4, SD = 0.15 mm).

Penion spatiosus† (Tate, 1888)
Figure 8.35
A – B: P.30701 [VIC] fossil from Bunga Creek, and P.30739 [VIC] fossil from Muddy Creek, Hamilton, both figured in Ponder 1973; C: P316912 [VIC] fossil from Muddy Creek, Hamilton.



Evidence used for change	Traditional examination of shell traits, geometric morphometric analysis of shells.
Comments	The shell morphology of *P. spatiosus* is a very unusual. *P. spatiosus* possibly represents a separate sister lineage to *Penion*, much like *Kelletia* or *Antarctoneptunea*. *P. spatiosus* has a large, top-heavy protoconch (the first protoconch whorl is larger than the following), and the axial ribs are positioned low on the slope of each teleoconch whorl. Specimens of *P. spatiosus* appear to reach shell maturity at 5 teleoconch whorls, whereas all other *Penion* reach maturity at a minimum of 6 whorls. Based on geometric morphometric analysis of shell shape and size, *P. spatiosus* is significantly different from other taxa classified as *Penion*, and the species is more different in shell shape to other *Penion* than species of *Kelletia, Antarctoneptunea,* and *Aeneator* (Chapter 7).
Geological range	Cheltenhamian to Kalimnan (3.4 – 5.0 Ma), Balcombian (15.5 – 15.0 Ma)
Fossil localities	Bunga Creek, Kalimna, Victoria (3.4 – 4.3 Ma). Meringa Creek, Kalimna, Victoria (5.0 – 4.3 Ma). Jemmys Point Formation, Lake Tyers, Victoria (5.0 – 4.3 Ma). Muddy Creek, Hamilton, Victoria (15.5 – 15.0 Ma).
Geological references	Ponder 1973 (taxonomic description)
Protoconch	2 ½ whorls, large and top-heavy (first protoconch whorl is larger than second).
Mean teleoconch length	7.41 cm (n = 4, SD = 1.89 cm).
Mean aperture length	5.55 cm (n = 4, SD = 1.68 cm).
Mean base protoconch width	2.16 mm (n = 4, SD = 0.23 mm).

Geological distribution of *Penion* and *Kelletia*

Here we summarise the geological distribution of *Penion*, *Kelletia* and *Antarctoneptunea* worldwide. Exact age estimates for geological localities are provided in tabled data above.

TABLE 8.1
Key for colours used in Tables 2 and 3.

TABLE 8.2

Summary of geological distribution of *Penion* shown against New Zealand geological timescale. Exact age estimates for localities are provided in tabled data above. ‘Extant’ obviously includes the present Haweran stage, but the distinction is made to clarify if there is a fossil record within the last 0.34 Ma.

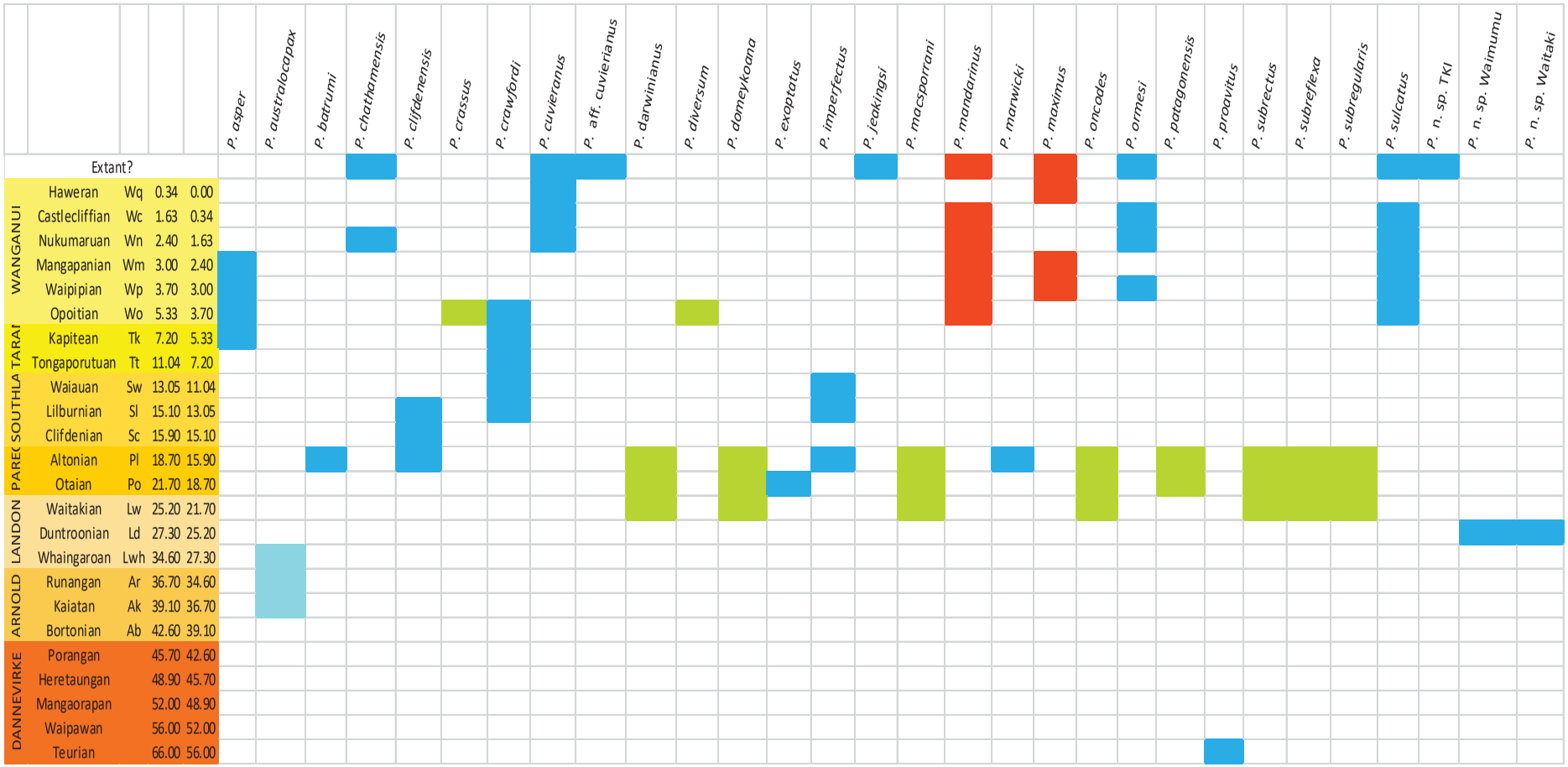
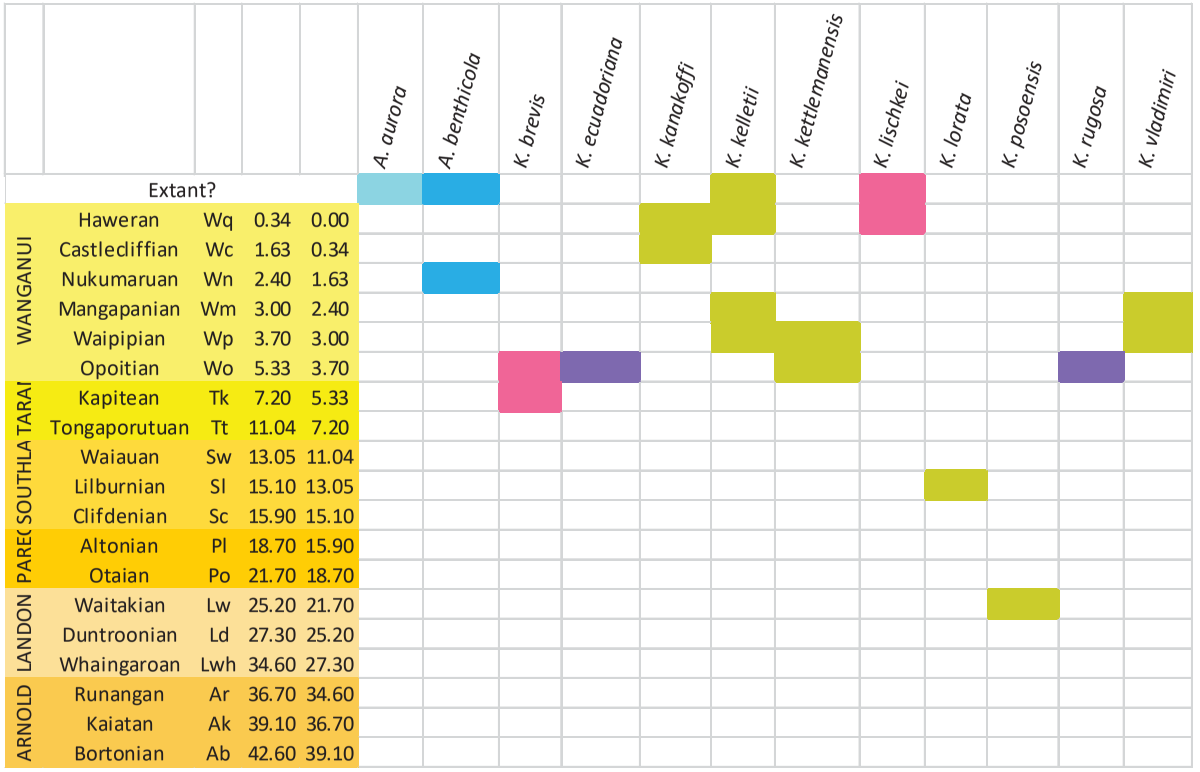


TABLE 8.3
Summary of geological distribution of *Kelletia* and *Antarctoneptunea* (including *P. benthicolus*) shown against New Zealand geological timescale. Exact age estimates for localities are provided in tabled data above. ‘Extant’ obviously includes the present Haweran stage, but the distinction is made to clarify if there is a fossil record within the last 0.34 Ma.



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Summation

Review

For this study I decided to treat species as arbitrary concepts and focus on the process of biological evolution in terms of genetic and phenotypic change exhibited by evolutionary lineages (Chapters 1 and 2). The aim was to consider evolutionary change from a perspective that can be understood by researchers across all fields using simple terms. Consequently, when I investigated the evolutionary relationships of marine snails putatively classified as *Penion* Fischer, 1884 from New Zealand and Australia, I attempted to analyse genetic and morphological variation naïvely with as few evolutionary assumptions as possible. Nuclear and mitochondrial DNA sequence was used to estimate evolutionary relationships (Chapters 3 and 4), and I used naïve as well as taxonomically informed analyses of morphological variation in shells (Chapters 5 – 7). Results indicated that in the case of *Penion*, there is a fairly close concordance between genetic and shell morphological variation (Chapters 3 and 4, 6), and that secondary sexual dimorphism is unlikely to be a confounding source of variation in at least some species (Chapter 5). As results developed, the evolution of *Antarctoneptunea* Dell, 1972 and *Kelletia* Bayle, 1884 was also considered alongside *Penion.* Molecular data suggested that previous taxonomic and biogeographic hypotheses regarding Southern Hemisphere true whelks were incorrect, but did identify a monophyletic New Zealand clade within *Penion* (Chapter 3). Naïve geometric morphometric methods allowed us to detect variation that agreed with genetic patterns that were unexpected based on current taxonomy (Chapters 3 and 4, 6). The interpretation of fossil sites, species and potential evolutionary lineages could also be revised using geometric morphometric analysis (Chapter 7). Depending on how these results were treated, and the underlying evolutionary assumptions made, different interpretations of the fossil record provided patterns of morphological change that fitted different models of evolutionary change (Chapter 7). A final section summarises the taxonomy of *Penion*, *Kelletia* and *Antarctoneptunea*, clarifying the operational taxonomic units (OTUs) used for this thesis and the revisions that are likely based on the results of the earlier chapters (Chapter 8). Overall therefore, this thesis presents a thorough investigation of the marine snail genus *Penion*, using living and extinct material, from an evolutionary perspective that should be interpretable and comparable even without knowledge of marine molluscs.

Future Research

Further genetic work

Although I sequenced individuals belonging to all recognised extant species of *Antarctoneptunea, Kelletia* and *Penion,* more genetic sequencing is required. In particular, sampling more individuals across the entire range of each species would be desirable to determine if any region hosts genetically distinct populations. In New Zealand the species complex of *P. ormesi* (Powell, 1927), *P. c. jeakingsi* (Powell, 1947) and *P.* n. sp. West Coast especially requires more comprehensive population genetic sampling (Chapter 4). In Australia, the morphological variation exhibited by shells of *P. mandarinus* (Duclos, 1832) warrants comparison to more extensive genetic data. Whelks recognised as *Aeneator* Finlay, 1926 in waters off Chile (McLean and Andrade 1982, Araya 2013), also should be sequenced to determine if this classification is accurate.

Future investigations would benefit if progress can be made with molluscan DNA extraction and purification methods, as many specimens sampled during this thesis exhibited DNA that was not easy to extract in good quality, or amplify and sequence using cost-effective methods such as Sanger sequencing. Although I noticed an improvement in DNA extraction quality using DNA purification methods, it seems that contaminants (likely mucopolysaccharides) continued to persist and interfere with downstream enzymatic reactions. This is a common experience among geneticists working with molluscs (e.g. Winnepenninckx *et al.* 1993, Skujienė and Soroka 2003, Pereira *et al.* 2011).

Further investigation of palaeontological evidence

I recommend that future researchers investigate the fossil record of *Penion* from Antarctica, Argentina and Chile (e.g. Frassinetti 2000, Frassinetti 2001, Nielsen 2003, Beu 2009, Reichler 2010), and *Kelletia* worldwide (e.g. Anderson and Martin 1914, Ozaki 1954, Olsson 1964, Addicott 1970), using the same geometric morphometric method employed for this thesis (Chapters 5 – 7). The morphology of fossil and living taxa could be compared worldwide, allowing an overall hypothesis to be formed for the evolution of the clade. It would also be interesting to analyse fossils from North America (e.g. Palmer and Bran 1965, Kollmann and Peel 1983, CoBabe and Allmon 1994), and Europe (e.g. Anderson 1973, Gilbert 1973, Moths and Albrecht 2010),

which have been putatively classified as *Penion, Kelletia* (sometimes *Boreokelletia* Anderson, 1964 in the Northern Hemisphere) in some past literature.

Community composition of the Wanganui fossil sites (within the last million years) could be compared with environmentally similar, modern-day locations such as Golden Bay. These two example locations are on opposite sides of the Cook Strait and Taranaki Bight, and both represent shallow-water, soft sediment basins populated with sponge and mussel beds. *Penion* has possibly undergone a change in species composition, perhaps driven by competitive niche displacement. The Wanganui fossil material appears to be dominated by *P. sulcatus* (Lamarck, 1816) with few *P. ormesi*, whereas present-day Golden Bay is dominated instead by *P. c. jeakingsi* with *P. sulcatus* restricted to rocky substrates. No previous research has focussed on the ecology of *Penion*, but *P. sulcatus* and *P. ormesi* (closely related to *P. c. jeakingsi*) have been hypothesised to be competing shelf species (Dell 1962). It is possible that *P. c. jeakingsi*, which potentially represents a recent speciation event (see Chapters 3 – 4), may be an evolutionary response from the *P. ormesi* clade to this competition. Are similar patterns observed for sympatric marine organisms (including other neogastropod snails such as *Aeneator, Alcithoe* H. Adams & A. Adams, 1853, and *Amalda* H. Adams & A. Adams, 1853) preserved in the fossil record at Wanganui?

Reproduction and behaviour of *Antarctoneptunea* and *Penion*

It is likely that developmental biology has had a significant impact upon the evolution of the *Antarctoneptunea, Kelletia* and *Penion* clade, influencing the potential for long-distance dispersal and speciation (Chapter 3). Future investigations are needed to experimentally demonstrate the developmental strategies of the Australian taxa *P. mandarinus* and *P. maximus* (Tryon, 1881), and representatives of the extant New Zealand species. The developmental strategy of *K. kelletii* (Forbes, 1850) has been demonstrated via laboratory rearing to be indirect development with facultative planktotrophic larvae (Vendetti 2009). However, the hypothesis that extant New Zealand *Penion* undergo direct development, and that Australian *Penion* potentially undergo indirect development, is based only upon protoconch and egg morphology (Ponder 1973). The large protoconchs exhibited by *A. benthicola* (Dell 1956), and *A. aurora* (Hedley, 1916) (Dell 1972), suggest direct development – but again no experimental data is available. If lineages with very large ranges such as *A. aurora*

undergo direct development, how have individuals been able to disperse such long distances?

The behaviour of *Antarctoneptunea* and *Penion* is undocumented, likely due to the significant depth at which most species occur. Nothing is known about the mating behaviour of either genus, in contrast to data from *Kelletia* (Rosenthal 1970), and other true whelk genera (e.g. Martel *et al.* 1986, Ilano *et al.* 2005, Morley 2013). Only limited anecdotal evidence exists for feeding behaviour (e.g. Willan *et al.* 2010). Given the large size of many species, it is possible that *Penion* may be capable of unusual behaviours such as kleptoparasitism from predatory echinoderms (e.g. as in *Buccinum undatum* Linnaeus, 1758; Rochette *et al.* 1995).

Environmental causes for shell morphological variation

Future investigations should compare variation in *Penion* shell morphology with environmental or ecological data. This is the missing major component to investigate variation in the shell morphology of whelks. Although I have established that there is a fairly close relationship between variation in shell size and shape with evolutionary relationships in *Penion* (Chapter 6), it is obvious that the variation observed within species is likely to be influenced by phenotypic plasticity.

Shells of *Penion* most likely differ in size and shape due to water depth. It has previously been hypothesised for *Buccinulum* Deshayes, 1830 that individuals occurring within shallow intertidal and subtidal environments (<20 m) are exposed to stronger wave action, which prevents the mantle for adhering to the shell at an adequate distance to produce a long siphonal canal (Ponder 1971). Based on the examination of shells and our geometric morphometric results, this patterns also seems likely within *Penion* as individuals belonging to species such as *P. sulcatus* and *P. ormesi* certainly appear exhibit shorter siphonal canals when they occur at shallow depths closer to the shore. Many species including *P. c. cuvierianus, P. maximus, P. ormesi* and *P. sulcatus* also appear to exhibit increasing shell size with depth. This likely interacts with substrate type as rocky environments are more common at shallow depths. Smaller shells and shorter siphonal canals may be advantageous to snails in shallow water habitats as they can more easily navigate rocky scree and are more robust to withstand the battering of waves against rocks. Conversely in the soft-sediment basins that dominate deep-water habitats, it is possible that snails partially submerged in sediment and can grow larger due to weaker wave action and potentially reduced risk of predation. A long siphonal

canal and proboscis may also be beneficial in deep-water soft-sediment basins for reaching embedded prey and detritus. At significant depths (>700 m), *P. benthicolus* (possibly *P. benthicolus delli* Powell, 1971) appears to decrease in size and exhibit more prominent teleoconch ridges. This morphotype may reflect a response to increased pressure – as has been hypothesised in the neogastropod genus *Alcithoe* based also upon geometric morphometric data (Hills *et al.* 2012).

Siphon whelks exhibit quite significant variation in protoconch size (Ponder 1973, Powell 1979), and this may reflect depth and the availability of food for hatch direct developing offspring. Generally speaking, protoconch size increases with depth based on observations made during the work for this thesis. It is possible that protoconch size is large at significant depths because larvae practice adelphophagy, where offspring cannibalise siblings and nurse cells within egg-cases. This behaviour is documented in other marine snails, and results in large protoconchs as a single larva gains the resources of multiple offspring, leading to an increased growth rate prior to hatching (e.g. Chaparro *et al.* 1999, Miloslavich and Penchaszadeh 2001, Thomsen *et al.* 2014). A similar hypothesis was previously proposed to explain protoconch variation in *Alcithoe* (Hills *et al.* 2012).

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Appendix I: Conferences

Findings from this thesis were presented at the academic conferences listed below.

2016	Vaux F., Hills S.F.K., Crampton J.S., Trewick S.A., Morgan-Richards M. (2016). Identifying evolutionary lineages of New Zealand marine snails. *19th Annual New Zealand Molecular Ecology Conference.* Auckland University, NZ. [20 minute presentation]
2015	Vaux F., Hills S.F.K., Marshall B.A., Crampton J.S., Trewick S.A., Morgan-Richards M. (2015). Paraphyly in New Zealand true whelks (Neogastropoda: Buccinoidea: Buccinulidae). *Molluscs 2015*. National Marine Science Centre, Southern Cross University, Australia. [20 minute presentation]
	Vaux F., Hills S.F.K., Crampton J.S., Trewick S.A., Morgan-Richards M. (2015). Testing for punctuated evolution in New Zealand marine snails. *Congress of the European Society for Evolutionary Biology XV.* University of Lausanne, Switzerland. [poster]
2014	Vaux F, Hills S.F.K., Crampton J.S., Trewick S.A., Morgan-Richards M. (2014). Integrated phylogenetics of a neogastropod genus. *GeoGenes V.* Museum of New Zealand Te Papa Tongarewa, NZ. [20 minute presentation]
	Vaux F, Hills S.F.K., Crampton J.S., Trewick S.A., Morgan-Richards M. (2014). Whelk phylogenetics as a test of punctuated evolution. *18th Annual New Zealand Phylogenomics Meeting.* University of Auckland, NZ. [20 minute presentation]