MOLLUSCAN RESEARCH
https://doi.org/10.1080/13235818.2021.1957552

Taylor & Francis
Taylor & Francis Group

Generation of large mitochondrial and nuclear nucleotide sequences and phylogenetic analyses using high-throughput short-read datasets for endangered Placostylinae snails of the southwest Pacific

Mathieu Quenu, Steven A. Trewick, Elizabeth E. Daly and Mary Morgan-Richards

Wildlife & Ecology, School of Agriculture & Environment, College of Sciences, Massey University, Palmerston North, New Zealand

ABSTRACT

Placostylinae are a sub-family of terrestrial land snails endemic to the southwest Pacific. Some species are harvested for food, and others are critically endangered. Here we assemble and characterise complete mitochondrial genomes, as well as three nuclear markers (partial 45S ribosomal cassettes and the histone genes H3 and H4) of five snail species from three geographical regions (New Zealand, New Caledonia and the Solomon Islands). Mitogenomes of Placostylinae snails ranged between 14,544 bp and 14,711 bp, with minor variation in the position of tRNA tyrosine (Y) and tRNA tryptophane (W). The 45S ribosomal cassette contained intra-genomic nucleotide variation in ITS2. Cassettes containing histone genes H3 and H4 and their non-transcribed spacer region were assembled for three species, with the two genes coded in the same direction. Phylogenetic analysis on this large dataset (mitochondrial genome + nuclear markers) supported geographical clustering of species but could not confidently infer monophyly of the four *Placostylus* species with respect to *Eumecostylus cleryi*. Analysis based on shorter cytochrome *c* oxidase sequences with a wider taxon sampling found species representing the genera *Eumecostylus* and *Placocharis* were nested within the phylogenetic diversity of *Placostylus*. Multi-locus phylogenetic analysis containing mitochondrial and nuclear sequences did not support monophyly of Placostylinae.

KEYWORDS

Histone genes; genetic resources; mitochondrial genome; phylogenetics; *Placostylus*; ribosomal cassette

Introduction

With approximately 85,000 recognised extant species, Mollusca is the second most diverse animal phylum on earth (MolluscaBase eds. 2021). Systematics within this phylum still need improvement, and many species complexes are yet to be fully described or understood (Lydeard *et al*. 2004). Molecular tools can be used to identify management units and complement morphological data (Moritz 1994). Short DNA sequences have been widely used for inferring phylogenetic relationships among molluscs in the last few decades (e.g., Wade *et al.* 2006; Herbert and Mitchell 2009; Ramirez *et al.* 2009), but the emergence of high-throughput Next Generation Sequencing (NGS) technology has made it possible to analyse significantly larger genetic data sets (Hunter *et al.* 2016). For phylogenetic inferences to be reliable, genomic regions targeted by NGS technologies must be both conserved enough to allow sequence alignment and at the same time display enough nucleotide variation to permit inference of statistically robust phylogenetic relationships. Technologies such as exon-based recapture (Teasdale *et al.* 2016) or anchored phylogenomics (Lemmon and Lemmon 2012) can now be used to sample up to hundreds of genomic regions with phylogenetic signal. They are, however, associated with a high methodological cost and require either the generation of transcriptomic data in parallel to generation of genomic libraries (exon-based recapture) or the use of probes to enrich genomic datasets in regions of interest (anchored phylogenomics). One simpler phylogenomic approach is to use mitochondrial and multi-copy nuclear gene data reconstructed from genomic libraries as input for phylogenetic analysis. Both types of markers are overrepresented in shotgun sequencing genomic libraries and therefore the methodological and economical cost associated with their generation is minimal. In addition, because they are present in high numbers they will usually be a good target in analysis of fossil shells, which are now providing opportunities to incorporate ancient DNA into tests of evolutionary change through phylogenetic analysis (Daly *et al.* 2020; Der Sarkissian *et al.* 2020; Ferreira *et al.* 2020). Here we use high-throughput short-read sequencing to assemble whole mitochondrial genomes, 45S nuclear ribosomal DNA cassettes and the histone genes H3 and H4 of five species of Placostylinae Pilsbry, 1946, a sub-family of giant terrestrial land snails endemic to the southwest Pacific region. We use these data to infer phylogenetic

CONTACT Mathieu Quenu mathieuquenu@hotmail.fr Wildlife & Ecology, School of Agriculture & Environment, College of Sciences, Massey University, Private Bag 11-222, Palmerston North 4442, New Zealand

Supplemental data for this article can be accessed at https://doi.org/10.1080/13235818.2021.1957552.

Published online 01 Aug 2021

relationships among Placostylinae species living on separate islands, and among other related species.

The Placostylinae belong to the superfamily Orthalicoidea (Breure *et al.* 2010; Breure and Romero 2012) and contain among others the three genera *Placostylus* (H. Beck, 1837), *Eumecostylus* (Martens in Albers, 1860) and *Placocharis* (Pilsbry, 1900) that are endemic to islands in the southwest Pacific. Most of the recognised species in these genera live on Melanesian islands from the Bismarck and Solomon archipelagos to Vanuatu, New Caledonia and Fiji but also on islands further south (Lord Howe, Three Kings, and the Far North of New Zealand). Many of these snail taxa are under threat of extinction from anthropogenic habitat change and predator introduction, and some are already considered extinct (Brescia *et al.* 2008; Stringer *et al.* 2014). In many places extensive fossil deposits of *Placostylus* provide a potential resource for studies of changing environmental conditions (Brook 1999). In New Caledonia at least six species are recognised (Dowle *et al.* 2015) and they are culturally and economically important as food (Quenu *et al.* 2020). On Grande Terre itself, the main island of New Caledonia, *Placostylus* populations have declined and some species are at risk (Brescia *et al.* 2008), but on Île Des Pins (Isle of Pines) just south of Grande Terre, *Placostylus* snails are still harvested for food by indigenous Kanak people. New Zealand *Placostylus* species are considered vulnerable and today survive only in highly fragmented remnant colonies (Parrish *et al.* 1995; Daly *et al.* 2020). On Lord Howe Island, *Placostylus bivaricosus* (Gaskoin, 1855) is critically endangered (Ponder *et al.* 2003). On other island systems such as Fiji and the Solomon Islands Placostylinae are subject to similar threats, although information on these populations is limited (Brodie 2012).

Previous molecular work on Placostylinae has relied on application of universal PCR primers to target short fragments (<850 bp) of mitochondrial genes. Using partial mtDNA cytochrome *c* oxidase subunit 1 (COXI) sequences, the Lord Howe species, *P. bivaricosus* (Gaskoin, 1855), was identified as sister to the two New Zealand mainland species, *Placostylus ambagiosus* (Suter, 1906) and *Placostylus hongii* (Lesson, 1830), while the third New Zealand species, *Placostylus bollonsii* (Suter, 1908), which is endemic to the Three Kings Islands is phylogenetically more distant (Ponder *et al.* 2003). A similar result was obtained using mtDNA COXI and ribosomal RNA16S sequences in a study (Trewick *et al.* 2009) that also included representatives of the New Caledonian species *Placostylus fibratus* (Martyn, 1789), *Placostylus porphyrostomus* (Pfeiffer, 1851) and *Placostylus caledonicus* (Petit, 1845). The New Caledonian species were inferred to be a sister group to New Zealand mainland and Lord Howe Island species. Further molecular analysis of New Caledonian *Placostylus* spp. used sequences of the faster evolving mtDNA NADH dehydrogenase 2 (ND2) gene in combination with shell shape analysis and nuclear SNP markers to explore population level diversity (Dowle *et al.* 2015). Deeper phylogenetic relationships have also been investigated within the superfamily Orthalicoidea using concatenated short DNA sequences (Breure and Romero 2012), including data from *P. ambagiosus* from New Zealand, *Placostylus eddystonensis* (Pfeiffer, 1855) from New Caledonia, and *Eumecostylus uligunosus* (Kobelt, 1891) and *Placocharis strangei* (Pfeiffer, 1855) from the Solomon Islands. The 2079 bp alignment of concatenated sequence (COXI, ITS2/28S, Histone H3) supported the Placostylinae as a clade within the Bothriembryontidae.

Until now, no full mitochondrial genome or 45S ribosomal RNA cassette has been assembled for any Placostylinae species, and the gene arrangement of histone H3 and H4 genes is not known. Mitochondrial DNA occurs in high copy numbers and therefore is likely to be most appropriate for ancient DNA studies from fossil shells (Shtolz and Mishmar 2019). Nuclear 45S ribosomal cassettes, which include the conserved regions 18S, 5.8S, 28S and the unconstrained Internal Transcribed Spacers 1 and 2 (ITS1 and ITS2), are multi-copy. The ribosomal subunits 18S, 5.8S and 28S are conserved by their function in the translation of cytoplasmic mRNA of eukaryotic cells (Kressler *et al.* 2010). ITS1 and ITS2 separate the different subunits within the 45S cassette and their nucleotide sequences are highly variable. The 45S ribosomal cassettes are present in numerous tandem copies, usually on several chromosomes in the nuclear genome (Dalet *et al.* 2014), and are thought to evolve via concerted evolution (Nei and Rooney 2005). Intra-individual variation of nucleotide sequences is common in ITS, and can produce statistical noise when ITS sequences are used in phylogenetic or population genetic analysis (Hillis and Dixon 1991; Nei and Rooney 2005; Sochorová *et al.* 2018). The two histone genes H3 and H4 code for protein subunits of the nucleosome complex. They are usually linked by a noncoding spacer region, and in molluscs they have been reported to be coded on opposing strands in some species (Armbruster *et al.* 2005; Harl *et al.* 2014), but not all, e.g., *Mytilus edulis* Linnaeus, 1758 (Albig *et al.* 2003). Due to their high copy number in animal cells all three loci (mitochondrial genome, 45S ribosomal cassette and Histone genes H3/H4) can be reconstructed using high-throughput short-read NGS data (Koot *et al.* 2020; Gemmell *et al.* 2020). Here we sample five species of Placostylinae: two from New Zealand (*P. ambagiosus*, *P. hongii*), two from New Caledonia (*P. fibratus*, *P. porphyrostomus*) and *Eumecostylus cleryi* (Petit de la Saussaye, 1850) from the Solomon Islands. By doing this we hope to provide genetic tools that will be useful in future population genetic analysis of endangered Placostylinae, and in analysis

of ancient DNA extracted from fossil shells. We compare phylogenetic inferences from few taxa with long sequences to phylogenetic inferences obtained using limited amounts of data but a larger taxon sample. To achieve this, three distinct phylogenetic analyses are generated. First, phylogenetic relationships between the five taxa are estimated using large mitochondrial and nuclear sequence datasets. Second, we extract a short COXI sequence for the five taxa and incorporate the new sequences in phylogenetic analysis of *Placostylus* and other closely related taxa (Trewick *et al.* 2009). Third, a multi-locus phylogenetic analysis containing mitochondrial and nuclear sequences is generated to investigate the relationships of sampled Placostylinae snails in relation to other Orthalicoidea sub-families (Breure and Romero 2012).

Material and methods

Tissue collection

We used foot muscle tissue samples from four species of land snail of the genus *Placostylus*. The samples of the New Zealand species, *P. ambagiosus* and *P. hongii*, were from a frozen tissue collection originally developed from whole body samples harvested for allozyme analysis (Triggs and Sherley 1993). *Placostylus ambagiosus* (PS185; NZ National Arthropod collection code GS4 of Triggs and Sherley 1993) was collected by G. Sherley from Cape Maria van Diemen, New Zealand. The specimen of *P. hongii* (PS257; NZ National Arthropod collection code WG865) came from eastern Far North, North Island. Although the precise provenance of PS257 is not recorded it was confirmed as *P. hongii* on the basis of shell morphology and region of origin. The two New Caledonian specimens, *P. fibratus* (PS28) and *P. porphyrostomus* (PS45), were collected in the field from populations on the Isle of Pines (Dowle *et al.* 2015). The Solomon Island specimen, *E. cleryi* (PS127), was collected in Honiara, Guadalcanal.

DNA extraction

Foot muscle samples of approximately 50 mg were cut from specimens using sterile scalpel blades. Each tissue was pressed in a clean paper towel to remove excess storage ethanol and cut into smaller pieces. Whole genomic DNA was extracted using incubation at 55°C in CTAB buffer (2% hexadecyltrimethyl ammonium bromide, 100 mM Tris-HCL pH 8.0 20 mM EDTA) with proteinase K (Trewick *et al.* 2009). Following tissue digestion the solution was purified using an equal volume of 24:1 chloroform-isoamyl alcohol and centrifugation. DNA was precipitated from the aqueous fraction using sodium acetate (3M NaOAC) and chilled 95% ethanol. This extraction method has been found to be the most effective for isolating high molecular weight DNA from neogastropods while avoiding the problems of mucopolysaccharide contamination (Winnepenninckx *et al.* 1993). DNA was re-suspended in 50 µl or 100 µl TE buffer (10 mM Tris, 0.1 mM EDTA) and quantified using Qubit fluorometry (Life Technologies, Thermo Fisher Scientific Inc).

Illumina sequencing

Total DNA extracts from the five Placostylinae specimens were processed through massive parallel, high-throughput sequencing using the ThruPLEX DNA-seq kit (Rubicon Genomics). Fragmented genomic DNA was pair-end sequenced on an Illumina Hiseq 2500. Reads were de-multiplexed using standard indexes. Resulting Illumina short reads were trimmed of adapters and passed through standard quality filters using the software fastp (Chen *et al.* 2018). Reads were paired in Geneious v8 (Kearse *et al.* 2012).

Genome and gene assemblies

Mitochondrial genomes were assembled from each of five sets of 101 bp paired-read data files. Initially the mitochondrial genome of *P. ambagiosus* PS185 was assembled starting with available Sanger sequenced partial sequences of *Placostylus* mtDNA COXI as a reference using the Geneious v8 mapping tools (Kearse *et al.* 2012). We then used iterative remapping to form consensus sequences from each previous mapping round using medium-low sensitivity with 25 iterations to assemble the full circular mitochondrial genome. Once assembled, this mitochondrial genome served as the initial reference for mapping of paired-end reads of other individuals, which were then iteratively remapped until maximum read coverage was achieved.

We used the MITOS web server (Bernt *et al.* 2013) to estimate gene annotations for one of the novel mtDNA genomes and used amino acid translation tools in Geneious to verify that each protein-coding sequence had an uninterrupted translation frame and the expected start and stop codons. We used ARWEN (Laslett and Canbäck 2008) to confirm secondary folding of transfer RNAs then transferred annotations to the other four similar genomes. Annotations were further checked for homology across the five genomes.

The same mapping approach was used to assemble the 45S nuclear ribosomal DNA cassettes of the five species, using available 5.8S and 28S sequences as our starting reference, and then mapping reads at medium-high sensitivity in Geneious (Kearse *et al.* 2012).

Fragments of histone 3 (H3) were also reconstructed by mapping reads of the five Placostylinae species onto reference sequences. Five 267 bp H3 fragments

were reconstructed based on GenBank data from the Placostylinae species *P. strangei* (JF514684, Breure and Romero 2012) and five 291 bp H4 fragments were reconstructed based on GenBank data from *Orcula dolium* (Draparnaud, 1801) (KY512728, Harl *et al.* 2014). To reconstruct whole histone H3/H4 complexes (containing their noncoding spacer region), we first used one of the H4 fragments as a reference (PS127, *E. cleryi*) and iteratively mapped corresponding reads at low sensitivity until the H3 fragment of the same individual could be mapped to a reference sequence. We then mapped paired-end reads of other specimens to the PS127 consensus sequence, but only reads of the New Caledonian species *P. fibratus* and *P. porphyrostomus* mapped continuously to the reference sequence. We identified coding direction of the histone genes using the Geneious amino acid translation tool.

Phylogenetic analysis

Mitogenome and combined nuclear markers analysis of five Placostylinae

Phylogenetic analysis was performed for mitochondrial genomes and nuclear markers separately. Whole mitochondrial genomes, ribosomal cassettes and histone genes were aligned using the software MUSCLE (Edgar 2004), with default options. Alignments were then checked visually and regions with ambiguous alignment were manually removed. For mitochondrial genome phylogenies we concatenated all coding DNA sequence (CDS) regions, and all the tRNA regions which are likely to hold phylogenetic signal (total length of this alignment was 10,981 bp). An additional phylogenetic analysis using only the CDS regions was also performed. We used BLAST (Madden 2013) to find the closest mitogenome to our dataset on the GenBank database [*Naesiotus nux* (Broderip, 1832)] and used it as an outgroup in the mitochondrial phylogeny (Hunter *et al.* 2016). Nuclear markers (45S cassette, H3, H4) were also concatenated after aligning each marker separately (total length of the alignment was 8216 bp). Nuclear sequences for closely related species were not found on GenBank and therefore nuclear analysis had to be run without an outgroup. Phylogenetic relationships among taxa were estimated using both Maximum Likelihood (ML) and Bayesian Inference (BI) approaches. PartitionFinder2 (Lanfear *et al.* 2017) was used to find the most appropriate models of nucleotide evolution, using the corrected Akaike Information Criterion (AICc) as a model evaluation metric. We used PhyML for the ML approach (Guindon *et al.* 2010) and MrBayes 3.0 for BI phylogenies (Huelsenbeck and Ronquist 2001). Bayesian analyses were run on the CIPRES science gateway 3.3 server (Miller *et al.* 2010), taking advantage of cloud computing processing power. For mitochondrial analyses we used a partition of three nucleotide models for the CDS concatenated region (GTR + gamma; GTR + I + gamma and GTR + gamma), and one model for the tRNA region (GTR + gamma). Only one model of nucleotide substitution was needed for the phylogenetic analysis of nuclear markers (GTR + I + gamma).

Mitochondrial COXI analysis of 10 species of Placostylinae

We extracted a 538 bp COXI fragment from our five mitochondrial genomes to produce phylogenetic trees containing a wider sampling of Placostylinae, based on much shorter DNA sequences. Sequences were extracted from GenBank for *Placostylus* species of New Zealand, New Caledonia, Three Kings Islands and Lord Howe Island (Trewick *et al.* 2009) and Placostylinae species of the Solomon Islands (Breure and Romero 2012). The total alignment included 10 putative species and 71 individuals. The same methods were applied for this phylogenetic analysis, we used both ML and BI approaches with only one model of nucleotide substitution (GTR + I + gamma).

Multi-locus analysis of multiple Orthalicoidea species

Finally, to assess phylogenetic relationships of the five Placostylinae species within the context of the wider Orthalicoidea superfamily we also performed a phylogenetic analysis using concatenated multi-locus alignment of COXI sequences, partial histone H3 sequences and partial 28S sequences (Table 1, Breure and Romero 2012). We used 13 sequences of total 1730 bp length for this analysis, using sequences of *Megaspira eliator* (Spix, 1827) as an outgroup, and only one model of nucleotide substitution (GTR + I + gamma).

Results and discussion

Mitochondrial genomes and nuclear loci characteristics

The five mitochondrial genomes ranged from 14,652 to 15,187 bp (Table 2, Supplementary Table 1). All contained the expected 37 genes found in most metazoans, including 13 protein-coding genes, 2 rRNAs and 22 tRNA genes. Of these genes 24 are encoded on the heavy strand and 13 on the light strand. Nucleotide composition is skewed towards a high proportion of adenine and thymine, which is a common observation for invertebrate mitochondrial genomes (Table 2; Shioiri and Takahata 2001). The size of the noncoding region varies from 539 to 1577 bp, i.e., 3.7–10.8% of the total mitochondrial genome. Arrangement of mitochondrial genes remains mostly unchanged across sampled taxa, with only the respective position of two tRNA genes differing among species (Figure 1).

Table 1. GenBank accession number and collection information of all sequences used in phylogenetic analysis in this study.

Species name	Locality	Collector	Mt genome	45S	COXI	H3	H4	Partial 28S
Naesiotus nux	San Cristobal Island, Galapagos	S. Hunter	KT821554.1		KT821554.1			
Placostylus fibratus	Isle of Pines, New Caledonia	F. Brescia	MT163270	MN567952	MT163270	MT559980	MT602525	MN567952
Placostylus porphyrostomus	Isle of Pines, New Caledonia	F. Brescia	MT163271	MN567955	MT163271	MT559981	MT602524	MN567955
Placostylus hongii	Far North, New Zealand	G. Sherley	MT163273	MN567954	MT163273	MT559984	MT602521	MN567954
Placostylus ambagiosus	Far North, New Zealand	G. Sherley	MT163272	MN567951	MT163272	MT559983	MT602522	MN567951
Eumecostylus cleryi	Guadalcanal, Solomon Islands	R. Richards	MT163274	MN567953	MT163274	MT559982	MT602523	MN567953
Eumecostylus uligunosus	Rokera, Solomon Islands	A. Delsaerdt			JF514642	JF514685		HM027505
Bothriembryon dux	Mt Caitlin, Western Australia				JF514643	JF514686		HM027490
Botrhriembryon indutus	Walyunga National Park, Australia	C. Whisson						EU622023
Prestonella bowkeri	Glen Avon, South Africa	D. Herbert			KF129392	JF514711		EU622021
Prestonella nuptialis	Craddock area, South Africa	D. Herbert			KF129349			EU622022
Discoleus aguirrei	Rio Negro, Argentina	M. Guezzo			KT371414			KT371389
Discoleus ameghinoi	Rio Negro, Argentina	M. Guezzo			KT371415	JF514698		JF514753
Placocharis strangei	New Georgia, Solomon Islands	A. Delsaerdt			JF514641	JF514684		HM027504
Megaspira eliator	Rio de Janeiro, Brasil	A. Galdino dos Santos			JF514610	JF514715		JF514721
Placostylus eddystonensis	Mount Koghis, New Caledonia	C. Wade						AY841297

Table 2. Summary statistics of five reconstructed mitochondrial genomes of Placostylinae species, assembled using 101 bp paired reads.

	P. ambagiosus (PS185)	*P. hongii* (PS257)	*P. fibratus* (PS28)	*P. porphyrostomus* (PS45)	*E. cleryi* (PS127)
Size (bp)	14,652	14,711	15,187	15,118	14,737
A + T proportion	0.76	0.76	0.73	0.73	0.75
Noncoding region (bp) and (%)	539 (3.7%)	900 (6.1%)	1041 (7.2%)	1026 (7.0%)	1577 (10.8%)
Genes	37	37	37	37	37
Gene regions (bp)	14,208	14,490	13,657	13,837	13,518
tRNA	22	22	22	22	22

Note: *P.* = *Placostylus*, *E.* = *Eumecostylus*.

In New Caledonian and Solomon Island species tRNA tyrosine (Y) is adjacent to Cox2 and followed by tRNA tryptophan (W), whereas in New Zealand species these two tRNAs are swapped and it is tRNA W that is adjacent to Cox2. Start codons for protein-coding genes include the standard ATG, ATA and ATT but also TTG and GTG. Start codons for invertebrate mitochondrial protein-coding genes are quite variable (Gaitán-Espitia *et al.* 2013; Shen *et al.* 2012) and within our dataset there is evidence of start codon mutations for the genes ND5, ND4L, ND3, ND4, Cox3 and ND2 (Table 2). Stop codons include the standard TAA and truncated T. The hardest region to reconstruct was ATP8, which also seems to be the most variable protein-coding gene of the mitochondrial genome. Length of this gene is extremely reduced in all five Placostylinae species (around 200 bp; Table 3). This observation suggests that ATP8 could be under relaxed selection in Placostylinae, which may be linked to the low mobility of the snails (Sun *et al.* 2017).

The 45S region contained the five expected components of the transcription unit: the three RNA coding regions 18S, 5.8S and 28S and two Internal Transcribed Spacers (ITS1 and ITS2). Mapped reads of the 45S ribosomal cassette displayed evidence of intra-genomic variation at some nucleotide positions of the ITS1 and ITS2 regions in all five sampled species genomes. This pattern of intraspecific variation has been reported for this marker in a number of other Gastropoda, and is widespread in metazoans (Stothard *et al.* 1996; Hoy and Rodriguez 2013; Davison 2002; Harris and Crandall 2000; Itskovich 2020). Relaxed selection combined with independent mutations in different sets of copies could lead to this pattern in ITS, while constraining selection minimises intra-genomic nucleotide variation in the transcribed 18S, 5.8S and 28S regions (Pereira and Baldwin 2016). This needs to be taken into consideration when using ITS1 and ITS2 in phylogenetic or population genetic analysis, as intra-genomic nucleotide variation could potentially confound conclusions reached from comparative ITS analyses. Sequencing methods based on polymerase chain reaction amplification are more likely to yield invariant sequences whereas high-throughput sequencing followed by mapping is more likely to make read variants apparent.

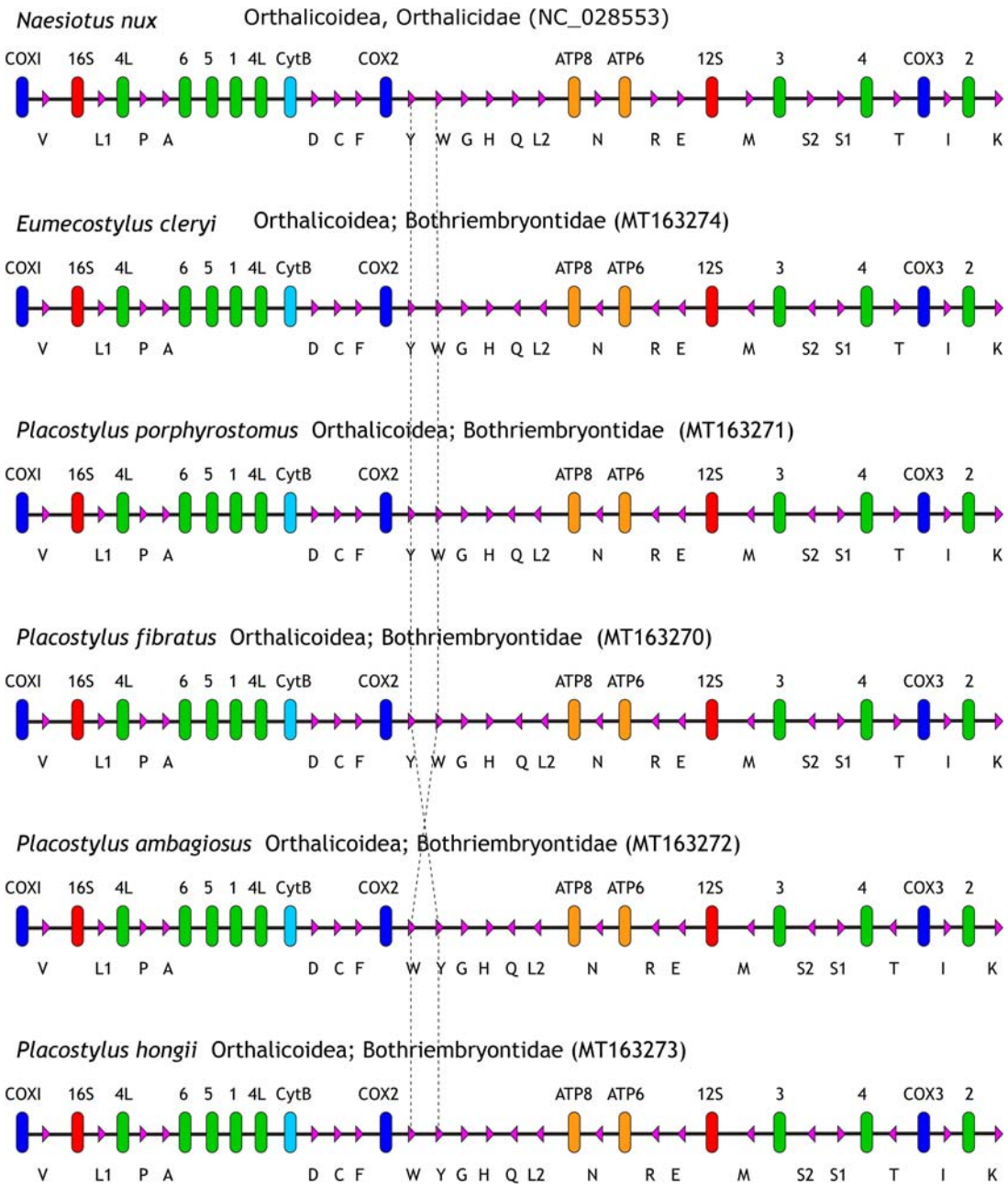

Figure 1. Comparison of mitochondrial genome gene order in five Placostylinae snail species. Full name of tRNA and CDS genes can be found in Table 3.

Histone genes H3 and H4 were reconstructed for the five Placostylinae species based on GenBank fragments from related species. Whole histone cassettes containing histone genes H3 and H4 and their noncoding spacer region could only be reconstructed for the Solomon Island species *E. cleryi* (GenBank accession number: MT726982) and the two New Caledonian species *P. fibratus* and *P. porphyrostomus* (GenBank accession numbers: MT726983 and MT726984). In these cassettes both genes were coded on the same strand, and were separated by a noncoding spacer region of 707 bp (*P. porphyrostomus*), 772 bp (*E. cleryi*) or 800 bp (*P. fibratus*). Histone genes H3 and H4 have been reported to be orientated in opposing directions in gastropod genomes (Armbruster *et al.* 2005; Harl *et al.* 2014), but other configurations have been reported in bivalves (Albig *et al.* 2003). Our findings confirm that histone gene cassettes are not configured consistently in gastropods. For the two New Zealand species, *P. ambagiosus* and *P. hongii*, the configuration of histone genes could not be retrieved from our data. Mapping reads from New Zealand species to consensus sequences of other species only led to discontinuous mapping reconstructions, with gaps and poor mapping resolution in the noncoding spacer region. It is hard to know if this result reflects read limitation in our data or indicates real biological information. If it were real, it would imply that histone genes H3 and H4 have physically distinct genome locations in

Table 3. Position and length of the 13 protein-coding genes and 2 rRNA genes found across five mitochondrial genomes of Placostylinae species.

Name	Type	Direction	Position					Length				
			PS127	PS45	PS28	PS185	PS257	PS127	PS45	PS28	PS185	PS257
COXI	CDS	f	1 → 1542 TTG/TAA	1 → 1494 TTG/TAG	1 → 1488 TTG/TAG	1 → 1533 TTG/TAA	1 → 1533 TTG/TAA	1542	1494	1488	1533	1533
16S	rRNA	f	1743 → 2665	1740 → 2658	1739 → 2660	1649 → 2684	1652 → 2685	923	919	922	1036	1034
ND6	CDS	f	2912 → 3388 ATG/TAG	2915 → 3394 ATG/TAA	2914 → 3393 ATG/TAA	2953 → 3433 ATG/TAG	2965 → 3450 ATG/TAA	477	480	480	481	486
ND5	CDS	f	3414 → 5057 GTG/TAG	3417 → 5057 GTG/TAG	3416 → 5056 ?/TAG	3455 → 5104 GTG/TAG	3470 → 5119 ATG/TAG	1644	1641	1641	1650	1650
ND1	CDS	f	5044 → 5964 ATG/TAA	5044 → 5964 ATG/TAG	5043 → 5963 ATG/TAG	5091 → 6008 ATG/TAA	5106 → 6023 ATG/TAA	921	921	921	918	918
ND4L	CDS	f	5972 → 6268 ATA/TAA	5972 → 6271 ATG/TAG	5971 → 6270 GTG/TAG	6016 → 6313 ATA/T	6031 → 6328 ATA/?	297	300	300	298	298
Cyt b	CDS	f	6276 → 7361 ATA/TAA	6276 → 7367 ATA/TAA	6275 → 7366 ATA/TAA	6320 → 7402 ATA/TAA	6332 → 7417 ATA/TAA	1086	1092	1092	1083	1086
COX2	CDS	f	7599 → 8260 ATG/TA	7588 → 8250 ATG/TAA	7588 → 8250 ATG/TAA	7637 → 8296 ATG/TAA	7662 → 8327 ATG/TAA	662	663	663	660	666
ATP8	CDS	r	8920 → 8747 ATG/TAA	9499 → 9293 ATG/TAA	9497 → 9291 ?/TAA	8987 → 8742 ?/TAA	9053 → 8802 ?/TAA	174	207	207	246	252
ATP6	CDS	r	9661 → 9008 ATG/TAA	10,239 → 9586 ATG/TAA	10,241 → 9588 ATG/TAA	9781 → 9128 ATG/TAA	9851 → 9198 ATG/TAA	654	654	654	654	654
12S	rRNA	r	10,627 → 9879	11,237 → 10,478	11,228 → 10,471	10,744 → 9979	10,832 → 10,048	749	760	758	766	1519
ND3	CDS	r	11,044 → 10,702 ATG/T	11,649 → 11,307 ATG/T	11,640 → 11,298 ATG/T	11,162 → 10,818 ATA/ATT	11,242 → 10,903 ATA/T	343	343	343	345	340
ND4	CDS	f	11,174 → 12,487 ATG/TAA	11,795 → 13,108 ATG/TAA	11,786 → 13,099 GTG/TAA	11,298 → 12,608 ATG/TAA	11,382 → 12,692 ATG/TAA	1314	1314	1314	1311	1311
COX3	CDS	r	13,409 → 12,561 ATG/TAG	14,022 → 13,183 ATG/TAG	14,013 → 13,174 ATG/TAG	13,538 → 12,687 ATT/TAG	13,601 → 12,756 ATT/T	849	840	840	852	846
ND2	CDS	f	13,552 → 14,478 ATG/TAG	14,165 → 15,088 ATG/TAG	14,157 → 15,080 ATA/TAG	13,628 → 14,614 ATT/TAA	13,690 → 14,673 ATG/TAA	927	924	924	987	984

Notes: For protein-coding genes, the nature of start and stop codons is indicated. Gene abbreviations COX: Cytochrome oxidase, ND: NADH dehydrogenase, Cyt: cytochrome b, ATP: ATP synthase. PS28: *Placostylus fibratus*, PS45: *Placostylus porphyrostomus*, PS127: *Eumecostylus cleryi*, PS185: *Placostylus ambagiosus*, PS257: *Placostylus hongii*.

P. ambagiosus and *P. hongii* rather than being organised in cassettes.

Phylogenies

Mitogenome and combined nuclear markers analysis of five Placostylinae

Phylogenetic relationships inferred from both mitochondrial and nuclear DNA sequences were similar and suggest geographical clustering of *Placostylus* species, with a sister relationship between the two New Zealand species, and between the two New Caledonian species (Figure 2). Using another bulimulid species, *Naesiotus nux* (Broderip, 1832), as an outgroup, *E. cleryi* (Solomon Islands) was placed in a sister relationship to the New Caledonian clade in the mitochondrial phylogeny (Figure 2). However, this branch was not strongly supported by ML bootstrapping (71) and BI posterior probability varied when incorporating tRNAs in the analysis (0.9675 in the phylogeny with both CDS and some tRNA regions; but only 0.8292 in a phylogeny using just CDS – tree not shown). Inferences of phylogenetic relationship among snail species from different geographical regions are therefore ambiguous with this dataset. Given the amount of data used in this analysis it is likely these uncertainties arise from either conflicting information in the different regions of the mitochondrial genome, and/or from insufficient taxon sampling (Jantzen *et al.* 2019).

Mitochondrial COXI analysis of 10 Placostylinae

Phylogenies based on short COXI sequences grouped *E. cleryi* and other snails from the Solomon Islands in a clade sister to *P. bivaricosus* (Lord Howe Island) with strong support (BI posterior probability = 1; Figure 3). Sister to this group is a monophyletic clade containing the *Placostylus* species from New Zealand, New Caledonia and Three Kings Islands. *Placostylus bollonsi*, the species from the Three Kings Islands, appears more closely related to species from New Caledonia than species from New Zealand. This is an odd result given the geographical setup of those islands (Three Kings Islands are 55 km north of New Zealand), but similar results have been found based on allozyme data (Triggs and Sherley 1993). Based on these data, the nesting of *Eumecostylus* and *Placocharis* within *Placostylus* suggests taxonomic

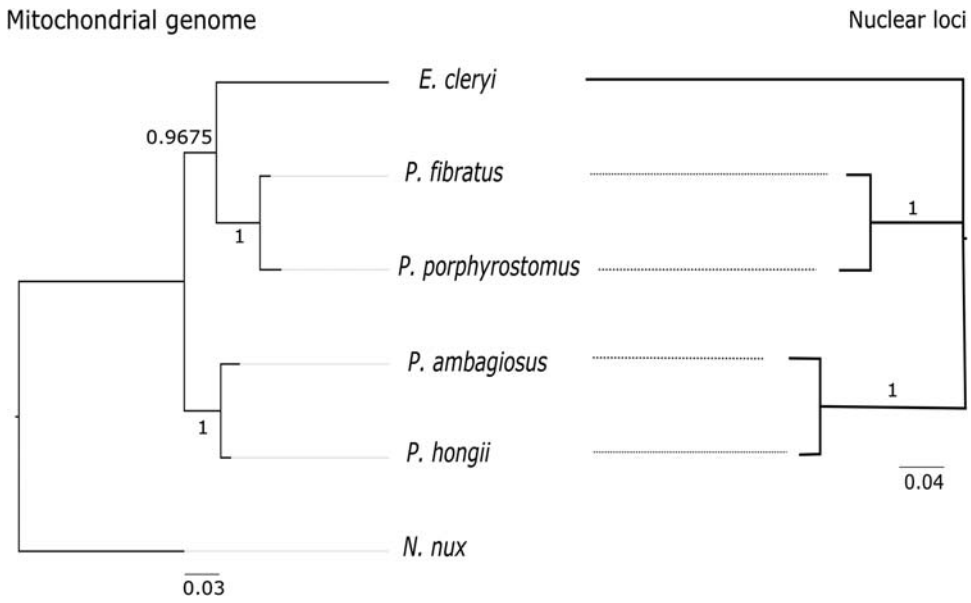

Figure 2. Phylogenetic relationships inferred for five giant terrestrial snails of the Pacific. *Naesiotus nux* (Bulimulidae) was used as the outgroup in the mitochondrial dataset analyses. *Placostylus fibratus* and *P. porphyrosotmus* are from New Caledonia, *P. ambagiosus* and *P. hongii* are from New Zealand and *E. cleryi* is from the Solomon Islands. The tree on the left is based on whole mitochondrial genome DNA sequences and that on the right is based on nuclear loci (ribosomal cassette 45S + histone genes). Both were inferred using a Bayesian phylogenetic approach.

redundancy, and using only one genus name for all species would seem more appropriate.

Multi-locus analysis of multiple Orthalicoidea species

Multi-locus analysis involving both mitochondrial (COXI) and nuclear (H3, 28S) sequences did not support the monophyly of the Placostylinae (Figure 4). Some Placostylinae species from the Solomon Islands (*P. strangei*, *E. uligunosus*, *E. cleryi*) were grouped with species from other sub-families [*Bothriembryon dux* (L. Pfeiffer, 1861); *Discoleus ameghinoi* (Ihering, 1908); *Prestonella nuptialis* (Melvill & Ponsonby, 1894); *Prestonella bowkeri* (G.B. Sowerby III, 1890); *Plectostylus peruvianus* (Bruguière, 1789)], but support for the corresponding branch is low (BI posterior probability 0.87). This differs from previous findings in this group, which used the same combination of loci, but fewer taxa (Breure and Romero 2012).

Overall, all phylogenetic relationships reported here supported monophyly within geographical regions (or

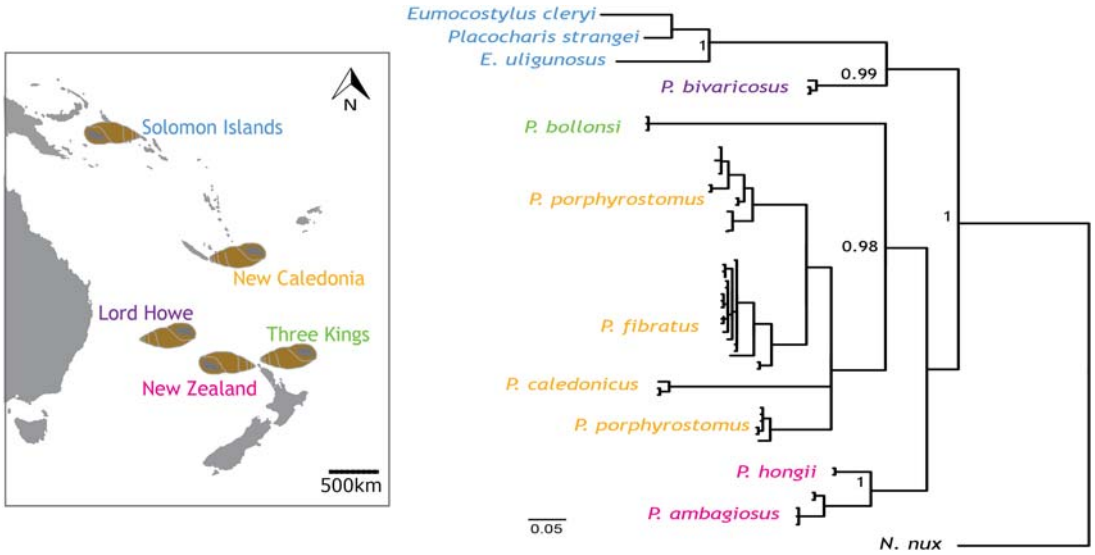

Figure 3. Phylogenetic relationships of 10 putative Placostylinae snail species from the southwest Pacific, inferred using a Bayesian phylogeny of partial mtDNA COXI (538 bp). Numbers at nodes refer to posterior probabilities indicating the level of support for clades. The shells of four *Placostylus* and one *Eumecostylus* species are shown. Species are colour coded on the map and phylogeny according to their geographical origin.

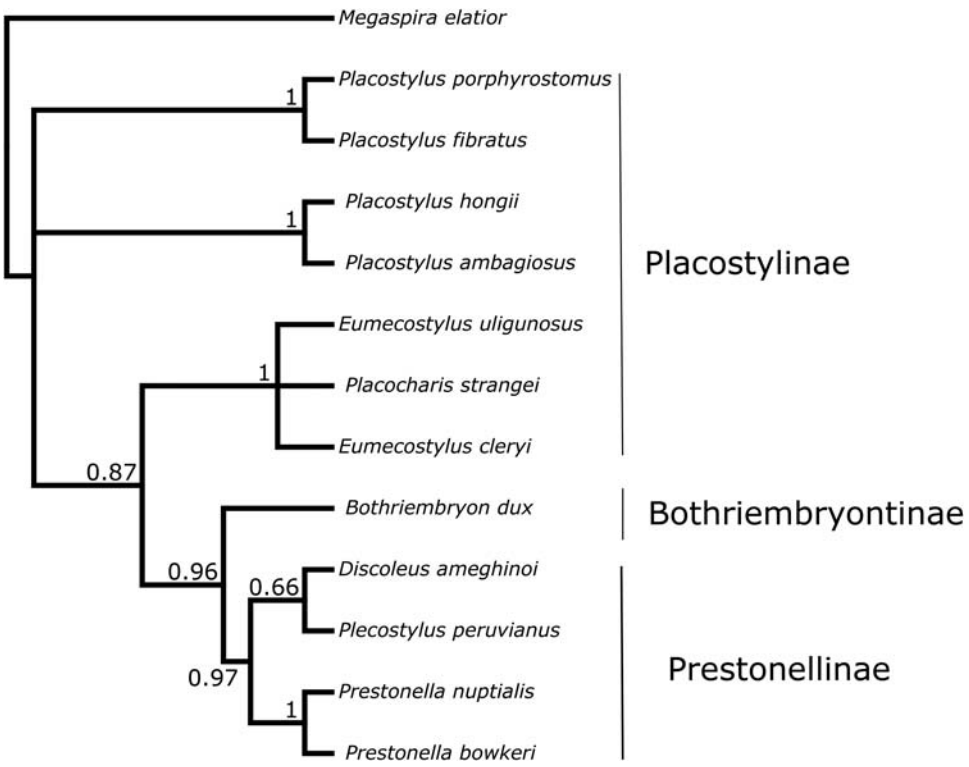

Figure 4. Multi-locus Bayesian phylogenetic relationships of 13 species of Orthalicoidea. The phylogeny is based on 1730 bp nucleotide alignment including concatenated sequences from COXI, H3 and 28S loci.

islands), but relationships among species in the wider Pacific rarely received strong branch support. Furthermore, phylogenetic relationships among the five Placostylinae species were different in each analysis. Long branch attraction caused by rapid molecular evolution within the Solomon Islands lineage and a relatively deep internode could be the source of this phylogenetic incongruence. Sampling of large NGS datasets for other species of Placostylinae will be needed to confidently infer phylogenetic relationships among these species (Uribe *et al.* 2019). From a biological perspective, dispersal from island to island is thought to be the main evolutionary process explaining the current distribution of Placostylinae species in the southwest Pacific (Trewick *et al.* 2009). This scenario provides no prediction of phylogenetic relationships as dispersal events to new locations could have happened in any order, and there is no limit to additional migration events that would overlay phylogenetic signal.

In conclusion, we provide full DNA sequences for the mitochondrial genome, and the nuclear 45S cassette, and Histone 3 and 4 for five giant land snails. These data provide the opportunity for developing better conservation genetic markers for a group of molluscs that are particularly vulnerable to human disturbance. In addition, because these genetic markers exist in multiple copies per cell, they provide an excellent resource for future study of a DNA from fossil snail shells.

Acknowledgements

We thank Fabrice Brescia and Margaret and Rhys Richards for collection of specimens in New Caledonia and the Solomon Islands; Abraham Breure for his help identifying the Solomon Island species; Eddy Dowle for DNA extractions; and members of the Phoenix group for Evolutionary Ecology and Genetics for helpful discussions (evolves.massey.ac.nz). We also would like to thank the two anonymous reviewers for their useful comments which greatly improved the quality of this manuscript, and the editor and associate editor for their help in the submission process.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This research was supported by a Massey University PhD scholarship (awarded to MQ) and a grant from the Royal Society of New Zealand Te Apārangi Marsden Fund 12-MAU-008.

Data availability statement

All sequences produced and used in this paper have been uploaded and are available to use in the NCBI GenBank database.

ORCID

Mathieu Quenu http://orcid.org/0000-0001-9392-6360
Mary Morgan-Richards http://orcid.org/0000-0002-3913-9814

References

Albig, W., Warthorst, U., Drabent, B., Prats, E., Cornudella, L. & Doenecke, D. (2003) *Mytilus edulis* core histone genes are organized in two clusters devoid of linker histone genes. *Journal of Molecular Evolution* 56, 597–606. doi:10.1007/s00239-002-2428-8.

Armbruster, G.F.J., Böhme, M., Bernhard, D. & Schlegel, M. (2005) The H3/H4 histone gene cluster of land snails (Gastropoda: Stylommatophora): TS/TV ratio, GC3 drive and signals in stylommatophoran phylogeny. *Journal of Molluscan Studies* 71, 339–348. doi:10.1093/mollus/eyi038.

Bernt, M., Donath, A., Jühling, F., Externbrink, F., Florentz, C., Fritzsch, G., Pütz, J., Middendorf, M. & Stadler, P.F. (2013) MITOS: Improved de novo metazoan mitochondrial genome annotation. *Molecular Phylogenetics and Evolution* 69, 313–319. doi:10.1016/j.ympev.2012.08.023.

Brescia, F.M., Pöllabauer, C.M., Potter, M.A. & Robertson, A.W. (2008) A review of the ecology and conservation of *Placostylus* (Mollusca: Gastropoda: Bulimulidae) in New Caledonia. *Molluscan Research* 28, 111–122.

Breure, A.S.H., Groenenberg, D.S.J. & Schilthuizen, M. (2010) New insights in the phylogenetic relations within the Orthalicoidea (Gastropoda, Stylommatophora) based on 28S sequence data. *Basteria* 74, 25–32.

Breure, A.S.H. & Romero, P.E. (2012) Support and surprises: Molecular phylogeny of the land snail superfamily Orthalicoidea using a three-locus gene analysis with a divergence time analysis and ancestral area reconstruction (Gastropoda: Stylommatophora). *Archiv Fur Molluskenkunde* 141, 1–20. doi:10.1127/arch.moll/1869-0963/141/001-020.

Brodie, G. (2012) *Placostylus kantavuensis. The IUCN Red List of Threatened Species*. Available online at https://dx.doi.org/10.2305/IUCN.UK.2012.RLTS.T195580A2389624.en. [Accessed on 15 July 2021.]

Brook, F.J. (1999) Stratigraphy, landsnail faunas, and paleoenvironmental history of coastal dunefields at Te Werahi, northernmost New Zealand. *Journal of the Royal Society of New Zealand* 29, 361–393. doi:10.1080/03014223.1999.9517603.

Chen, S., Zhou, Y., Chen, Y. & Gu, J. (2018) Fastp: An ultra-fast all-in-one FASTQ preprocessor. *Bioinformatics* 34, i884–i890. doi:10.1093/bioinformatics/bty560.

Dalet, J.T., Saloma, C.P., Olivera, B.M. & Heralde, F.M. (2014) Karyological analysis and FISH physical mapping of 18S rDNA genes, (GATA)n centromeric and (TTAGGG)n telomeric sequences in *Conus magus* Linnaeus, 1758. *Journal of Molluscan Studies* 81, 274–289. doi:10.1093/mollus/eyu090.

Daly, E.E., Trewick, S.A., Dowle, E.J., Crampton, J.S. & Richards, M.M. (2020) Conservation of pupu whakarongotaua, the snail that listens for the war party. *Ethnobiology and Conservation* 9. doi:10.15451/EC2020-05-9.13-1-27.

Davison, A. (2002) Land snails as a model to understand the role of history and selection in the origins of biodiversity. *Population Ecology* 44, 129–136. doi:10.1007/s101440200016.

Der Sarkissian, C., Möller, P., Hofman, C.A., Ilsøe, P., Rick, T.C., Schiøtte, T., Sørensen, M.V., Dalén, L. & Orlando, L. (2020) Unveiling the ecological applications of ancient DNA from mollusk shells. *Frontiers in Ecology and Evolution*, e000037. doi:10.3389/fevo.2020.00037.

Dowle, E.J., Morgan-Richards, M., Brescia, F. & Trewick, S.A. (2015) Correlation between shell phenotype and local environment suggests a role for natural selection in the evolution of *Placostylus* snails. *Molecular Ecology* 24, 4205–4221. doi:10.1111/mec.13302.

Edgar, R.C. (2004) MUSCLE: A multiple sequence alignment method with reduced time and space complexity. *BMC Bioinformatics* 5, article 113. doi:10.1186/1471-2105-5-113.

Ferreira, S., Ashby, R., Jeunen, G.J., Rutherford, K., Collins, C., Todd, E.V. & Gemmell, N.J. (2020) DNA from mollusc shell: A valuable and underutilised substrate for genetic analyses. *PeerJ* 8, e9420. doi:10.7717/peerj.9420.

Gaitán-Espitia, J.D., Nespolo, R.F. & Opazo, J.C. (2013) The complete mitochondrial genome of the land snail *Cornu aspersum* (Helicidae: Mollusca): Intra-specific divergence of protein-coding genes and phylogenetic considerations within Euthyneura. *PLoS One* 8, e67299. doi:10.1371/journal.pone.0067299.

Gemmell, M.R., Trewick, S.A., Hills, S.F.K. & Morgan-Richards, M. (2020) Phylogenetic topology and timing of New Zealand olive shells are consistent with punctuated equilibrium. *Journal of Zoological Systematics and Evolutionary Research* 58, 209–220. doi:10.1111/jzs.12342.

Guindon, S., Dufayard, J.F., Lefort, V., Anisimova, M., Hordijk, W. & Gascuel, O. (2010) New algorithms and methods to estimate maximum-likelihood phylogenies: Assessing the performance of PhyML 3.0. *Systematic Biology* 59, 307–321. doi:10.1093/sysbio/syq010.

Harl, J., Duda, M., Kruckenhauser, L., Sattmann, H. & Haring, E. (2014) In search of glacial refuges of the land snail *Orcula dolium* (Pulmonata, Orculidae) – An integrative approach using DNA sequence and fossil data. *PLoS One* 9, e96012. doi:10.1371/journal.pone.0096012.

Harris, D.J. & Crandall, K.A. (2000) Intragenomic variation within ITS1 and ITS2 of freshwater crayfishes (Decapoda: Cambaridae): Implications for phylogenetic and microsatellite studies. *Molecular Biology and Evolution* 17, 284–291. doi:10.1093/oxfordjournals.molbev.a026308.

Herbert, D.G. & Mitchell, A. (2009) Phylogenetic relationships of the enigmatic land snail genus *Prestonella*: The missing African element in the Gondwanan superfamily Orthalicoidea (Mollusca: Stylommatophora). *Biological Journal of the Linnean Society* 96, 203–221. doi:10.1111/j.1095-8312.2008.01109.x.

Hillis, D.M. & Dixon, M.T. (1991) Ribosomal DNA: Molecular evolution and phylogenetic inference. *Quarterly Review of Biology* 66, 411–446. doi:10.1086/417338.

Hoy, M.S. & Rodriguez, R.J. (2013) Intragenomic sequence variation at the ITS1-ITS2 region and at the 18S and 28S nuclear ribosomal DNA genes of the New Zealand mud snail, *Potamopyrgus antipodarum* (Hydrobiidae: Mollusca). *Journal of Molluscan Studies* 79, 205–217. doi:10.1093/mollus/eyt016.

Huelsenbeck, J.P. & Ronquist, F. (2001) MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* 17, 754–755. doi:10.1093/bioinformatics/17.8.754.

Hunter, S.S., Settles, M.L., New, D.D., Parent, C.E. & Gerritsen, A.T. (2016) Mitochondrial genome sequence of the Galápagos endemic land snail *Naesiotus nux*. *Genome Announcements* 4, e01362-15. doi:10.1128/genomeA.01362-15.

Itskovich, V. (2020) Intragenomic variation of rDNA internal transcribed spacers in the endemic Baikal sponge *Lubomirskia baikalensis* (Pallas, 1776) (Spongillida, Lubomirskiidae): Implications for Porifera barcoding. *Journal of Great Lakes Research* 46, 62–66. doi:10.1016/j.jglr.2019.10.009.

Jantzen, J.R., Whitten, W.M., Neubig, K.M., Majure, L.C., Soltis, D.E. & Soltis, P.S. (2019) Effects of taxon sampling and tree

reconstruction methods on phylodiversity metrics. *Ecology and Evolution* 9, e3.5425. doi:10.1002/ece3.5425.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Meintjes, P. & Drummond, A. (2012) Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* 28, 1647–1649. doi:10.1093/bioinformatics/bts199.

Koot, E.M., Morgan-Richards, M. & Trewick, S.A. (2020) An alpine grasshopper radiation older than the mountains, on Kā Tiritiri o te Moana (Southern Alps) of Aotearoa (New Zealand). *Molecular Phylogenetics and Evolution* 147, e 106783. doi:10.1016/j.ympev.2020.106783.

Kressler, D., Hurt, E. & Baßler, J. (2010) Driving ribosome assembly. *Biochimica et Biophysica Acta – Molecular Cell Research* 1803, 673–683. doi:10.1016/j.bbamcr.2009.10.009.

Lanfear, R., Frandsen, P.B., Wright, A.M., Senfeld, T. & Calcott, B. (2017) PartitionFinder 2: New methods for selecting partitioned models of evolution for molecular and morphological phylogenetic analyses. *Molecular Biology and Evolution* 34, 772–773. doi:doi:10.1093/molbev/msw260.

Laslett, D. & Canbäck, B. (2008) ARWEN: A program to detect tRNA genes in metazoan mitochondrial nucleotide sequences. *Bioinformatics* 24, 172–175. doi:10.1093/bioinformatics/btm573.

Lemmon, A.R. & Lemmon, E.M. (2012) High-throughput identification of informative nuclear loci for shallow-scale phylogenetics and phylogeography. *Systematic Biology* 61, 745–761. doi:10.1093/sysbio/sys051.

Lydeard, C., Cowie, R.H., Ponder, W.F., Bogan, A.E., Bouchet, P., Clark, S.A., Cummings, K.S., Frest, T.J., Gargominy, O., Herbert, D.G., Hershler, R., Perez, K.E., Roth, B., Seddon, M., Strong, E.E. & Thompson, F.G. (2004) The global decline of nonmarine mollusks. *BioScience* 54, 321–330. doi:10.1641/0006.

Madden, T. (2013) The BLAST sequence analysis tool. In: *The NCBI Handbook*. NCBI, Bethesda, Chapter 16, pp. 1–15.

Miller, M.A., Pfeiffer, W. & Schwartz, T. (2010) Creating the CIPRES Science Gateway for inference of large phylogenetic trees. *2010 Gateway Computing Environments Workshop, GCE 2010*. doi:10.1109/GCE.2010.5676129.

Moritz, C. (1994) Defining "evolutionarily significant units" for conservation. *Trends in Ecology and Evolution* 9, 373–375. doi:10.1016/0169-5347(94)90057-4.

Nei, M. & Rooney, A.P. (2005) Concerted and birth-and-death evolution of multigene families. *Annual Review of Genetics* 39, 121–152. doi:10.1146/annurev.genet.39.073003.112240.

Parrish, R., Sherley, G. & Aviss, M. (1995) *Giant land snail recovery plan, Placostylus spp., Paryphanta sp.* Threatened Species Recovery Plan Series No. 13. Department of Conservation, Wellington.

Pereira, T.J. & Baldwin, J.G. (2016) Contrasting evolutionary patterns of 28S and ITS rRNA genes reveal high intragenomic variation in *Cephalenchus* (Nematoda): Implications for species delimitation. *Molecular Phylogenetics and Evolution* 98, 244–260. doi:10.1016/j.ympev.2016.02.016.

Ponder, W.F., Colgan, D.J., Gleeson, D.M. & Sherley, G.H. (2003) Relationships of *Placostylus* from Lord Howe Island: An investigation using the mitochondrial cytochrome c oxidase 1 gene. *Molluscan Research* 23, 159–178. doi:10.1071/MR03001.

Quenu, M., Trewick, S.A., Brescia, F. & Morgan-Richards, M. (2020) Geometric morphometrics and machine learning challenge currently accepted species limits of the land snail *Placostylus* (Pulmonata: Bothriembryontidae) on the Isle of Pines, New Caledonia. *Journal of Molluscan Studies* 86, 35–41. doi:10.1093/mollus/eyz031.

Ramirez, J., Ramírez, R., Romero, P., Chumbe, A. & Ramírez, P. (2009) Posición evolutiva de caracoles terrestres peruanos (Orthalicidae) entre los Stylommatophora (Mollusca: Gastropoda). *Revista Peruana de Biologia* 16, 51–56. doi:10.15381/rpb.v16i1.193.

Shen, X., Tian, M., Meng, X., Liu, H., Cheng, H., Zhu, C. & Zhao, F. (2012) Complete mitochondrial genome of *Membranipora grandicella* (Bryozoa: Cheilostomatida) determined with next-generation sequencing: The first representative of the suborder Malacostegina. *Comparative Biochemistry and Physiology – Part D: Genomics and Proteomics* 7, 248–253. doi:10.1016/j.cbd.2012.03.003.

Shioiri, C. & Takahata, N. (2001) Skew of mononucleotide frequencies, relative abundance of dinucleotides, and DNA strand asymmetry. *Journal of Molecular Evolution* 53, 364–376. doi:10.1007/s002390010226.

Shtolz, N. & Mishmar, D. (2019) The mitochondrial genome–on selective constraints and signatures at the organism, cell, and single mitochondrion levels. *Frontiers in Ecology and Evolution* 7, e342. doi:10.3389/fevo.2019.00342.

Sochorová, J., Garcia, S., Gálvez, F., Symonová, R. & Kovařík, A. (2018) Evolutionary trends in animal ribosomal DNA loci: Introduction to a new online database. *Chromosoma* 127, 141–150. doi:10.1007/s00412-017-0651-8.

Stothard, J.R., Hughes, S. & Rollinson, D. (1996) Variation within the Internal Transcribed Spacer (ITS) of ribosomal DNA genes of intermediate snail hosts within the genus *Bulinus* (Gastropoda: Planorbidae). *Acta Tropica* 61, 19–29. doi:10.1016/0001-706X(95)00137-4.

Stringer, I.A.N., Parrish, G.R., Sherley, G.H. & MacKenzie, D.I. (2014) The biology of *Placostylus ambagiosus* (Pulmonata: Bulimulidae) in New Zealand: Part 2. Population changes, growth, mortality and life expectancy. *Molluscan Research* 34, 155–175. doi:10.1080/13235818.2014.888985.

Sun, S., Li, Q., Kong, L. & Yu, H. (2017) Limited locomotive ability relaxed selective constraints on molluscs mitochondrial genomes. *Scientific Reports* 7, e10628. doi:10.1038/s41598-017-11117-z.

Teasdale, L.C., Köhler, F., Murray, K.D., O'Hara, T. & Moussalli, A. (2016) Identification and qualification of 500 nuclear, single-copy, orthologous genes for the Eupulmonata (Gastropoda) using transcriptome sequencing and exon capture. *Molecular Ecology Resources* 16, 1107–1123. doi:10.1111/1755-0998.12552.

Trewick, S., Brescia, F. & Jordan, C. (2009) Diversity and phylogeny of New Caledonian *Placostylus* land snails; evidence from mitochondrial DNA. *Mémoires du Muséum national d'histoire naturelle* 198, 421–436.

Triggs, S.J. & Sherley, G.H. (1993) Allozyme genetic diversity in *Placostylus* land snails and implications for conservation. *New Zealand Journal of Zoology* 20, 19–33. doi:10.1080/03014223.1993.10423239.

Uribe, J.E., Irisarri, I., Templado, J. & Zardoya, R. (2019) New patellogastropod mitogenomes help counteracting long--branch attraction in the deep phylogeny of gastropod mollusks. *Molecular Phylogenetics and Evolution* 133, 12–23. doi:10.1016/j.ympev.2018.12.019.

Wade, C.M., Mordan, P.B. & Naggs, F. (2006) Evolutionary relationships among the Pulmonate land snails and slugs (Pulmonata, Stylommatophora). *Biological Journal of the Linnean Society* 87, 593–610.

Winnepenninckx, B., Backeljau, T. & De Wachter, R. (1993) Extraction of high molecular weight DNA from molluscs. *Trends in Genetics* 9, 407.