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Lineage Identification Affects Estimates of Evolutionary Mode in Marine Snails

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Abstract.—In order to study evolutionary pattern and process, we need to be able to accurately identify species and the evolutionary lineages from which they are derived. Determining the concordance between genetic and morphological variation of living populations, and then directly comparing extant and fossil morphological data, provides a robust approach for improving our identification of lineages through time. We investigate genetic and shell morphological variation in extant species of *Penion* marine snails from New Zealand, and extend this analysis into deep time using fossils. We find that genetic and morphological variation identify similar patterns and support most currently recognized extant species. However, some taxonomic over-splitting is detected due to shell size being a poor trait for species delimitation, and we identify incorrect assignment of some fossil specimens. We infer that a single evolutionary lineage (*Penion sulcatus*) has existed for 22 myr, with most aspects of shell shape and shell size evolving under a random walk. However, by removing samples previously classified as the extinct species *P. marwicki*, we instead detect morphological stasis for one axis of shell shape variation. This result demonstrates how lineage identification can change our perception of evolutionary pattern and process. [Genotyping by sequencing; geometric morphometrics; morphological evolution; Neogastropoda; phenotype; speciation; stasis.]

Our perception of trait evolution and change in biodiversity is strongly influenced by our identification of species as delineated segments of an evolutionary lineage in time (de Queiroz 1998; Sites and Marshall 2003). Unsurprisingly, if living and fossil species derived from these lineage segments are not identified consistently, analyses that rely upon taxonomic identification (i.e., most) are likely to be inaccurate. This issue is especially important for the investigation of evolutionary tempo and mode (Lande 1986; Agapow et al. 2004), where change can be described differently (e.g., stasis, random walk or punctuated) depending on whether one or many species are recognized over a given length of a lineage.

Morphology is the predominant evidence for species delimitation preserved in the fossil record, which means that the majority of all organisms that have existed are likely to be defined exclusively using phenotype (Allmon and Yacobucci 2016; Marshall 2017). Fossils can preserve morphology for many different internal and external anatomical components, and "morphospecies" can be distinguished based on variation for a wide variety of continuous and discrete morphological characters. Shape and size are usually key for species delimitation though because these morphological features are typically preserved best in the fossil record. However, diversity among living organisms assessed using molecular markers frequently demonstrates the existence of crypsis, polymorphism, and morphological convergence (e.g., Trewick 1998; Bickford et al. 2007; Coppard and Lessios 2017). Consequently, a perennial interest for evolutionary biologists and paleontologists is the degree of concordance between morphological species and separately evolving lineages as revealed by genetic data. This relationship matters in particular for extinct species (Stanley and Yang 1987; Gould 1991; Eldredge 1993; Forey et al. 2004). Restricting analyses of the fossil record to only generic-level classification can help to avoid this issue, as morphology is more likely to accurately reflect deeper evolutionary splits (Forey et al. 2004; Foote et al. 2007; Foote 2011; Eronen et al. 2011). In addition, generic-level analyses can help to circumvent statistical limitations due to the incompleteness of the fossil record, as it is easier to sample a greater proportion of genera than species. However, estimates of evolutionary change using genera and species can be highly discrepant, and it can be theoretically difficult to integrate data from well-defined living species and fossil genera (Roy et al. 1996; Wilkinson 2011; Hendricks et al. 2014; Weise et al. 2016).

It is probably appropriate to recognize distinct species where fossils are separated by geologically significant periods of time, or if they originate from geographically distant provenances. However, many extinct morphospecies are classified from adjacent or overlapping geological time intervals, and it is common for fossil sites to contain multiple species from the same genus. Previous studies have highlighted sampling biases (e.g., Guillerme and Cooper 2018; Hopkins et al. 2018), and arbitrary limits set for the temporal duration of a species (e.g., Aze et al. 2013), as potential sources of error when attempting to analyze evolutionary change and fossil diversity. Where there

is no clear temporal or spatial disjunction, or substantial morphological differences between fossil taxa, a genetic context for morphological variation is advantageous. Studies can gather this context by integrating genetic data from living relatives and the morphological analysis of both extant and fossil specimens (e.g., Stanley and Yang 1987; Jackson and Cheetham 1994; Hills et al. 2012; Smith and Hendricks 2013; Hopkins et al. 2018).

Siphon whelks from New Zealand provide an opportunity to identify evolutionary lineages using genetic and morphological data, and this knowledge can be applied to fossil material. Siphon whelks of the genus *Penion* P. Fischer, 1884 are large marine snails (Fig. 1a) with a rich fossil record in New Zealand, including 19 extinct species, found in high abundance from many paleontological sites spanning the last 66 myr (Beu and Maxwell 1990). Although many fossils are well-preserved (Fig. 1c), evolutionary relationships among extant and fossil taxa are unclear and the taxonomic identity of specimens at many fossil sites is uncertain (Beu and Maxwell 1990). Shells exhibit considerable morphological variation in shape, size, and sculpture within and among the six extant species (and one subspecies) currently recognized in New Zealand waters (Powell 1979; Supplementary Fig. S1 available on Dryad at http://dx.doi.org/10.5061/dryad.gqnk98sh6), but phylogenetic analysis of mitochondrial and nuclear ribosomal DNA sequence data (mtDNA and rDNA) has resolved most extant *Penion* species as distinct evolutionary lineages (Vaux et al. 2017b; Vaux et al. 2018; Fig. 1, Supplementary Fig. S1 available on Dryad). We focus on species that in previous studies did not show concordance between genetic and morphological data, and investigate morphological evolution in an evolutionary lineage through 20 myr.

Geometric morphometric analyses of shell shape and size have revealed that shell morphology is concordant with deep phylogenetic splits reflecting genus-level classification (Vaux et al. 2018), and that secondary sexual dimorphism is unlikely to be a confounding source of variation in *Penion* (Vaux et al. 2017a). However, the distinction of *Penion* species using shell morphology is less certain. Two phenotypically dissimilar but related species *P. sulcatus* (Lamarck, 1816) and *P. chathamensis* (Powell, 1938) are easily distinguished using naïve morphometric cluster analysis (Vaux et al. 2017a). However, the same statistical method applied to a larger data set with sampling across three genera was unable to accurately distinguish individual species (Vaux et al. 2018). In that case, species may not have been distinguished because generic-level differences in shell morphology among the three sampled taxa dominated variance in the data set (Vaux et al. 2018), which is equivalent to a phylogenetic scale effect (Graham et al. 2018). The distinction of many species therefore may be possible in a focused data set, sampling a reduced set of taxa.

In this study, we use New Zealand *Penion* as an exemplar to integrate extinct and extant material in order to identify evolutionary lineages and estimate evolutionary mode. Multilocus nuclear genetic variation among six extant *Penion* taxa is investigated, which is used to determine species delimitation between *P. chathamensis* and *P. fairfieldae* (Powell, 1947), and between *P. jeakingsi* (Powell, 1947) and *P. ormesi* (Powell, 1927). Using a geometric morphometric method, we assess the extent of shell shape and size variation within extant species and apply this knowledge to identify fossil material. Two subsampled data sets are used to analyze morphological variation among extant: 1) *P. chathamensis* and *P. fairfieldae* and 2) *P. jeakingsi* and *P. ormesi*. These morphometric results are compared with variation in nuclear DNA sequence data and the signal from previous molecular phylogenetics results (Vaux et al. 2018; Fig. 1b). The taxonomic identification of fossil specimens is investigated at two paleontological sites that putatively exhibit multiple species of *Penion* (Beu and Maxwell 1990): 1) Wanganui Beach (0.97–0.38 Ma) with putative fossils of *P. sulcatus*, *P. ormesi*, and *P. jeakingsi* and 2) Te Piki, Cape Runaway (2.40–1.63 Ma) with specimens currently classified as *P. sulcatus* and *P. cuvierianus* (Powell 1927) (Fig. 1a). Fossil specimens from these sites are analyzed along with extant populations from the surrounding geographic regions.

Lastly, we investigate whether shells of the extinct species *P. clifdenensis* (Finlay, 1930) (18.70–13.05 Ma), *P. marwicki* (Finlay, 1930) (18.7–15.9 Ma), and *P. exoptatus* (Powell & Bartrum, 1929) (21.7–18.7 Ma) (Beu and Maxwell 1990) are morphometrically different from extant and fossil *P. sulcatus*. Although *P. sulcatus* is currently only recognized in the fossil record from 5.3 Ma onwards (Beu and Raine 2009), the genetically estimated most recent common ancestor of *P. sulcatus* and other *Penion* predates these extinct taxa (median 40.6 Ma; 95% HPD 49.8–32.1 Ma; Vaux et al. 2017b). The fossil taxa and *P. sulcatus* have been hypothesized to represent the same evolutionary lineage (Beu and Maxwell 1990), but alternatively they may be independent lineages with morphological convergence, or multiple sister lineages with minimal morphological divergence. We consider morphological variation for these four species within the context of the genetic and morphometric findings from the extant taxa and paleontological site analyses. We investigate to what extent the inclusion of fossil entities impacts the inference of evolutionary mode.

Materials and Methods

Sampling

All extant New Zealand *Penion* species were sampled previously for mtDNA, rDNA, and partial mtDNA *cox1* sequence variation (Vaux et al. 2018). The north-eastern taxon *P. cuvierianus cuvierianus* (Powell, 1927) was found to be genetically distinct from *P. cuvierianus jeakingsi* on the west coast (mtDNA and rDNA; Vaux et al. 2018).

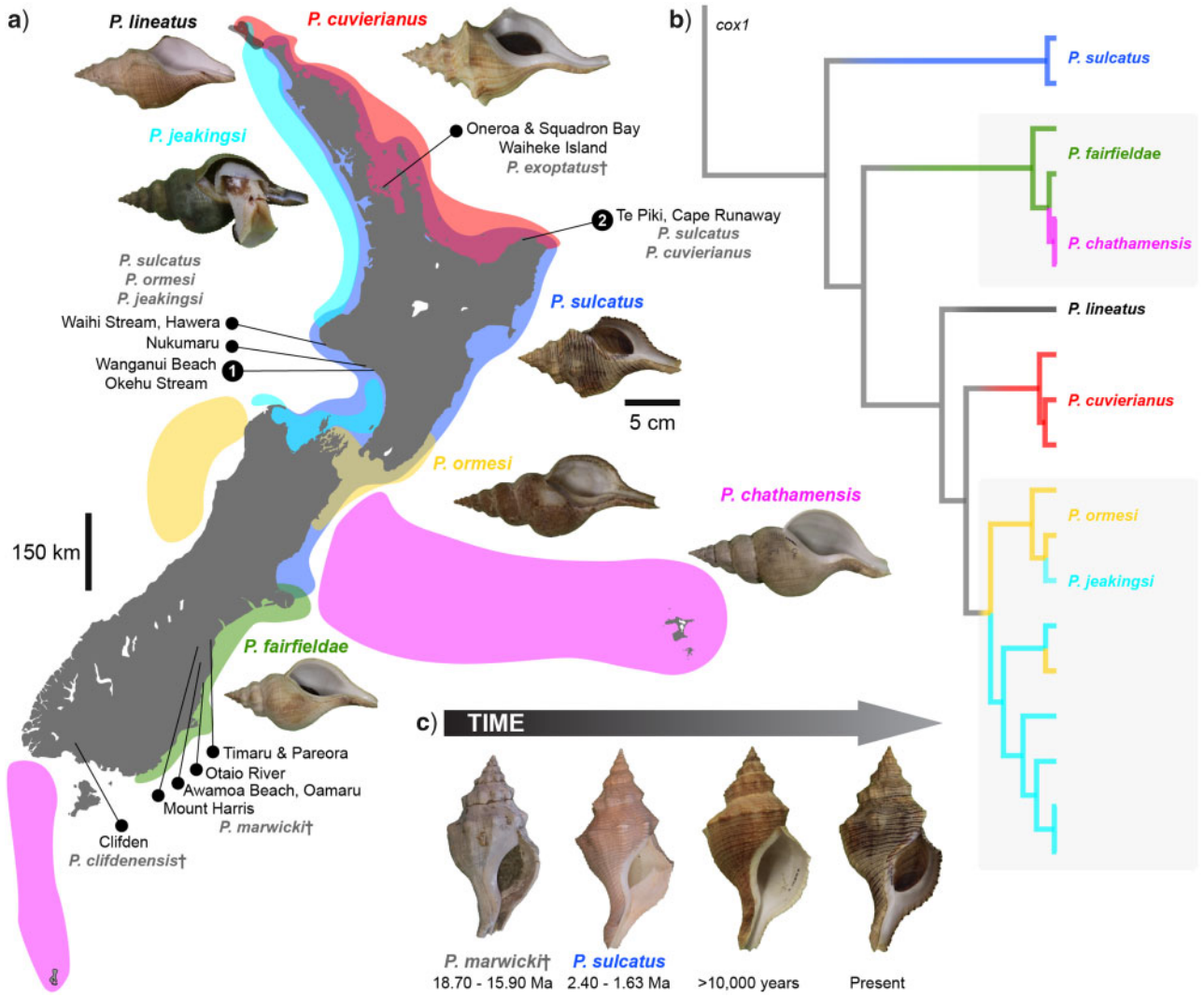

FIGURE 1. a) Distribution of extant *Penion* species in New Zealand waters. Shells of each taxon are shown to scale, with names and geographic ranges illustrated in matching colors. Geographic ranges often overlap and are shown as translucent layers. Sampled fossil localities are labeled with black markers and their preliminary classification in gray. b) Evolutionary relationships of these species, using a Bayesian phylogeny based on mtDNA (cox1; 500 bp; Vaux et al. 2018). c) Extant and fossil specimens demonstrating the preservation of *Penion* shells (not to scale) over millions of years.

Here, we refer to them as different species, as *P. jeakingsi* was estimated to be more closely related to *P. ormesi* than *P. cuvierianus*. Only two pairs of recognized taxa were genetically indistinguishable: 1) *P. chathamensis* and *P. fairfieldae* and 2) *P. jeakingsi* and *P. ormesi* (Vaux et al. 2018; Fig. 1b). *Penion benthicolus* Dell, 1956 is not closely related to the other living New Zealand *Penion* species (Vaux et al. 2018) and is now referred as *Antarctoneptunea benthicola* (Dell 1956) (MolluscaBase 2020), and so we did not include samples within this study. We generated new nuclear DNA sequence data for 18 individual snails representing these four putative species plus *P. cuvierianus and P. sulcatus* (Table 1).

A total of 889 shells were used for morphometric analysis, consisting of a data set of 741 shells from all extant New Zealand *Penion* taxa (Vaux et al. 2018) and new sampling of 148 fossil shells (Table 1, Supplementary Table S1 available on Dryad). Sampling locations for shells were organized into 12 marine biogeographic regions (Supplementary Fig. S2 available on Dryad). The fossils ranged in age from 21.70 to 0.45 Ma (Supplementary Table S1 available on Dryad). Age estimates are considered to be reliable due to the well-studied chronostratigraphy of New Zealand Cenozoic localities such as Wanganui Basin (Carter and Naish 1998; Cooper and editors 2004; Raine et al. 2015).

Specimens were classified based on current taxonomy by experienced molluscan taxonomists (B.A. Marshall and A.G. Beu) using a large collection (NMNZ) and the traditional examination of shell morphology that considers the holistic assessment of shell shape, size, sculpture, protoconch morphology, and color, supported by sympatry or syntopy (e.g., Ponder 1973; Powell 1979). Fossil taxonomy in particular considers relative

TABLE 1. Sampling for New Zealand *Penion* for genetic analysis

#	Taxon	Voucher ID	Origin
1	*P. chathamensis*	M.190355/1	Auckland Islands
2	*P. chathamensis*	M.190355/2	Auckland Islands
3	*P. chathamensis*	M.190065/2	N of Mernoo Bank, Chatham Rise
4	*P. chathamensis*	M.190070/3	E of Mernoo Bank, Chatham Rise
5	*P. chathamensis*	M.274013/3	Auckland Islands
6	*P. cuvierianus*	M.183792/1	Red Mercury Island, Coromandel
7	*P. cuvierianus*	M.183927	Mayor Island, Bay of Plenty
8	*P. cuvierianus*	Phoenix1	Auckland
9	*P. jeakingsi*	M.279432/1	Tasman Bay
10	*P. jeakingsi*	M.279432/2	Tasman Bay
11	*P. jeakingsi*	M.279432/3	Tasman Bay
12	*P. jeakingsi*	M.279432/4	Tasman Bay
13	*P. jeakingsi*	M.279432/5	Tasman Bay
14	*P. fairfieldae*	M.316052/2	Karitane Canyon, Otago Peninsula
15	*P. ormesi*	M.299869/1	White Bluffs, Cloudy Bay
16	*P. sulcatus*	Phoenix9	Auckland
17	*P. sulcatus*	M.329188	Mahia Peninsula, Hawke's Bay
18	*P. sulcatus*	M.329187	Karaka Bay, Wellington

Notes: New Zealand siphon whelks (*Penion*) used for ddRAD sequencing. Voucher IDs are provided for specimens held at the Museum of New Zealand Te Papa Tongarewa.

sculptural prominence of the teleoconch nodules and spiral cords (Beu and Maxwell 1990). Some of the species names used in this study can be considered operational taxonomic units, as some extinct fossil species recorded from a small number of paleontological localities were combined with other extant or fossil species based on the traditional examination of shell traits (Supplementary Table S2 available on Dryad; for further detail see Vaux 2017). The taxonomic identity of young fossils (<3.0 Ma) from two sites requires examination (Beu and Raine 2009). *Penion* fossils from Wanganui are classified as *P. sulcatus*, *P. ormesi*, or *P. jeakingsi*, whereas those from Te Piki are thought to be either *P. sulcatus* or *P. cuvierianus* (Fig. 1a, Table 2; Supplementary Table S1 available on Dryad). Sampling also included fossils from several other paleontological sites across New Zealand that are classified as three extinct species: *P. marwicki*, *P. clifdenensis*, and *P. exoptatus* (Fig. 1a, Table 2; Supplementary Table S1 available on Dryad).

ddRAD Sequencing

We investigated nuclear genetic variation among 18 *Penion* individuals at numerous loci using double digest restriction-site associated DNA sequencing (ddRAD sequencing; Peterson et al. 2012), with modifications described elsewhere (Gemmell et al. 2018). Two high-fidelity restriction enzymes were used, meaning that each retained DNA fragment was flanked by a restriction site for *NsiI-HF* (6 bp cutter, 5′…ATGCAT… 3′) and *MboI* (4 bp cutter, 5′…GATC… 3′; NEB) on either end. This combination of enzymes was expected to yield only nuclear markers as the *NSiI* restriction sites does not occur in the mtDNA genomes of *Penion* (Vaux et al. 2018). After the size selection of fragments between 250 and 350 bp and the ligation of barcodes, samples were pooled and sequenced using the Illumina Hi-Seq 2500 high-throughput platform (via New Zealand Genomics Limited) with 150 bp single-end sequencing chemistry.

RAD sequencing has previously been used to study genetic variation in other marine gastropods (Chu et al. 2014; Gleason and Burton 2016; Miller et al. 2016; Abdelkrim et al. 2018; Gemmell et al. 2018) and bivalves (Araneda et al. 2016; Lal et al. 2016; Van Wyngaarden et al. 2017; Xu et al. 2018; Silliman 2019). Often, the number of shared nuclear loci among highly divergent species is low (Pante et al. 2015), however, a small number of loci can be adequate to distinguish genetic lineages (Rubin et al. 2012; Leaché et al. 2014).

Genotypic Data

DNA sequence reads for the 18 ddRAD sequenced *Penion* individuals were processed using the Stacks 1.01 pipeline (Catchen et al. 2011; Catchen et al. 2013). Following the recommendation of Mastretta-Yanes et al. (2015), the influence of variance in parameter settings was explored in order to optimize the analysis. Our objective was to maximize the number of conserved loci among the six sampled *Penion* species without including a high proportion of likely erroneous reads. In ustacks, the minimum coverage depth required to create a stack was 3 (-m 3), the maximum distance (in nucleotides) allowed between stacks was 5 (-M 5), and the maximum distance allowed to align secondary reads to primary stacks was 7 (-N 7). In cstacks, the number of mismatches allowed between sample loci was set to 15 (-n 15). All

TABLE 2. Sampling of extant and fossil *Penion* shells

Species	Total sampling	Extant NZ *Penion*	Extant species comparisons		Fossil site analyses		*P. sulcatus* lineages	
			P. chathamensis vs. *P. fairfieldae*	*P. jeakingsi* vs. *P. ormesi*	Wanganui	Te Piki	With *P. marwicki*	Without *P. marwicki*
P. chathamensis	125	125 (50)	125					
P. cuvierianus	238	221 (71)				200 17†		
P. clifdenensis†	7						7†	7†
P. exoptatus†	4						4†	4†
P. jeakingsi	85	78 (50)		78	69 7†			
P. fairfieldae	48	48	48					
P. lineatus	25	25						
P. marwicki†	64						64†	
P. ormesi	61	54		54	33 7†			
P. sulcatus	232	190 (50)			51 23†	138 19†	190 56†	190 56†
Totals	889	741	173	132	190	374	321	257

Notes: Sampling of extant and fossil *Penion* shells used for geometric morphometric analyses. Extinct species and fossils are marked with a cross (†). The numbers in brackets for several species record their sampling in the subsampled analysis of New Zealand *Penion*.

other ustacks and cstacks parameters were set to default values for Stacks 1.01.

In the populations component of Stacks, all individuals were grouped into one population so that the representation of loci (and genotypic results for species delimitation) was not influenced by *a priori* taxonomic interpretation. Only the first SNP for each variant locus was retained for analysis to minimize genetic linkage (−−write_single_snp). The minimum coverage depth required for individuals at a locus was set at five (-m 5). Two SNP data sets were produced, differing in the representation of each locus among individuals (-p 1 with -r 0.9 or -r 0.5). In the small, more stringent data set with fewer loci, each locus had to be present in at least 90% of individuals (16/18 snails), whereas in the large, more lenient data set each locus had to be present in at least 50% of individuals (9/18 snails). The small data set was likely restricted to variant loci shared among all species. The large data set was expected to include variant loci for closely related taxa but also a higher proportion of missing data as such loci may not occur among more distantly related taxa. The Stacks settings maximized the number of loci available for our small sample of individuals, 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). The settings probably masked some intraspecific variation but were similar to other studies investigating multiple congeneric species (e.g., Wagner et al. 2013; Dowle et al. 2014; Dowle et al. 2015; Rodríguez-Expeleta et al. 2016). Since no reference genome for *Penion* exists, the genomic identity of ddRAD loci was not investigated and SNPs were treated as anonymous nuclear markers.

Genotypic SNP variation among individual was explored using principal components analysis (PCA) implemented in R package adegenet 2.1.1 (Jombart 2008; Jombart and Ahmed 2011; R Core Team 2018). We determined the number of "meaningful" principal components (PCs) to retain for interpretation and downstream analyses using the broken-stick test on PC Eigenvalues in the R package vegan 2.5-2 (Oksanen et al. 2018), such that retained PCs are axes that explain more variance among samples than expected by chance alone (Jackson 1993; Cangelosi and Goriely 2007). Genotypes were assigned to clusters using a Bayesian approach in Structure 2.3.4 (Pritchard et al. 2000), which was used to investigate genetic differentiation among individuals. Ten replications of the admixture model with independent allele frequencies were used, with an MCMC length

of 100,000 generations and a 10% burn-in. Up to nine potential genotypic clusters were tested for ($K = 1$–9). The optimal number of clusters was determined by examining estimates of posterior probability for a given value of K (Pritchard et al. 2000), and ΔK, the rate of change in logarithmic probability of the data (Evanno et al. 2005) implemented in Structure Harvester 0.6.8 (Earl and vonHoldt 2012). Clustering for the optimal K value across the 10 replications was averaged using Clumpp 1.1.2 (Jakobsson and Rosenberg 2007), and assignment probability structure graphs were generated in DISTRUCT 1.1 (Rosenberg 2004).

Geometric Morphometric Analysis of Shells

Extant and fossil shells were photographed and digitized using the same two-dimensional landmark-based geometric morphometric described in previous studies of *Penion* (Vaux et al. 2017a; Vaux et al. 2018). Shells were photographed with the aperture facing upward and a total of 45 landmarks were digitized (Vaux et al. 2017a) using tpsUtil, tpsDig (Rohlf 2013) and CoordGen8 (Sheets 2014). Six landmarks and 39 semi-landmarks were used to capture the shell shape (Vaux et al. 2017a). Using this approach, experimental error during photography and digitization, respectively represented just 1.2% and 0.08% of shape variation among samples (Vaux et al. 2017a).

PCA implemented in MorphoJ 1.06c (Klingenberg 2011) was used to analyze shell shape. The PCs generated by PCA reflect mathematically independent variation in the shape of objects and centroid size acts as a proxy for size variation independent of shape (hereafter "size"). The number of PCs retained for analysis was determined using the broken-stick test on Eigenvalues for each PC. The similarity of taxa and sampling regions for shell shape was determined using ordination of the retained PCs using 90% mean confidence ellipses. In some cases, PCs were scaled with shell size using the base "scale" function in R (Becker et al. 1988; see Vaux et al. 2018), and three-dimensional scatterplots were produced using the R package car 3.0-2 (Fox and Weisberg 2011) to visualize shell shape and size variation together, with 50% confidence ellipsoids. The capacity for shell shape to distinguish groups was determined using cross-validation scores estimated by canonical variates analysis (CVA) implemented using the R package MASS 7.3-26 (Venables and Ripley 2002; R Core Team 2018) and based on PCs that in sum accounted for 95% of variation among specimens (see methods Vaux et al. 2018). CVA ordination plots were produced in MorphoJ.

To infer structure in our data sets without *a priori* identification, we employed Gaussian mixture modeling using the R package mclust 5.2 (Fraley and Raftery 2002). This approach treats the morphometric data set as sampling from a mixture of populations with different Gaussian distributions, and the modeling process divides the data into different clusters, each one corresponding to a single Gaussian distribution (Fraley and Raftery 2006). Different models created for a range of hypothetical clusters were compared using Bayesian information criteria scores to determine which model was the best fit to the data, therefore estimating an optimal number of clusters in our sample. The clusters of individuals identified by this approach were derived from variation in shell shape and size, and the analysis was naïve to the putative classification of specimens (Fraley and Raftery 2012). Each Gaussian mixture model tested by mclust is named after a parameterization (see Fraley and Raftery 2012). Where shell size was included with the significant number of PCs (variable) for Gaussian mixture modeling, the PCs were scaled with size using the base "scale" function in R and weighted equally in order to remove the effects of different magnitudes of measurement of shape and size variables (Vaux et al. 2017a; Vaux et al. 2018).

Time Series Analysis

We investigated patterns of morphological change in two putative evolutionary lineages associated with *P. sulcatus* using the R package paleoTS 0.4.4 (Hunt 2006; Hunt 2007). Specifically, we tested the fit of shell shape and size variables (retained PCs and size) to three different models of evolutionary change: 1) evolutionary stasis, meaning constrained fluctuations from a mean state through time; 2) an unbiased random walk, representing stochastic change in a trait through time; and 3) a generalized random walk, representing directional change in a trait with some stochastic variation considered. Under stasis, drift is more limited than expected under the unbiased random walk model, indicating that constraining (stabilizing) selection or gene flow are limiting fluctuations. Due to a limited number of time intervals (6 or 7), we did not attempt to fit more complex mode-shift models (Hunt et al. 2015). Support for models was determined using the Akaike information criterion with correction for finite sample sizes (AICc), utilizing Akaike weights.

Results

Genotypic Variation

The smaller ddRAD data set, where each retained locus had data for >90% of individuals, contained 36 SNPs, whereas the larger data set with more missing data retained 1885 SNPs. Species generally occupied distinct regions of genotypic space in PCA ordination (Fig. 2a, Supplementary Fig. S3a available on Dryad), with some overlap on certain axes (Fig. 2a). However, the *P. fairfieldae* individual clustered with samples of *P. chathamensis* (Fig. 2a) and the genotype of the *P. ormesi* individual was similar to samples of *P. jeakingsi* (Fig. 2). The small and large ddRAD data sets had a best fit to three and five clusters, respectively, according to the Structure analysis (Fig. 2c,d, Supplementary Fig. S3b,c available on Dryad). The genotypic clusters identified for the small data set aligned closely with mtDNA clades; 1) *P. sulcatus*, 2) *P. chathamensis* plus *P. fairfieldae*, 3) *P. cuvierianus*, *P. ormesi*,

and *P. jeakingsi* (Fig. 2b). A suboptimal model for the small data set with five clusters separated all taxa, except for *P. fairfieldae* that was grouped with *P. chathamensis* (Fig. 2c). The optimal five clusters identified for the large data set were messier with reduced assignment confidences, but supported the similar relationships and did not differentiate *P. fairfieldae* from *P. chathamensis* or *P. jeakingsi* from *P. ormesi* (Fig. 2d).

Shell Morphology of Extant Species

The three retained PCs of shell variation mostly reflected variation in the relative height of the teleoconch spire and width of the siphonal canal and the lower part of the aperture (Supplementary Fig. S4b available on Dryad). The seven extant New Zealand *Penion* taxa could not be easily distinguished using shape for 889 shells based on PCA (Table 3; Supplementary Fig. S4a,b available on Dryad). Naïve cluster analysis using Gaussian mixture models could not distinguish the seven New Zealand *Penion* taxa using shape and size (Supplementary Fig. S5 available on Dryad). The optimal Gaussian model for the data had just three clusters (EEE3 and VEE3; Supplementary Fig. S5 available on Dryad), which partially matched the three genotypic clusters (Supplementary Fig. S6 available on Dryad). However, using 12 PCs that represented 95% of shape variation among samples, CVA separated most taxa (Fig. 3; Supplementary Table S3 available on Dryad). For example, >90% of the *P. sulcatus* specimens were correctly identified in cross-validation using the cross-validation scores (Supplementary Table S3 available on Dryad). The grouping of taxa by shell shape under CVA broadly reflected genotypic clusters and phylogenetic reconstructions (Fig. 3), for example, shells of *P. fairfieldae* were often confused with those of *P. chathamensis* (37.5%, 18/48 specimens), as were samples of *P. ormesi* with *P. jeakingsi* (28.0%, 14/60; Supplementary Table S3 available on Dryad). PCA, CVA, and naïve cluster analysis for a subsampled data set with nearly even sampling of taxa did not significantly improve the distinction of taxa or clades (Supplementary Table S4, Figs. S7–S9 available on Dryad). In contrast, when we analyzed the shell shape and size of only *P. sulcatus* and *P. chathamensis*, which clearly represent distinct genetic lineages (Figs. 1b and 2), naïve cluster analysis supported an optimal model with two clusters and high assignment probabilities that closely matched taxonomic classification (Fig. 4a; Vaux et al. 2017a).

Is there over-splitting of extant species?—Neither nuclear nor mitochondrial DNA sequence data could distinguish *P. chathamensis* and *P. fairfieldae* (Fig. 2, Supplementary Fig. S3a available on Dryad; Vaux et al. 2018). We therefore investigated morphological variation among 173 shells (125 *P. chathamensis* shells, 48 *P. fairfieldae*; Table 2). Although different in size, the two species were only partially separated by shell shape (Fig. 5a, Supplementary Fig. S9a,b available on Dryad). A large proportion of shape variance captured by the four retained PCs (79.0%; Table 3) reflected differences in the relative height of the teleoconch spire and length of the siphonal canal (Fig. 5b, Supplementary Fig. S9d available on Dryad). Using 12 PCs for 95% of shell shape variance (Table 3), CVA with cross-validation had moderate success for the distinction of the two species (Supplementary Table S5 available on Dryad). Using only shape variation, naïve cluster analysis was unable to accurately distinguish the species (VEI2 model), but the inclusion of shell size significantly improved the distinction of the taxa (EEI2 model; Fig. 4b, Supplementary Fig. S11 available on Dryad). Exceptions were among *P. fairfieldae* from the Auckland Islands, where approximately half of the sampled individuals clustered with *P. chathamensis* specimens.

Genotypic variation within the large nuclear data set (1885 SNPs) indicated that *P. ormesi* and *P. jeakingsi* were similar but not identical (Fig. 2a,d). A suboptimal clustering model for the smaller genotype data set (36 SNPs; $K = 5$) did successfully distinguish the taxa though (Fig. 2b,c), but mtDNA haplotypes did not (Fig. 1b). We therefore investigated morphological variation among 132 shells (54 *P. ormesi*, 78 *P. jeakingsi*; Table 2). Shell shape and size variation for the two species overlapped considerably (Fig. 5c, Supplementary Fig. S12a,b available on Dryad). A large proportion of shape variance captured by the four retained PCs (77.3%; Table 3) reflected differences in the width of the aperture (Fig. 5d, Supplementary Fig. S12d available on Dryad). Using 14 PCs representing 95% of shape variance (Table 3), CVA distinguished *P. ormesi* and *P. jeakingsi* with moderate success (~80% of specimens; Supplementary Table S6 available on Dryad). Naïve cluster analysis using only shell shape found highest support for three clusters (VEI3 model), whereas analysis including shell size supported two groups (VVI2 model; Fig. 4c, Supplementary Figs. S12e and S13 available on Dryad). Most specimens of *P. jeakingsi* were separated under these models, and accuracy improved somewhat with the inclusion of shell size, although specimens from Northland and the Three Kinds Islands were grouped separately under both optimal models. The third cluster identified under the VEI3 model using only shell shape comprised a small number of individuals representing both species (Supplementary Fig. S12e available on Dryad).

Integration of Data From Fossil Penion

With the new biological context provided by the genetic and morphometric results for extant *Penion* species, we investigated the capacity to identify species and evolutionary lineages in the fossil record using shell shape and size. We analyzed morphometric variation among fossils of *Penion* from two sites and assessed the taxonomic identity of specimens. Afterwards, we analyzed morphometric variation among extant and fossil specimens of *P. sulcatus* and three extinct taxa,

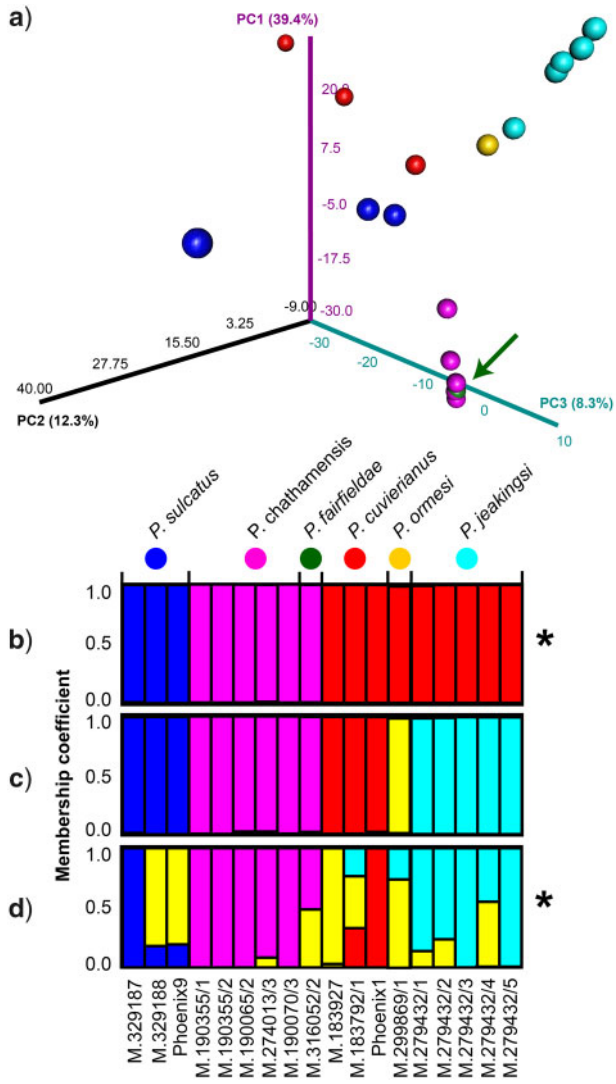

FIGURE 2. Genetic structure among a subset of New Zealand *Penion* species. a) Three principal components (60% of nuclear genetic variation) separate most taxa using the large ddRAD sequencing data set of 1885 SNPs. A green arrow identifies the *P. fairfieldae* individual that overlaps with *P. chathamensis* samples. The six currently recognized *Penion* taxa are colored separately. b–d) Structure plots showing the assignment probabilities of the 18 ddRAD sequenced *Penion* individuals from six taxa among estimated genotypic clusters. b and c) The optimal clustering model ($K = 3$) and an alternative model ($K = 5$), respectively for the small data set of 36 SNPs. d) The optimal model ($K = 5$) for the large data set of 1885 SNPs.

and interpreted the likelihood of whether these four taxa belong to the same evolutionary lineage.

Identity of Wanganui fossils.—We investigated morphological variation among 190 shells from Wanganui, comparing 37 fossil specimens with extant shells from 51 *P. sulcatus*, 33 *P. ormesi*, and 69 *P. jeakingsi* snails (Table 2). Four PCs were retained (79.6% of variance; Table 3). We were able to distinguish the majority of extant and fossil specimens classified as *P. sulcatus* using PC1 (Supplementary Figs. S14a and S15a,b available on Dryad), which captured variation in the width of the lower half of the shell (Supplementary Fig. S15c available on Dryad). Almost all the *Penion* fossils from Wanganui resembled *P. sulcatus* more than *P. ormesi* or *P. jeakingsi* (Supplementary Fig. S14a available on Dryad). Naïve cluster analysis with and without the inclusion of shell size found highest support for three clusters (Fig. 4d, Supplementary Figs. S15d and S16 available on Dryad). Almost all fossils (35/37), regardless of previous classification, were assigned with high confidence to a cluster dominated by *P. sulcatus* (VEE3 model; Fig. 4d).

Identity of Te Piki fossils.—We investigated morphological variation among 374 shells from Te Piki, comparing 36 fossil specimens with extant shells from 138 *P. sulcatus* and 200 *P. cuvierianus* snails (Table 2). Three PCs were retained (83.9% of variance; Table 3). Most

TABLE 3. Retained principal components

Retained PCs	Extant NZ *Penion*	Extant species comparisons: *P. chathamensis* vs *P. fairfieldae*	Extant species comparisons: *P. jeakingsi* vs *P. ormesi*	Fossil site analyses: Wanganui	Fossil site analyses: Te Piki	Potential *P. sulcatus* lineages: With *P. marwicki*	Potential *P. sulcatus* lineages: Without *P. marwicki*
1	56.2%	42.5%	37.7%	53.2%	66.5%	34.1%	35.2%
2	15.0%	18.0%	21.7%	13.4%	11.1%	21.8%	21.7%
3	7.1%	12.6%	10.5%	7.5%	6.3%	14.1%	10.8%
4	—	5.9%	7.4%	5.5%	—	—	5.7%
Total significant	78.3%	79.0%	77.3%	79.6%	83.9%	70.0%	73.4%
# PCs for 95% variance	12	12	14	13	10	16	16

Notes: Retained principal components (PC) of morphological variation within each *Penion* shell shape data set, and percentage variance explained by each PC. The number of PCs to retain was determined by the broken-stick test. PCs representing 95% of variance among samples in each data set were used for CVA.

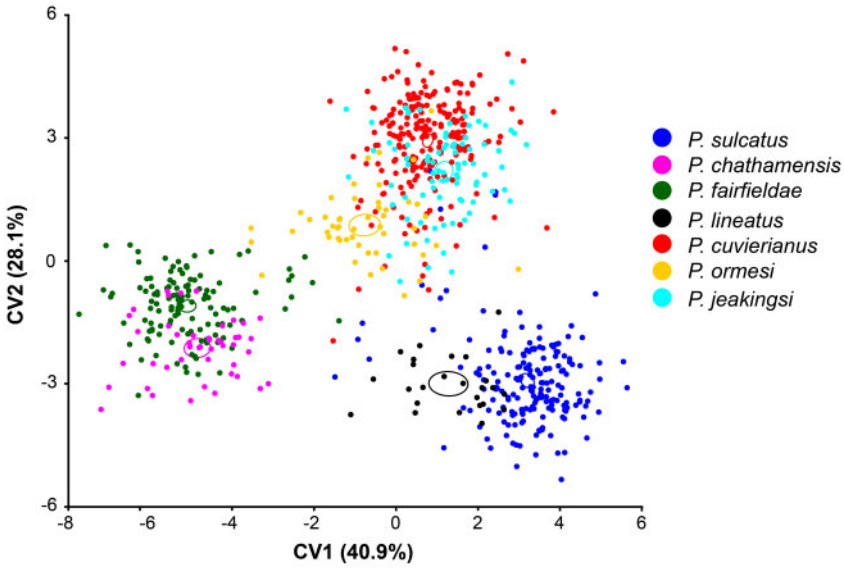

FIGURE 3. Shell shape variation among all extant *Penion* taxa (816 shells) from New Zealand using geometric morphometrics and CVA, in which taxonomy is provided as *a priori* information and differences among groups for shell shape are maximized. Taxa are color coded and 90% mean confidence ellipses shown.

extant specimens of *P. sulcatus* and *P. cuvierianus* could be distinguished from one another based on PC1 (Supplementary Fig. S14b available on Dryad), which reflected variation in the width of the shell and the angle of the teleoconch spire (Supplementary Fig. S17c available on Dryad). There was considerable overlap between the fossils and extant specimens of *P. sulcatus* across all other retained PCs (Supplementary Figs. S14b and S17a,b available on Dryad). Although the fossil specimens were slightly different in shape from the extant population sample, the majority of *Penion* fossils from Te Piki more closely resembled *P. sulcatus* than *P. cuvierianus*. Naïve cluster analysis of shell shape found highest support for two clusters (EEI2 model) and four clusters with the inclusion of shell size (EEE4 model; Fig. 4e, Supplementary Figs. S17d and S18 available on Dryad). The groups identified under both models presented a similar pattern, where 80% of fossil specimens (29/36) were clustered with extant specimens of *P. sulcatus*, with the remainder clustered with *P. cuvierianus* (Fig. 4e, Supplementary Fig. S17d available on Dryad).

Potential fossil lineages.—We analyzed morphological variation among 321 specimens, comparing 75 fossils from the extinct species *P. clifdenensis*, *P. marwicki*, and *P. exoptatus*, with 246 extant and fossil shells of *P. sulcatus* (Table 2). Some fossils from Wanganui and Te Piki newly identified as *P. sulcatus* were included in this analysis (Table 2). Three PCs were retained (70.0% of variance; Table 3), which captured most possible aspects of shell shape variation (Supplementary Fig. S19d available on Dryad). Shell shape was partially able to distinguish *P. marwicki* but there was considerable overlap among samples of *P. clifdenensis*, *P. exoptatus*, and *P. sulcatus* (Supplementary Figs. S14c and S19a,b available on Dryad). Using 16 PCs for 95% of shell shape variance

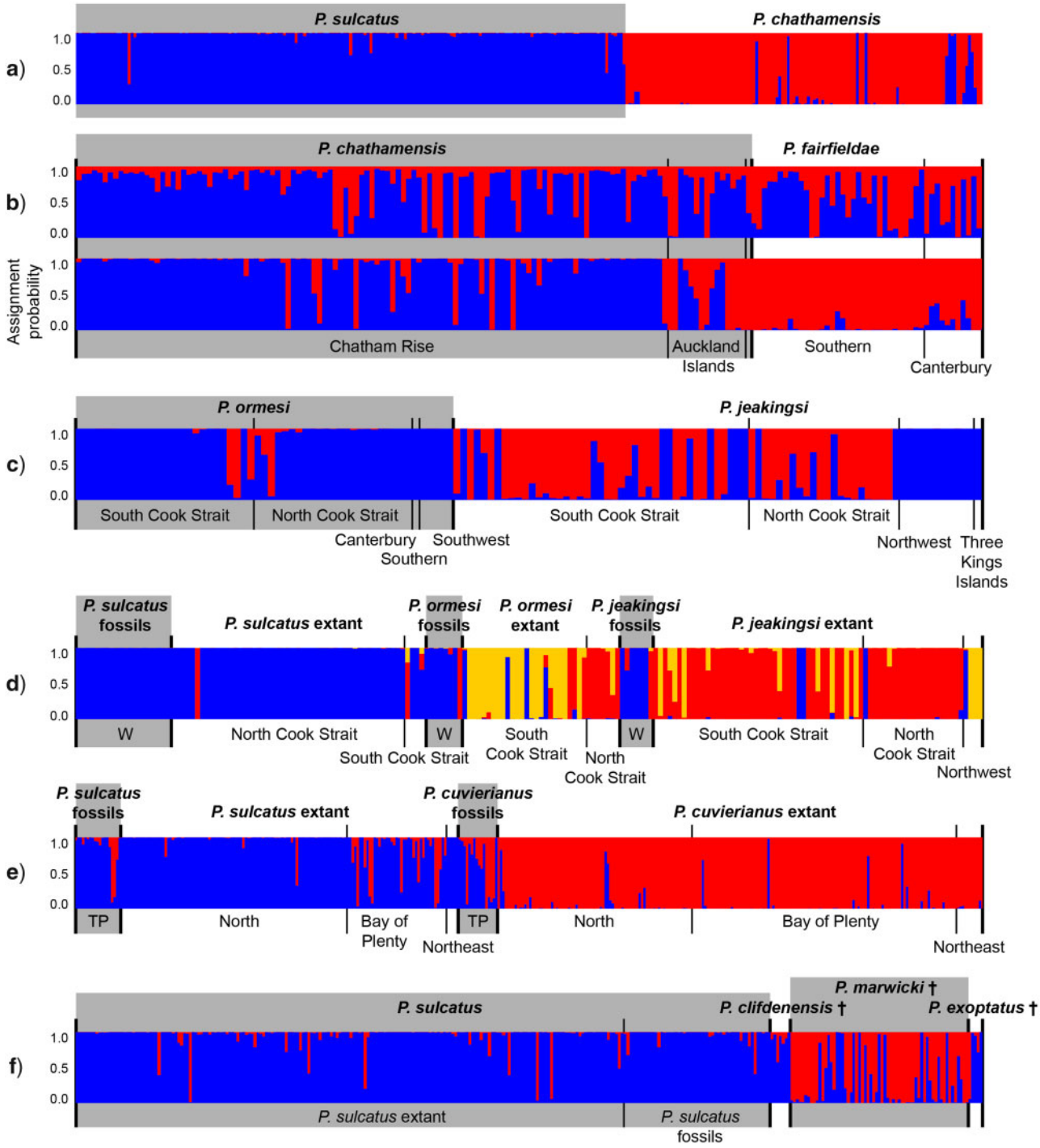

FIGURE 4. Clustering subsets of New Zealand *Penion* species using Gaussian mixture modeling with shell shape and size. The optimal cluster model for each data set was estimated naïvely using the R package mclust. The preliminary classification of specimens and where they were collected is shown above and below each plot. (a) Comparing *P. sulcatus* and *P. chathamensis* revealed clear phenotypic distinction of these two taxa (optimal VEV2 model, using PCs 1–3 of shape, and shell size; Vaux et al. 2017a). b) Shells of *P. chathamensis* and *P. fairfieldae* were analyzed without (above) and with (below) shell size (optimal VEI2 using PCs 1–4; EEI2 using PCs 1–4 and size). c) Analysis of *P. ormesi* and *P. jeakingsi* had poor discrimination of two taxa (optimal VVI2 model using PCs 1–4 and size). d) An analysis combining fossils from Wanganui with modern shells of *P. sulcatus*, *P. ormesi*, and *P. jeakingsi* from adjacent regions (optimal VEE3 model using PCs 1–4 and size). e) An analysis combining fossils from Te Piki with modern shells of *P. sulcatus* and *P. cuvierianus* from adjacent regions (EEI2 using PCs 1–3). f) An analysis combining reclassified shells of extant and fossil *P. sulcatus*, and fossils of the extinct species *P. clifdenensis*, *P. marwicki*, and *P. exoptatus* (EEE2 using PCs 1–3). Alternative Gaussian mixture (mclust) models and Bayesian information criteria support for each data set are provided in the Supplementary Material available on Dryad.

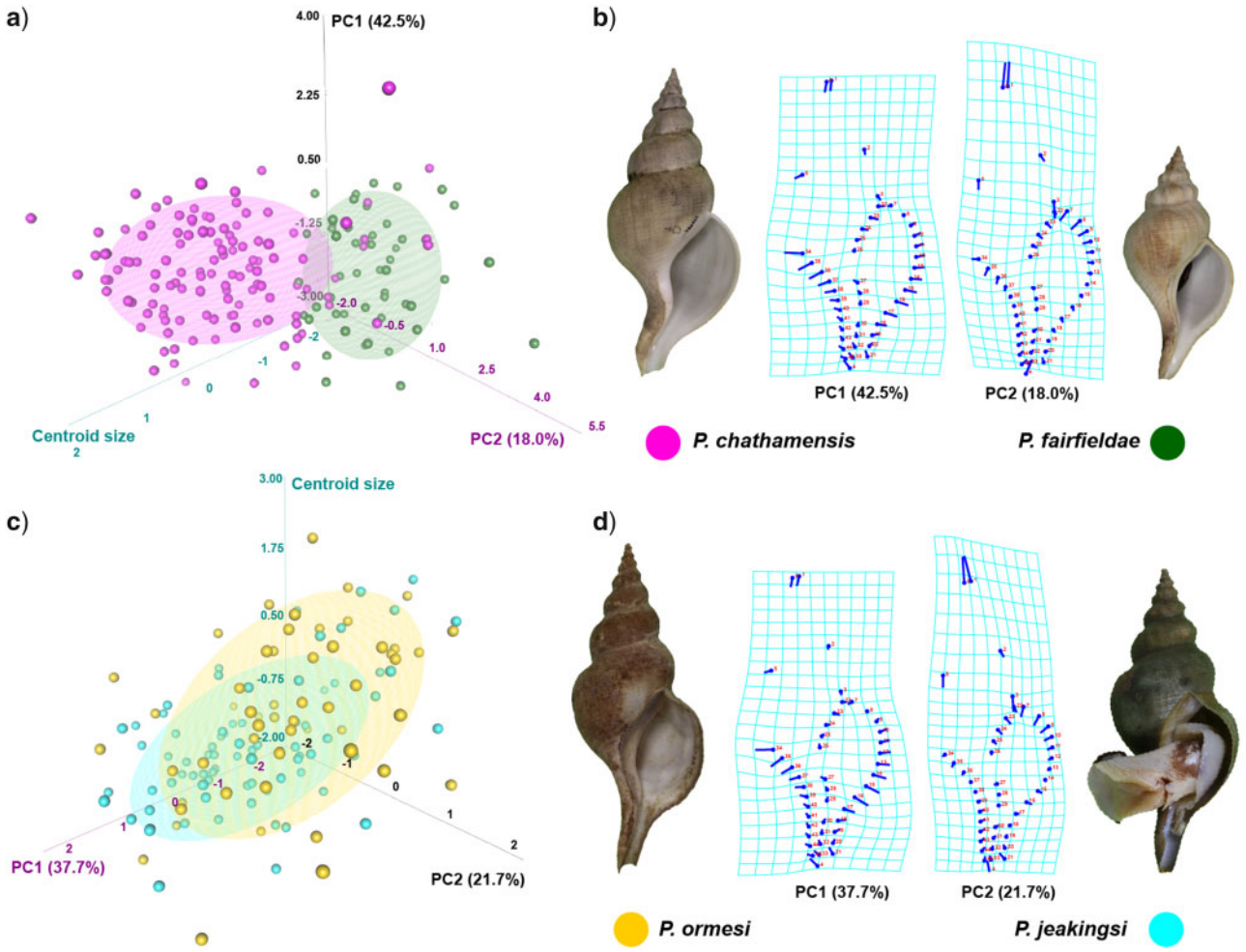

FIGURE 5. Can shell morphology distinguish two pairs of extant *Penion* taxa? a and c) Three-dimensional scatterplots showing shell shape and size variation and 50% mean confidence ellipsoids. Samples and ellipsoids for groups are coloured according to *a priori* classification. b and d) Thin plate spline diagrams and example shells (not shown to scale) for each comparison. The length of lollipop lines for each numbered landmark illustrates the deformation of shape space for each presented PC.

(Table 3), CVA cross-validation success was high for the distinction of *P. sulcatus* and *P. marwicki*, but low for the separation of *P. clifdenensis* and *P. exoptatus* from *P. sulcatus* (Supplementary Table S7 available on Dryad). Naïve cluster analysis with and without the inclusion of shell size found highest support for two clusters (EEE2 and VEV2 models; Fig. 4f, Supplementary Figs. S19e and S20 available on Dryad). Most specimens of *P. marwicki* formed a separate cluster, whereas specimens of *P. clifdenensis* and *P. exoptatus* were grouped with extant and fossil specimens of *P. sulcatus*. Groups identified in both models were similar, although assignment probabilities varied—indicating uncertainty about the distinction of *P. marwicki*.

We investigated the impact of including *P. marwicki* within a putative evolutionary lineage (containing *P. sulcatus*, *P. clifdenensis*, and *P. exoptatus*), specifically testing how this affected a time series analysis in paleoTS. With all taxa included within the lineage, there were seven time intervals available for analysis, and there were six where *P. marwicki* was excluded (Fig. 6). Where *P. marwicki* was included (321 specimens; Table 2), two of the three statistically retained shape variables (PCs 1 and 3) and shell size were fitted best to the unbiased random walk model (Akaike weight 0.69–0.84; Fig. 6a; Supplementary Table S8 available on Dryad). In contrast, variation for the second retained axis of shape variation (PC2) had an equally poor fit to both unbiased random walk and evolutionary stasis (Akaike weight <0.55; Fig. 6a; Supplementary Table S8 available on Dryad). With *P. marwicki* removed (257 specimens; Table 2), we re-analyzed samples and four PCs of shell shape variation were retained (73.4% of variance; Table 3). Using these four shape PCs and shell size, all traits fitted best to an unbiased random walk model, except for PC2 that had best support for evolutionary stasis (Akaike weight = 0.69; Fig. 6b; Supplementary Table S9 available

on Dryad). PC2 for each data set represented almost identical amounts of shell shape variation (21.8% with *P. marwicki*, 21.7% without *P. marwicki*; Table 3), and both reflected variation in the relative height of the teleoconch spire and aperture (with *P. marwicki* included shown in Supplementary Fig. S19d available on Dryad). We therefore consider PC2 in each data set to be comparable. Overall, the choice of whether to include *P. marwicki* within the evolutionary lineage appeared to be the key determinant of whether a signature of morphological stasis was detected.

Discussion

Integrating data from living and extinct species will help to determine the extent to which species diversification and phenotypic evolution are coupled in the natural world, which is required before the drivers of evolutionary change can be investigated (Hunt and Rabosky 2014). Our approach was to study the level of concordance between genetic and morphometric variation in extant populations, followed by comparison of morphological variation between modern and fossil populations, in order to resolve uncertain species identification in fossil deposits. We believe that our work with the *Penion* genus provides a useful case study for the integration of extant and extinct material for research on evolutionary mode.

In the more stringent ddRAD data set, using fewer anonymous nuclear SNPs, most of the currently recognized extant species of New Zealand *Penion* were supported (Fig. 2b,c; Supplementary Fig. S3a available on Dryad), in agreement with previous mtDNA and rDNA phylogenetic reconstructions (Vaux et al. 2018). In the larger ddRAD data set with more SNPs, the optimal clustering model in Structure suggested some confusion among taxa (Fig. 2d), but PCA results indicated again that most extant species could be distinguished (Fig. 2a). We note that the results for the ddRAD analysis should be treated with some caution, as only one individual was sampled for *P. fairfieldae* and *P. ormesi*, however, these results were congruent with the previous mtDNA genome and *cox1* phylogenetic reconstructions that sampled multiple individuals of both species (Vaux et al. 2018; Fig. 1b). Future research based on nuclear DNA would obviously benefit from more frequent sampling. The occurrence of interbreeding among *Penion* species has not been investigated, and so it is possible that some of the mixed genetic identities observed in the large SNP data set (Fig. 2d) could indicate some level of past introgression among lineages. However, the increased proportion of missing data in the large ddRAD data set could also lead to a result of mixed genetic identity.

Morphometric analysis did not readily identify all New Zealand taxa, but results from *a priori* informed analyses showed considerable concordance between shell morphology and genetic data (Fig. 3; Supplementary Table S3 available on Dryad), and separately analyzing subsets of taxa was more informative for species delimitation. However, both the ddRAD and phylogenetic evidence brings into question the taxonomic distinction of allopatric *P. chathamensis* from *P. fairfieldae*, and *P. ormesi* from *P. jeakingsi*. Shell shape variation could not readily distinguish *P. chathamensis* from *P. fairfieldae*, although they could be partially distinguished using shell size (Figs. 4b and 5a). We conclude that *P. chathamensis* and *P. fairfieldae* represent the same genetic lineage and that these names should be considered synonymous.

Morphometric analysis of the questionable species *P. ormesi* and *P. jeakingsi* showed concordance with the large SNP data set (1885 SNPs) and previous evidence from mtDNA and nuclear markers (Vaux et al. 2018), indicating that individuals identified as these species are part of the same evolutionary lineage. Substantial overlap in both shell shape and size was observed between the two taxa (Figs. 4c and 5c). Based on these results, we conclude that *P. jeakingsi* and *P. ormesi* are part of variation of a single species, and that these taxa are synonymous. However, the disjunct distribution of these snails, and the inference of three mitochondrial lineages and morphometric clusters unaligned with the current taxonomic distinction of *P. jeakingsi* or *P. ormesi* (Fig. 1a,b and Supplementary Fig. S12e available on Dryad), suggests that further biological diversity may be present within this lineage. Subsequent, more detailed, examination of this lineage will be required to resolve any undescribed, recently diverged subclades, or population structure, within *P. ormesi*. A map with updated distributions for extant New Zealand *Penion* is illustrated in Supplementary Figure S21 available on Dryad.

Interpretation of the Fossil Record

Although shell shape and size are generally good characters to distinguish *Penion* species, our analysis of extant specimens revealed some over-splitting in the current taxonomy. This is, of course, also a potential problem in the fossil record where the incorrect classification of fossil specimens, or entire extinct species, based on shell size or unique localities, possibly could obscure our ability to trace single evolutionary lineages through time. Morphometric analysis of fossils from Wanganui and Te Piki indicated that most specimens are *P. sulcatus*, rather than *P. ormesi* or *P. cuvierianus* (Fig. 4d,e; Supplementary Fig. S14a,b available on Dryad). The variation observed between these young fossils (<2.4 Ma) and modern specimens was similar to the variation observed among living populations (Fig. 4).

We addressed the classification of extinct species and the identification of an evolutionary lineage over a longer time period (~21.7 myr). Morphometric results did not separate *P. clifdenensis* and *P. exoptatus* from *P. sulcatus* (Fig. 4f; Supplementary Fig. S14c available on Dryad). Although the temporal duration of a species along an evolutionary lineage is arbitrary (de Queiroz 1998; de Queiroz 2007; Vaux et al. 2016), our morphometric results

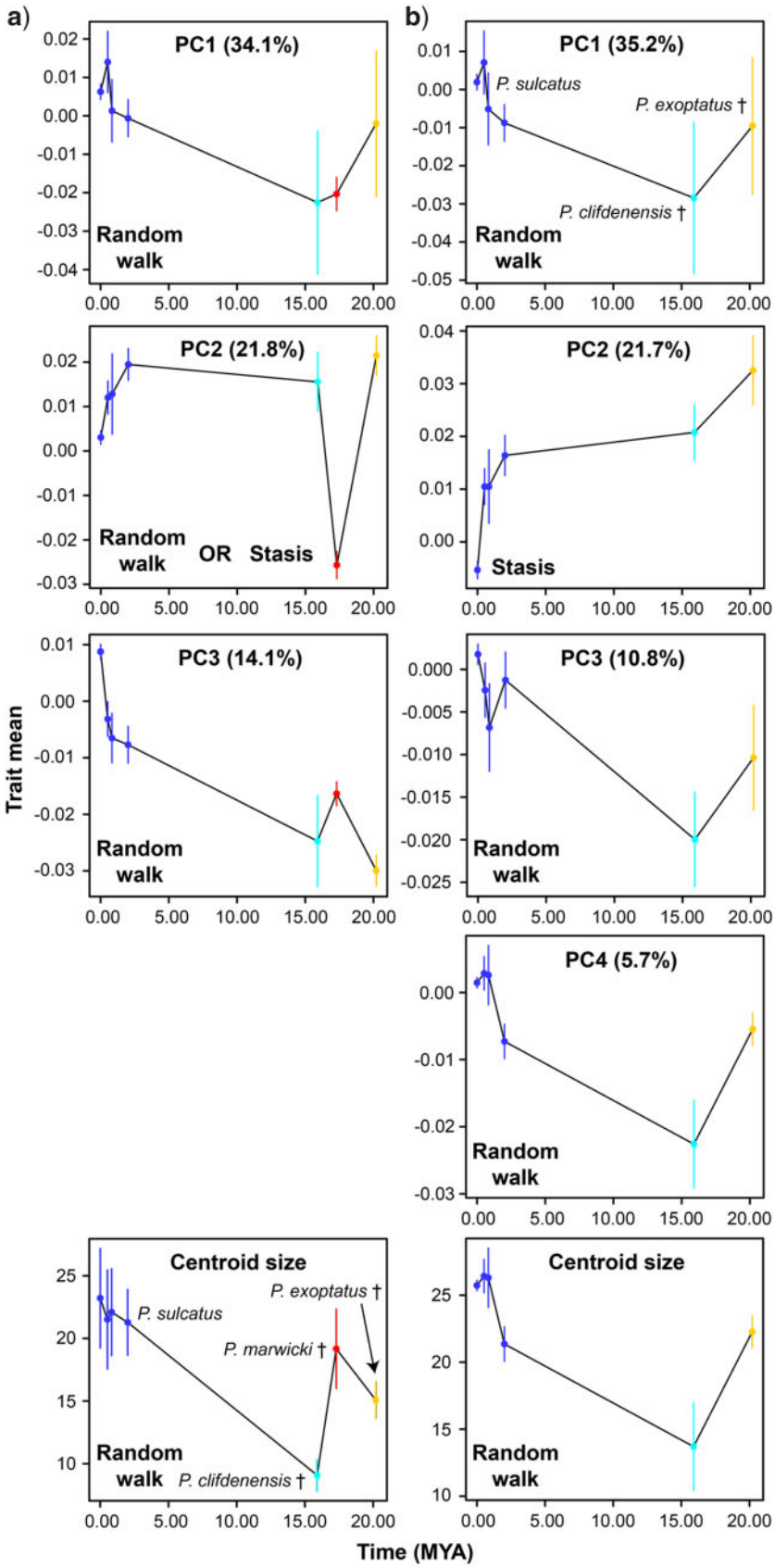

FIGURE 6. Time series analyses of shell shape and size evolution estimated for two alternative *Penion sulcatus* lineages over 20 myr (results from paleoTS). The best fitting model of evolutionary mode is labeled for each analyzed trait (retained shell shape PCs and shell size). a) Evolutionary lineage consisting of *P. sulcatus*, *P. clifdenensis*, *P. marwicki*, and *P. exoptatus*; each shown in a different color. b) Evolutionary lineage consisting of *P. sulcatus*, *P. clifdenensis*, and *P. exoptatus*. Full paleoTS results including support values for each evolutionary model per trait are provided in Supplementary Tables S8 and S9 available on Dryad.

suggest that these fossils represent a single lineage, compatible with molecular clock analysis that estimated the age for the most recent common ancestor of *P. sulcatus* and other New Zealand *Penion* species long before the oldest fossil *P. clifdenensis* or *P. exoptatus* (median 40.6 Ma; 95% HPD 49.8–32.1 Ma; Vaux et al. 2017b). Considering that shell morphology and evolutionary relationships appear to be concordant in *Penion*, this result is remarkable as it suggests that no substantial morphological change has occurred in the *P. sulcatus* lineage since the fossils recognized as *P. exoptatus* in the New Zealand regional Otaian stage (late Aquitanian–early Burdigalian international stages, early Miocene, 21.7–18.7 Ma). In contrast, distinction of *P. marwicki* from the *P. sulcatus* specimens is more ambiguous; with morphometric analyses separating about 60% of fossil specimens (Fig. 4f; Supplementary Fig. S14c available on Dryad). The most parsimonious interpretation of this degree of shape differentiation is that *P. marwicki* represents a separate evolutionary lineage. Interestingly, *P. marwicki* fossils date to a similar age as specimens of *P. clifdenensis*, and the apparent start of morphological conservatism, in the New Zealand regional Altonian stage (Burdigalian international stages, early Miocene, 18.7–15.9 Ma fossils). However, no fossils resembling *P. sulcatus* are known to overlap in time with *P. marwicki* lineage material (Beu and Maxwell 1990).

Impact of Lineage and Species Identification on Time Series Analysis

The inclusion or removal of *P. marwicki* from the putative *P. sulcatus* lineage had little effect on estimates of evolutionary mode for shell size and most axes of shell shape variation, with these variables fitting an unbiased random walk model (Fig. 6a). However, the exclusion of *P. marwicki* affected one shape trait (PC2), causing it to fit a model of morphological stasis (Fig. 6b). We acknowledge that our time series analysis with *P. sulcatus* is simplistic—a relatively small number of time intervals are available in our fossil sequence, which might favor a best fit to a random walk model even when the true evolution of a trait follows an alternative model (Hunt 2008). We have also not investigated re-binning samples (Guillerme and Cooper 2018), tested alternative time series methods, or simulated fitted models for comparison to empirical data (Cooper et al. 2016; Voje 2018). However, our results are sufficient to demonstrate that lineage identification and the taxonomic classification of fossils can significantly change estimates of evolutionary mode.

This change in results highlights the importance of taxonomic classification when selecting specimens, as well as time intervals, for testing the fit of trait variation to models of evolutionary change through time. This influence upon time series analysis is similar to the effect of taxonomic "lumping and splitting" upon interpretations of the fossil record diversity. Like Michaux (1989), we note that 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 variation can be observed as gradual change. Likewise, the magnitude of evolutionary change contained within a single evolutionary lineage or taxonomic species is clearly relevant for concepts such as evolutionary stasis (Lande 1986).

Despite previous discussion of this point, we note that methods papers for time series analysis have not directly addressed the issue of lineage identification (Hunt 2007; Hopkins and Lidgard 2012), although differences observed between clade and species-level time series analyses may have been affected by lineage identification (Hunt and Slater 2016). Likewise, time binning is known to bias analyses of evolutionary change (Guillerme and Cooper 2018), but the outcome of this effect for the temporal duration of taxonomic units needs further investigation. Studies of evolutionary mode and tempo are likely to benefit if the boundaries of morphological variation within a species or lineage are first estimated within a genetic context, before attempting to fit models of evolutionary change to samples.

In conclusion, our understanding of trait evolution and changes in biodiversity through time relies on the identification of evolutionary lineages, and the consistent classification of taxonomic units such as species. Lineage identification is especially important for investigations of evolutionary mode and tempo, which is why more example systems that incorporate extant variation into time series analyses are needed (Gould 1991; Hunt 2010).

Supplementary Material

Data available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.gqnk98sh6.

Demultiplexed forward DNA sequence reads for the 18 *Penion* individuals sequenced in this study are openly available on the NCBI sequence read archive (SRA) under: PRJNA564825, http://www.ncbi.nlm.nih.gov/bioproject/564825.

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