

Article

Do Deep Mitochondrial DNA Divergences within Intertidal Gastropods Reveal Phylogeographic Signals from Earlier Glacial Cycles?

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Abstract: Climatic cycles have frequently been hypothesised to influence the phylogeography of temperate marine organisms through such factors as hydrological changes and landbridge formation at glacial maxima. However, it is rarely considered whether observed phylogeographic patterns are predominantly influenced by the most recent cycle or those that preceded it. Whether high genetic divergences within intertidal taxa provide an opportunity to investigate such questions is studied here. Three southeastern Australian gastropod taxa that exhibit such divergence were studied, namely, *Ascorhis tasmanica*, *Phallomedusa solida* and the regions' two species of the genus *Nerita*. Maximum likelihood phylogenetic analyses revealed bootstrap-supported clades within *Nerita atramentosa*, *N. melanotragus* and *P. solida* each of which may have been influenced by climatically induced isolation in previous glacial cycles. These clades are all now very widely distributed within the ranges of their respective species. The loss of variants resulting in the divergence of the haplotypes in the clades may be stochastic but was more likely due to selection, at least for *P. solida*. *Ascorhis tasmanica* was revealed to have a comparatively large number of sporadically distributed divergent groups; however, their evolution may have been more influenced by factors other than climate cycles.

Keywords: ATP synthase subunit α ; Bassian Isthmus; cytochrome *c* oxidase; histone H3; New South Wales; Tasmania; Victoria



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1. Introduction

Climatic cycles have frequently been hypothesised to influence the phylogeography of temperate marine organisms through such factors as hydrological changes and landbridge formation at glacial maxima. However, it is less often considered whether observed phylogeographic patterns are predominantly due to the most recent cycle or those that preceded it. The best systems for approaching such questions would be expected to be well-studied regions that have taxa showing patterns of deep genetic divergences likely to have been generated at earlier times.

The phylogeography of southeastern Australian coasts has been extensively studied as a model system for investigating the evolutionary effects of climatic cycles. A considerable fraction of the studied taxa exhibits genetic divergence between populations east and west of the transect from Wilsons Promontory to northeastern Tasmania [1,2]. This partitioning is usually discussed in relation to the geographic isolation potentially arising during climate cycles. A number of factors might contribute to such isolation. Because Bass Strait to the south of Victoria is shallow, it can be divided by the formation of landbridges between the mainland and the northeast of Tasmania at glacial maxima, of which there have been at least nine in the last 800,000 years [3]. At glacial maxima, the populations of temperate-adapted species may be genetically isolated by the lowering of sea surface temperatures, especially by northwards migration of the Southern Subtropical Front, the boundary between warmer sub-tropical and cooler Antarctic surface waters [4–6]. This could displace the range of the species so far north that they no longer have contact around the southern tip of Tasmania.

Another complication that may prevent population connectivity is that emergent land extends to the south of Tasmania during glacial maxima. Furthermore, a long sandy stretch of coastline to the east of Wilsons Promontory in Victoria may act as a strong barrier to contemporary dispersal and gene flow [7]. Genetic isolation caused by such factors may induce geographical differences in the partition of variation, for instance through the appearance of new variants in only one of the isolated areas, or the loss of pre-existing types. When the isolating barriers are removed, gene flow may occur between the formerly separated populations as their ranges expand, if they have not become distinct species.

The patterns of variation in marine and estuarine taxa in southeastern Australia has been reviewed [1,2]. Some taxa do not show patterns that are clearly due to climate-cycle-induced isolation. However, the region also has a number of taxa exhibiting patterns of genetic variation which may be associated with climate cycles. These include the gastropods *Scutus antipodes* Montfort, 1810, *Austrolittorina unifasciata* (Gray, 1826) (both in [8]), *Bembicium nanum* (Lamarck, 1822), *Haustrum vinosa* (Lamarck, 1822) and *Cellana tramoserica* (Holten, 1802) (all in [7]) in which closely related genetic variants occur in very different frequencies to the east and west of Wilson's Promontory. The transect between this promontory and northeastern Tasmania is more shallow than other parts of the Bass Strait, meaning that this is the first part of the landbridge across it to emerge as glaciations increases, and the last to be submerged as they recede. Microsatellite analysis of the bivalve *Donax deltooides* Lamarck, 1818 has also revealed clear demarcations of populations to the east and west of the promontory [9].

Southeastern Australia also has taxa that phylogeographic investigations have revealed to have high genetic divergence. These include the region's two species in the gastropod *Austrocochlea constricta* (Lamarck, 1822) complex [10,11], its two species of the gastropod genus *Nerita* Linnaeus, 1758, *N. melanotragus* E. A. Smith, 1884 and *N. atramentosa* Reeve, 1855 [12], the chiton *Plaxiphora albida* (Blainville, 1825) [7], the estuarine panpulmonate *Phallomedusa solida* (E. von Martens, 1878) [13] and the barnacle *Catomerus polymerus* (Darwin, 1854) [7,14]. Another species which may have deep intra-specific divergences is the estuarine caenogastropod micromollusc *Ascorhis tasmanica*, which is represented in GenBank by very distinct sequences from the studies of Hershler et al. [15] and Zielske et al. [16]. However, the species has not yet been studied phylogeographically; thus, it has been included in this study.

Some of the taxa with deep divergences have been proposed to have had their evolution influenced by earlier climatic cycles [2,13,14]. However, this has not been critically examined. Consequently, this investigation was performed to examine whether the phylogenetic relationships, demographic history or spatial distribution of deeply divergent clades or sub-clades within them, could potentially reveal phylogeographic signals of earlier climatic cycles. The investigation focussed on the gastropod taxa with divergent sequences, *Nerita*, *Ascorhis tasmanica* and *P. solida*. The phylogeography of the *Austrocochlea constricta* complex has been previously investigated. The two deeply divergent clades in the complex have been shown to be distinct species with largely non-overlapping ranges.

The observation of deeply divergent clades reflects the evolutionary loss of haplotypes that were intermediate in sequence between those that are now extant in a population. The loss may have occurred through stochastic processes, possibly with an increased rate due to bottlenecks if range shifts or reductions have been occasioned by climatic cycles. This process may have been repeated in different climatic cycles. In general, the random nature of this loss would suggest that the relative lengths of the branches leading to the clades in phylogenies would tend to not be much greater than other branches in the topologies. The loss of haplotypes may also be related to selective sweeps leading to the fixation of a sequence in one of the regions geographically isolated during glacial maxima. This would be hastened by the occurrence of simultaneous sweeps by different haplotypes in each of the isolated regions. A combination of stochastic loss on one side of a barrier and a selective sweep on the other could contribute to the development of deep divergences between clades, but the process would be much slower than if there were simultaneous sweeps.

Contemporary spatial patterning of genetic variation has been observed not only in the examples of closely related variants listed above, but also in the separation of divergent clades in *Nerita*, *Plaxiphora* and *Catomerus* [9,12,13]. Teske et al. [2] proposed that, generally, the persistence of a structure after the dissolution of isolating mechanisms may depend on the interactive effects of multiple factors including hydrology, temperature-related adaptation and diversifying selection and fitness-related competition. However, the applicability of the proposed interactive effects to deeply divergent clades is problematic. If the clades represent distinct species, the maintenance of geographic patterns after barrier dissolution may be explained by inter-species competition [17]. However, it is difficult to explain the maintenance of geographic divergent clade localisation within a single species. If the localisation has persisted through multiple climate cycles, there would have been repeated opportunities for its erosion via gene flow during each cycle. The localisation might have developed during the most recent cycle(s), but unusual circumstances seem to be needed for this to have occurred, for example, by selective sweeps occurring on both sides of an isolating barrier during a single cycle.

The *Nerita* of southeastern Australia were long thought to be only one species but are now proposed to be two, *N. melanotragus* and *N. atramentosa*, on the basis of opercular colour differences and high divergence of cytochrome *c* oxidase subunit I (COI) sequences [12,18]. There are very high divergences between populations on the mainland east (*N. melanotragus*) and west (*N. atramentosa*) of Wilsons Promontory in this gene [12]. Only one COI sequence from Tasmania [19] is presently available in GenBank, although Teske et al. [2] reported some analyses based on unpublished mitochondrial DNA data. Most but not all *Nerita* individuals from the east of the Tasmanian main island have the opercular colour characteristic of *N. melanotragus*, and most of those are from the north of *N. atramentosa* [20]. There is no apparent spatial separation on the islands between Wilsons Promontory and Tasmania based on opercular colour [21]. Hales [22] showed that the eastern species *N. melanotragus* is sometimes common somewhat to the west of Wilsons Promontory, at different tidal levels to *N. atramentosa*. Further investigation of DNA sequences in *Nerita* is desirable to confirm a species status by establishing the independent assortment of mtDNA and nuclear gene variation, to collect more data from Tasmania and to assess whether the distribution of mitochondrial DNA clades within each of the species is phylogeographically informative. More data are also needed for more northerly populations of *N. melanotragus* as few specimens from north of Sydney have been sequenced.

Phallomedusa solida is one of two species in the genus, the other being *P. austrina* Golding, Ponder and Byrne, 2007 which is found in northern Tasmania, western Victoria and South Australia [13]. *Phallomedusa solida* is found in Queensland, New South Wales, eastern and northern Tasmania and eastern Victoria [13]. *Phallomedusa solida* comprises two deeply divergent clades of COI sequences with almost complete range overlap. The limited available nuclear DNA data suggest that the clades belong to a single species but do not conclusively show this [13]. Further investigations are needed to confirm the species status of the clades.

The morphology of *Ascorhis tasmanica* was intensively studied by Ponder and Clark [23]. Ponder and Clark [23] described a related species *A. occidua*, Ponder and Clark, 1988, from the southern and western coasts of Australia, but found no evidence of additional species in populations on the eastern seaboard. There are large differences between the pair of sequences in GenBank from New South Wales [15] and the pair from Victoria [16].

Genetic divergences in these gastropod taxa were examined using DNA sequences from the nucleus and the mitochondria. The assortment of the variants in these two sequence classes were initially used to study species status. Then, the patterns of variation were examined phylogeographically and demographically to search for evidence of the possible effects of earlier glacial cycles. Such signals might potentially be revealed in the distribution of the clades within species, or sub-clades within them, or in differences between them in their demographic history. The distribution of the clades was particularly

considered during the investigation to ascertain whether there were any relictual variation in localised regions that would be at threat from contemporary climate change.

2. Materials and Methods

2.1. Collections and DNA Sequencing

Specimens were collected from estuaries, lagoons or intertidal rocks in localities in New South Wales, Tasmania and Victoria (Table 1, Figure 1) and stored in 95% ethanol or frozen at $-80\text{ }^{\circ}\text{C}$. DNA was extracted using QIAGEN DNeasy™ kits from the whole animal, including the shell, for *A. tasmanica* or from small amounts of tissue at the posterior end of the body (mantle, gills and foot muscle) for specimens of *Nerita*. DNA was eluted at the completion of the extraction into the QiagenAE buffer. DNA from specimens of *P. solida* was available from the study of Golding et al. [13].

Table 1. Population locations for newly collected sequences. The second column provides the location number used in Figure 1 and the third specifies the collection event identifier (“MAL” number) in the collections of the Australian Museum. The final column lists the number of new sequences at each site: figures in plain font are for ATPS α and those in bold for COI. Italic figures are for ND1 in *Nerita* and for H3 in other taxa.

Species	Location Number	Collection Event (MAL#)	Locality	Latitude	Longitude	Collection Date	No. of Sequences
<i>Ascorhis tasmanica</i>							
	A1	MAL71148	North Avoca	33°27'27"	151°26'30"	11 May 2007	3, 3
	A2	MAL88537	Lake Nangudga,	36°17'	150°08'	13 June 2006	1, 2, 1
	A3	MAL72686	Merimbula, Back Lagoon,	36°53'08"	149°55'01"	1 March 2008	1, 2, 1
	A4	MAL72687	Moulding Lagoon	41°59'	148°14'	3 April 2007	2, 3
	A5	MAL72688	Swansea,	42°07'52"	148°04'35"	3 April 2007	2, 3, 1
<i>Nerita atramentosa</i>							
	Na1	MAL72701	Bridport	40°58'54"	147°23'08"	3 April 2007	2, 1
	Na2	MAL71157	Georgetown	41°06'33"	146°48'39"	4 April 2007	2, 2, 1
<i>Nerita melanotragus</i>							
	Nm1	MAL71145	Hastings Point	28°21'46"	153°34'46"	1 April 2008	1
	Nm2	MAL71144	Skennars Head	28°49'20"	153°36'26"	1 April 2008	2, 2, 2
	Nm3	MAL71148	Norh Avoca Rock Platform	33°27'27"	151°26'30"	11 May 2007	2
	Nm4	MAL71138	Bottle and Glass Rocks	33°50'51"	151°16'12"	28 March 2007	2, 2, 2
	Nm5	MAL71152	Shelly Beach, Cronulla	33°04'01"	159°09'31"	12 May 2009	3, 6, 3
	Nm6	MAL71140	Bicheno	41°52'18"	148°17'54"	3 April 2007	3
	Nm7	MAL71151	Deviot	41°15'	146°56'	4 April 2007	3
<i>Phallomedusa solida</i>							
	P1	MAL76554	Clarence	29°25'59"	153°14'17"	31 March 2008	1
	P2	MAL72691	Ballina, North Creek	29°28'44"	153°21'50"	1 April 2008	1
	P3	MAL71151	Angourie	28°10'34"	153°32'27"	2 April 2008	1
	P4	MAL76555	Roseville,	33°46'07"	151°11'57"	28 March 2007	2
	P5	MAL76552	Little Salt Pan Creek	33°58'17"	151°02'24"	3 February 2006	1
	P7	MAL71160	Narooma, at highway bridge	36°12'35"	150°07'20"	13 June 2006	2
	P8	MAL76559	Falmouth	41°30'	148°16'	3 April 2007	1

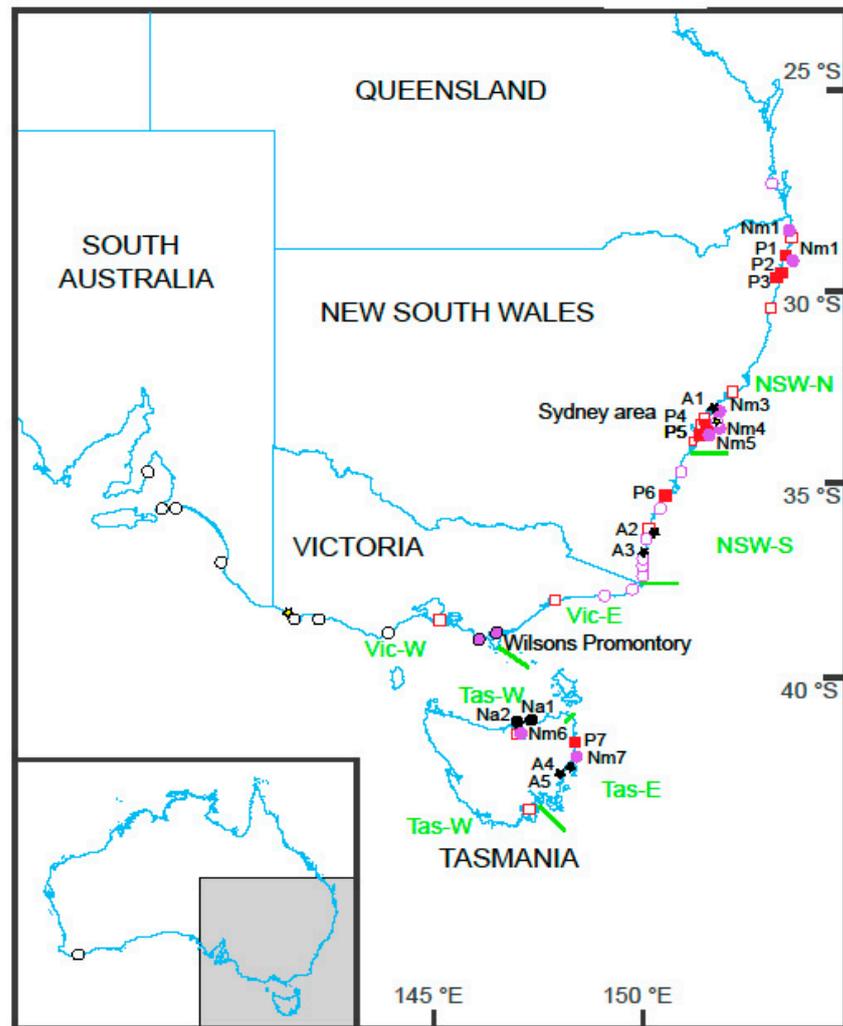


Figure 1. Map of sample localities. Localities are identified by black circles for *Nerita atramentosa*, pink circles for *N. melanotragus*, black stars for *A. tasmanica* and red squares for *P. solida*. Filled symbols indicate localities from which new data were collected for this article. These are referenced by letters as detailed in the legend to Table 1. Empty symbols indicate data from the literature (details in text). The localities for *Nerita* with black outline and pink fill have data for both species in the genus. The superimposed symbols for *A. tasmanica* and *N. melanotragus* in the Sydney area of New South Wales denote two and three nearby localities of the species, respectively. Boundaries between regions are indicated by green bars.

PCR was conducted for the nuclear sequences ATP synthase subunit α , including parts of the CDS and the intron (abbreviated as ATPS α) and histone H3 (H3) and the mitochondrial sequences COI and nicotinamide adenine dinucleotide dehydrogenase subunit 1 (ND1).

PCRs were performed in 25 μ L of BIOTAQ RED Taq Master Mix (Bio-Line, London, UK), 0.125 μ L of each primer (at 100 pM per μ L), 23.75 μ L of purified water (from the RED Taq kit) and 1 μ L of DNA (the original elution was diluted 1 in 8 for a few samples that required further dilution) in a total reaction volume of 50 μ L.

The cycling profile for COI was: 93 $^{\circ}$ C for 3 min, 43 $^{\circ}$ C for 1 min, 72 $^{\circ}$ C for 1 min for one cycle; 93 $^{\circ}$ C for 30 s, 43 $^{\circ}$ C for 1 min, 72 $^{\circ}$ C for 1 min for 32 cycles; and 72 $^{\circ}$ C for 5 min for the final cycle. Aliquots (3 μ L) of PCR products were run on 2% agarose gels and visualised using UV-fluorescence. Single-banded products were treated with ExoSAP-IT (USB Corporation, Cleveland, Ohio) and sequenced in both directions by Macrogen Inc (Geumchun-Gu, Korea) using the original primers individually. Amplifications of the other

sequences were similar to annealing temperatures of 50 °C for ND1 and H3, and 54 to 56 °C for ATPS α .

PCR amplifications of COI were performed with the Folmer et al. [24] primers, those for ATPS α with the primers in Jarman et al. [25], and those for H3 with the H3NF/H3NR set of Colgan et al. [26] and ND1 with primers designed here: NAD1F: ACTTCATARGAAATNGTYTG; and NAD1R: TTTACYTTATTAGAACGNAARGG.

GenBank accession numbers for the COI sequences are OQ194189–OQ194192 for *N. atramentosa*, OQ1941869–OQ194188 for *N. melanotragus*, OQ300061 for *P. solida* and OQ192103–OQ192115 for *A. tasmanica*. Accession numbers for ATPS α are OQ291304 and OQ291305 for *N. atramentosa*, OQ291301–OQ291303 and OQ291306–OQ291308 for *N. melanotragus*, OQ302183–OQ302191 for *P. solida* and OQ291295–OQ291298 for *A. tasmanica*. Numbers for *A. tasmanica* H3 are OQ207667–OQ207671 and those for *Nerita* ND1 are OQ207657–OQ207659 for *N. atramentosa* and OQ207660–OQ207666 for *N. melanotragus*.

2.2. Sequence Editing and Alignment

Electropherograms were examined and sequences assembled using Sequencher, version 4.9 (Gene Codes Corporation, Ann Arbor, Michigan). Alignments were made with CLUSTALX [27] using the default parameters, and inspected in BioEdit [28]. A base in ATPS α was considered heterozygous if the peak height of the second most abundant nucleotide in the chromatogram was at least two-thirds of the height of the most abundant. Phase [29] as implemented in DnaSP ver. 5.1 [30] was used to predict haplotypes for variable positions of ATPS α using default parameters except that the number of iterations was increased to 200.

Datasets were compiled from newly collected sequences and those available in GenBank. Four COI sequences for *A. tasmanica* were added from GenBank [16,17]. Two were published by Hershler et al. [16]. AF129330 is from Careel Bay, near Sydney. AF129329 is recorded in GenBank as being from “13 Mile Creek” but is probably from Manly Lagoon, which is given as the locality of one of the sequences (accession number not specified) by Hershler et al. [16]. No ATPS α sequences were available for this species in GenBank.

For *N. atramentosa* and *N. melanotragus*, most GenBank COI sequences were from Waters et al. [12], with two others of each species from Frey and Vermeij [19], one *N. melanotragus* from Amin et al. [31] and one *N. atramentosa* from Williams and Ozawa [32]. Three ATPS α sequences were available from Frey and Vermeij [19], one from *N. atramentosa* and two from *N. melanotragus*. The ND1 analyses were based only on the sequences collected for this study as no GenBank sequences were available. The *P. solida* COI sequences of Golding et al. [13] were re-analysed here, with the addition of one newly collected sequence. No ATPS α sequences were available for this species in GenBank to add to those collected here; thus, analyses were restricted to the data collected here.

The provenance of specimens in the figures is generally listed by state. However, the three more intensively sampled states were each divided into two regions. NSW-N included localities in Sydney and northwards in New South Wales, and NSW-S included localities in southern New South Wales; the other regions were Vic-E (eastern Victoria), Vic-W (western Victoria), Tas-E (eastern Tasmania) and Tas-W (western Tasmania) (Figure 1).

2.3. Analyses

Phylogenetic analyses were conducted using maximum likelihood (“ML”) performed on the CIPRES data portal [33] using the RAxML Blackbox [34] with default assumptions (not using empirical data frequencies, no invariable sites). The three sets of codon positions were treated as separate partitions in the COI analyses. Partitioning was not applied to the ATPS α data because of the small size of the partial exon sequence included in the alignment. The numbers of required rapid bootstrap replicates was calculated by the majority rules extended (“MRE”) bootstopping criterion [35]. Trees were examined using Figtree v. 1.4.2. [<http://tree.bio.ed.ac.uk/software/figtree/>] (accessed on 18 November 2016).

Pairwise Kimura 2-parameter genetic distances [36] between groups or species were calculated in MEGA 7 [37]. The rate variation among sites was modelled with a gamma distribution (shape parameter = 1). All ambiguous positions were removed for each sequence pair. Standard deviations were estimated using 200 bootstrap replicates.

DnaSP ver. 5.10.01 [30] was used to calculate measures of genetic variability including haplotype and nucleotide diversity and the Tajima D statistic [38]. The probability of obtaining observed values of the D statistic was reported directly by DnaSP. Mismatch analyses [39] were conducted in DnaSP and summarised by the R2 statistic [40], and the Θ and τ parameters of population size were calculated in DnaSP ver. 5.10.01 [30].

POPART [41] was used to calculate haplotype networks shown in the Supplementary Figures. The TCS procedure [42] was used to construct these networks. Sequences with missing data were retained in the analyses, but sites lacking data for more than 5% of sequences were excluded.

Automatic partitioning of COI variation by ASAP [43] was used to investigate species status where this could not be determined by joint consideration of nuclear and mitochondrial sequences.

3. Results

3.1. *Nerita*

Specimens identified as *N. melanotragus* or *N. atramentosa* by COI sequences were distinguished by eight substitutions in the 410 base pair alignment of ATP5 α sequences (Treebase). One of the differences was found in the coding region. The strict co-varying of COI and ATP5 α sequence classes constituted a non-independent assortment of nuclear and mitochondrial genes which supports the recognition of *N. melanotragus* and *N. atramentosa* as two species. The two species were resolved as robustly supported clades in the ML analysis (Figure 2). Specimens identified genetically as *N. melanotragus* or *N. atramentosa* were also distinguished by the characteristic operculum colours of these species. Nucleotide divergences within each of the two *Nerita* species were similar for both COI and ATP5 α (Table 2).

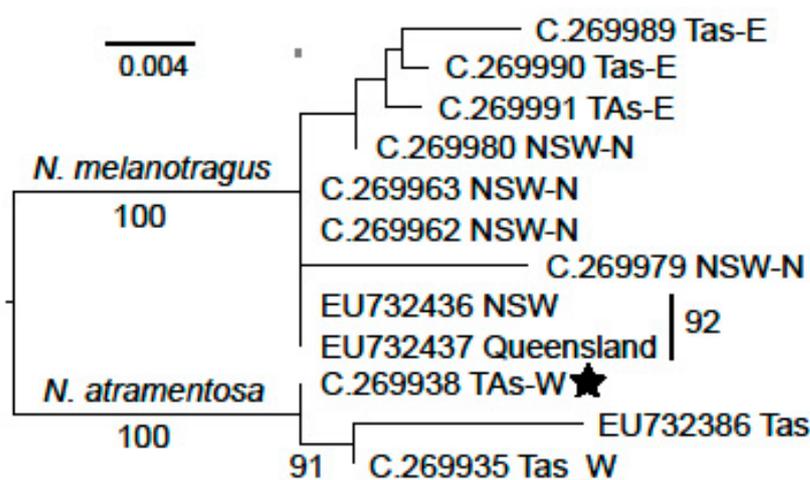


Figure 2. Maximum likelihood topology for *Nerita atramentosa* ATP5 α sequences. Sequences are identified by Australian Museum registration number (e.g., C. 269935) or GenBank accession number. Bootstrap percentages above 70% are written on branches. The *N. atramentosa* specimen with a star belongs to the clade identified by this symbol in Figure 3. The provenance of each sequence is given according to states, or the regions within states specified in Figure 1. The scale bar shows the number of changes per site.

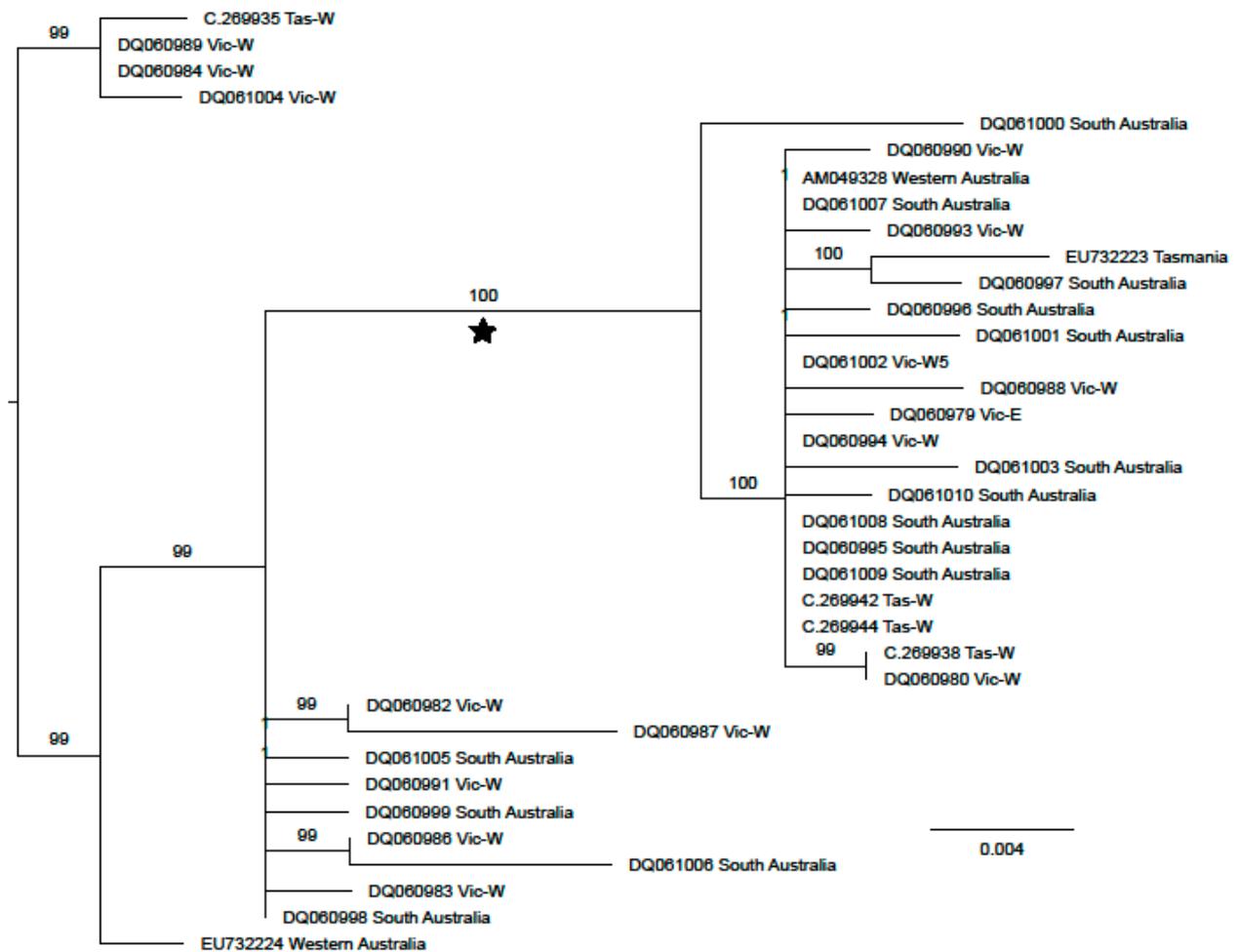


Figure 3. Maximum likelihood topology for *Nerita atramentosa* COI sequences. Sequences are identified by Australian Museum registration number (e.g., C. 269935) or GenBank accession numbers. Bootstrap percentages above 70% are written on branches. The robustly supported clade specified by a star is discussed in the text. The provenance of each sequence is given according to states, or the regions within states specified in Figure 1. The scale bar shows the number of changes per site. The root is placed at the location determined by a combined analysis of the *N. melanotragus* and *N. atramentosa* sequences.

Data were collected from ND1 to explore whether they might be more variable than COI sequences and provide a finer scale resolution of mitochondrial DNA networks in *Nerita*. These preliminary data did not suggest this to be the case. The K2P mean pairwise distance between the species was considerable (0.142 \pm 0.023) but was low within species, being 0.006 \pm 0.003 for both *N. melanotragus* and *N. atramentosa*. Three haplotypes were observed in *N. atramentosa* (among three sequences), and four in *N. melanotragus* (seven sequences).

Some summary statistics for the COI sequence data (haplotype and nucleotide divergence) did not show great differences between *N. atramentosa* and *N. melanotragus* (Table 2). However, significant differences were apparent in the average pairwise K2P distances for the species which were 0.018 \pm 0.082 for the former and 0.008 \pm 0.002 for the latter. The species also appeared to have different demographic histories. The Tajima D value was negative in both species, but was significantly so only in the latter, in which Θ_i and τ parameters were notably lower and higher, respectively, than in *N. atramentosa*.

Table 2. Estimates of evolutionary divergence within groups. The statistics are the number of haplotypes (column 4: No. Hap); haplotype diversity (column 5: Hap. Div.), Nucleotide diversity \pm standard deviations (column 6: π), average pairwise difference between sequences (column 7: Av. Diff.), Tajima's D (column 8, with probability), the Θ_i and τ population size parameters (column 9: Popn Growth) and the Ramos-Onsins and Rozas R2 statistic (column 10). The Θ_i value for all analyses was set at 1000 to allow comparison of Θ_i values. The lengths of the sequences (column 3) differ from the overall lengths of the alignment as DnaSP performs complete deletion of alignment positions with missing data in any sequence. The figures in the number of sequences column for ATPS α refer to the number of haplotypes (two for each individual).

Species/Gene	Number of Sequences	Length	No. Hap.	Hap. Div.	π	Av. Diff.	Tajima's D	Popn Growth	R2
<i>Ascorhis tasmanica</i>									
ATPS α	10	365	2	0.356	0.009 \pm 0.004	3.2	0.026, $p > 0.1$	Θ_i 13.973 τ 0.000	0.178
COI	17	577	11	0.941	0.024 \pm 0.003	13.618	-0.252, $p > 0.1$	Θ_i 6.506 τ 7.112	0.125
<i>Nerita atramentosa</i>									
ATPS α	6	393	3	0.800	0.007 \pm 0.002	2.667	1.219, $p > 0.1$	Θ_i 1.309, τ 1.788	0.267
COI	36	571	22	0.917	0.008 \pm 0.001	4.724	-1.673, $p > 0.05$	Θ_i 2.2267 τ 2.4971	0.051
<i>Nerita melanotragus</i>									
ATPS α	18	393	10	0.85	0.007 \pm 0.002	2.817	-1.783, $p > 0.05$	Θ_i 2.2260 τ 0.557	0.119
COI	69	512	48	0.974	0.008 \pm 0.001	4.179	-2.066, $p < 0.05$	Θ_i 0.525 τ 0.654	0.034
<i>Phallomedusa solida</i>									
ATPS α -overall	18	225	9	0.869	0.007 \pm 0.0001	1.601	-0.723, $p > 0.1$	Θ_i 0.000 τ 1.601	0.119
COI-overall	72	593	52	0.960	0.018 \pm 0.002	10.967	-1.133, $p > 0.1$	Θ_i 9.105 τ 1.862	0.066
COI-clade A	48	613	36	0.957	0.005 \pm 0.001	3.124	-2.591, $p < 0.001$	Θ_i 0.512 τ 2.612	0.024
COI-clade B	20	637	16	0.983	0.007 \pm 0.001	4.579	-1.797, $p > 0.05$	Θ_i 0.780 τ 0.799	0.058

ML analyses conducted separately for each of the *Nerita* species revealed some evidence of structuring within the topologies (Figures 3 and 4). This was more marked for *N. atramentosa* in which many sequences were resolved in a derived, strongly supported group, the members of which were separated by at least five mutational steps from all other *N. atramentosa* sequences (ca. 0.78% of the positions in the alignment). The average pairwise K2P distances between members of supported clade and other *N. atramentosa* sequences were 0.015 \pm 0.004. Within the clade, it was 0.003 \pm 0.001 and within non-members of the clade it was 0.007 \pm 0.002. Both members and non-members of the supported clade were distributed across the studied range of the taxon in Tasmania, Victoria, South Australia and Western Australia (Supplementary Figure S1).

No strong phylogenetic structure was apparent in the analyses of *N. melanotragus* sequences. Only one group had bootstrap support of more than 70%. This was separated from the closest sequences by four mutational steps (Figure 4). There was no apparent geographic partitioning in the *N. melanotragus* phylogeny (Figure 4) or haplotype network (Supplementary Figure S2): all main groups were found in all eastern Australian regions. Few east coast Tasmania haplotypes have been sequenced but these are widely distributed in the network. The samples from northern New South Wales were also dispersed in the phylogeny.

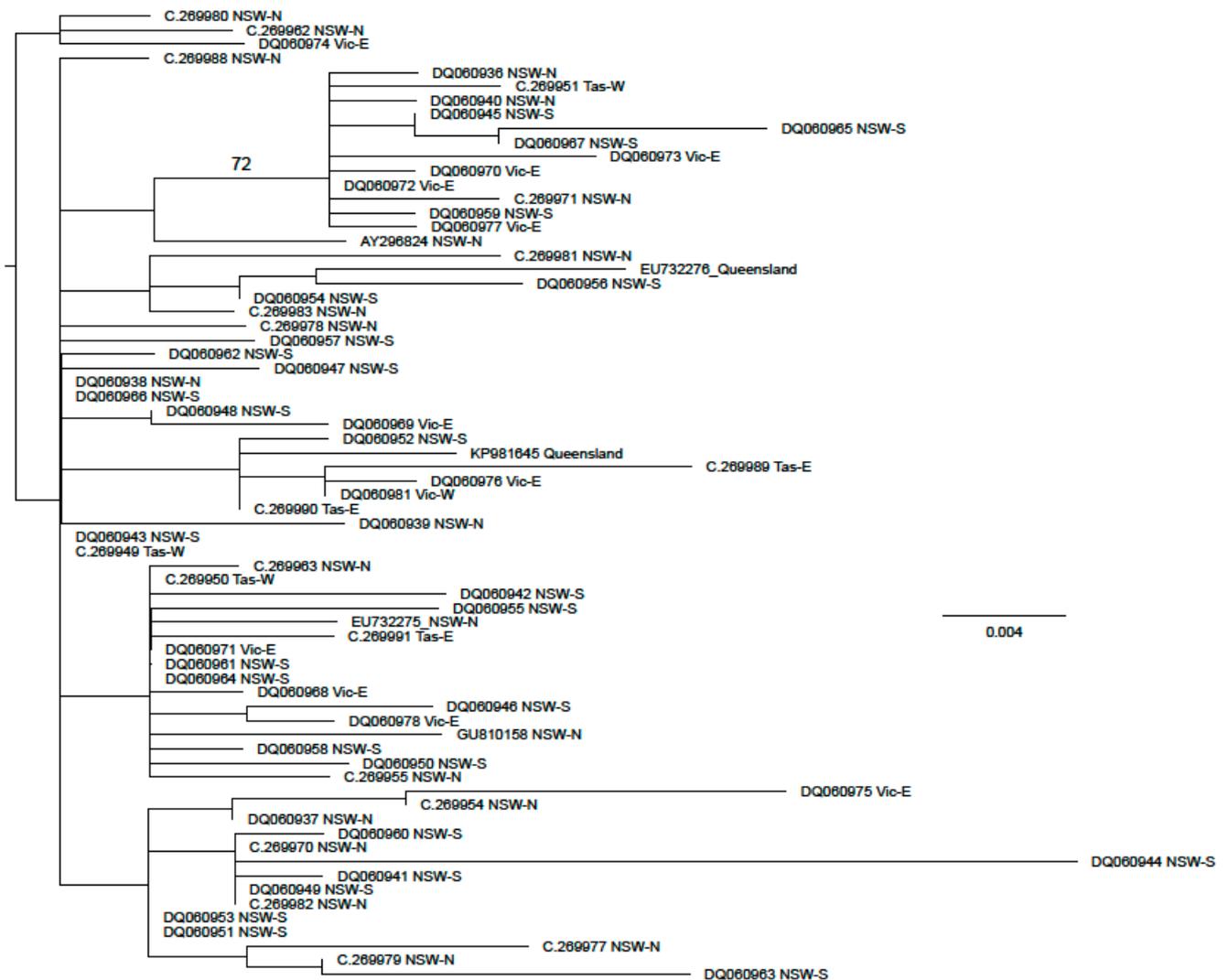


Figure 4. Maximum likelihood topology for *Nerita melanotragus* COI sequences. Sequences are identified by Australian Museum registration number (e.g., C. 269929) or GenBank accession number. Bootstrap percentages above 70% are written on branches. The provenance of each sequence is given according to states, or the regions within states specified in Figure 1. The scale bar shows the number of changes per site. The root is placed at the location determined by a combined analysis of the *N. melanotragus* and *N. atramentos* sequences.

3.2. *Ascorhis tasmanica*

Four groups (labelled A–D, Figure 5) were defined following maximum likelihood analysis of the *A. tasmanica* COI sequences. Three were distinct, robustly supported clades. There were also four sequences that were closely related to each other, but which did not form a clade. All four groups were genetically distant from each other (Table 3, Supplementary Figure S3). The new specimens from the Sydney area (locality A1) differed from those previously collected from this area and deposited in GenBank (clade D). Rather, the new sequences were more similar to sequences from southern New South Wales and Tasmania (groups A and B). Group C was seen in both eastern Tasmania and Victoria.

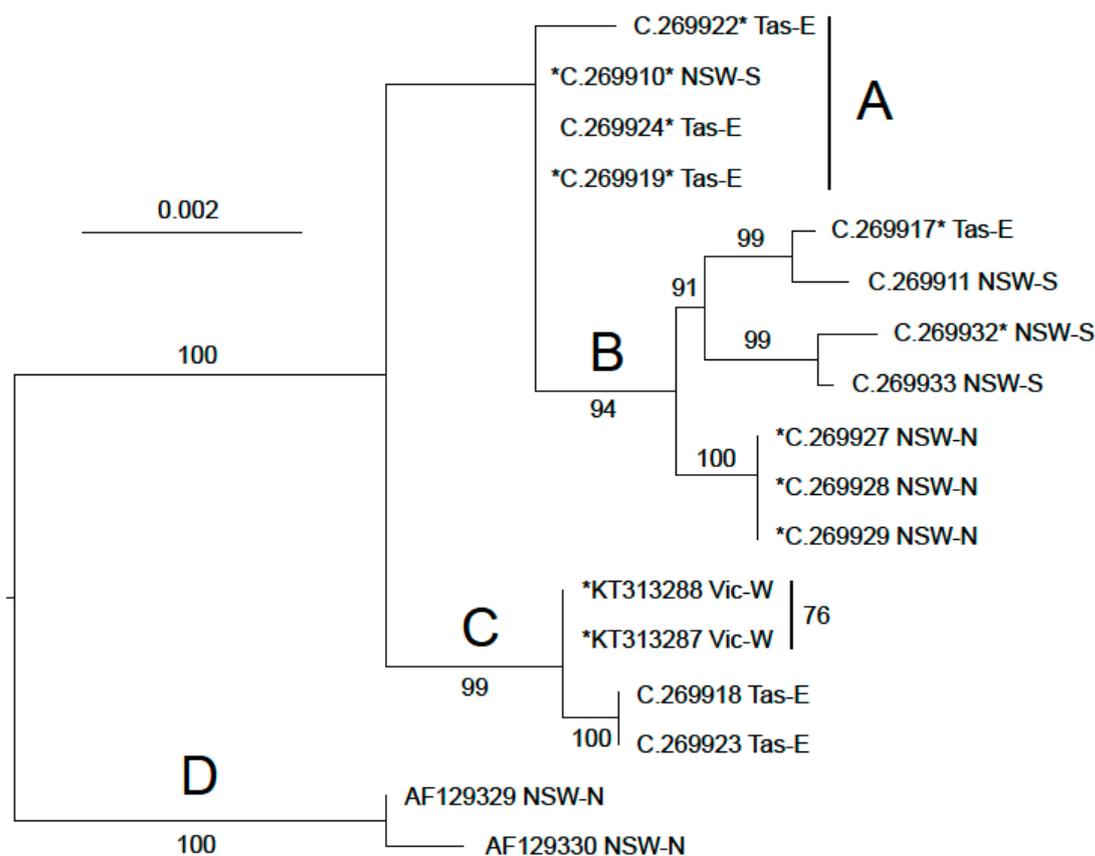


Figure 5. Maximum likelihood topology for *Ascorhis tasmanica* COI sequences. Sequences are identified by Australian Museum registration number (e.g., C. 269929) or GenBank accession number. Bootstrap percentages above 70% are written on branches. The large letters near branches define the groups in this species referred to in the text. Specimens with identifiers preceded by an asterisk have also been sequenced for H3 and those with a following asterisk for ATPS α . The provenance of each sequence is given according to states, or the regions within states specified in Figure 1. The scale bar shows the number of changes per site.

Table 3. Average pairwise base substitutions per site between groups in *Ascorhis tasmanica* based on the Kimura 2-parameter model. Average pairwise distances are shown below the diagonal and standard error estimates are shown above it. Rate variation among sites was modelled with a gamma distribution (shape parameter = 1). All ambiguous positions were removed for each sequence pair. Groups are designated as in Figure 5.

Group	Group A	Group B	Group C	Group D
Group A		0.004	0.008	0.005
Group B	0.017		0.008	0.006
Group C	0.041	0.047		0.008
Group D	0.019	0.030	0.041	

There was little variation in four of the five sequences scored for ATPS α . These four were from clades A and B and had only one variable base which had different nucleotides in the pairs of samples from localities A4 and A5. The fifth sequence AMS C.269910 from A2 had an insert of five bases and up to nine other base differences from the other sequences. This may represent divergent northern ATPS α sequences which would imply that the nuclear and mitochondrial markers assorted independently, as the COI sequence from this specimen was identical to some sequences from localities A4 and A5 in Tasmania. H3 sequences showed very little variation suggesting that the groups in which it was

sequenced (A, B and C) were conspecific. The only notable variant was that there was a polymorphism at one base position in the sequences from locality A1. The status of clade D was assessed by ASAP analysis [43]. The separation of the sequences into two partitions, clade D and the other sequences, was the third most likely outcome of the analysis. The two more likely outcomes, were contradicted by assortment analysis, also suggested that clade D may be a distinct species.

3.3. *Phallomedusa solida*

The ATPS α sequences in this species displayed low variation. Only three base positions had two or more variants each found in multiple individuals. Heterozygotes were observed at all three of these positions. All other variants were found in only one individual and as heterozygotes with one exception which was homozygous. Both COI clades were represented in the ATPS α sequences (seven clade A and two clade B). One clade B individual did have a unique sequence at the polymorphic sites, but all three of its variants at these were seen in at least one clade A individual. The ML analysis of the ATPS α sequences (Figure 6) illustrates the independent assortment of the nuclear and mitochondrial genes, indicating that the specimens were conspecific.

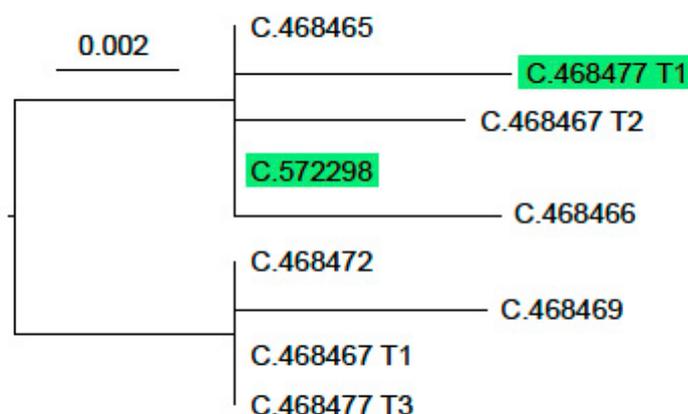


Figure 6. Maximum likelihood topology for *Phallomedusa solida* ATPS α sequences. Sequences are identified by Australian Museum registration number (e.g., C. 468465) No branch was supported by bootstrap percentages of more than 70%. The scale bar shows the number of changes per site. Specimens highlighted with green belong to COI clade B and other specimens belong to clade A.

The re-analysis of the overall COI data showed two divergent groups of sequences in very close agreement with Figure 3 of Golding et al. [13] so it is not reproduced here. Statistical comparisons for the clades generally suggested that their demographic histories were similar. It was, however, notable that the average pairwise difference between sequences was lower in clade A and its Tajima's D value was negative and highly significant, suggesting that it may have grown in size or that it had experienced positive selection on (some of) its variants. This was also suggested by the Tajima D value for clade B, but not significantly.

4. Discussion

The first aim of this study was to determine the implications of phylogenetic study of the studied nuclear and mitochondrial sequences for species status. The analyses showed that assortment of nuclear and mitochondrial genes in *Nerita* was not independent. This supports the distinction of two species of the genus in southern Australia as proposed by Waters et al. [12]. Assortment of the sequence types was independent within each of *N. atramentosa* and *N. melanotragus* for the clades in which this could be tested, particularly the large robustly supported clade in the former species. The observation of independent assortment supported the recognition of only one species in *P. solida* and in *A. tasmanica*. The possible exception to this was the divergent sequences of *A. tasmanica* found by Hershler

et al. [15] from around Sydney for which neither ATPS α nor H3 sequences are available, and which ASAP analyses [43] suggested might be a distinct species.

There is considerable geographical separation of the two *Nerita* species in southeastern Australia, but it is not complete. Both species are found east and west of the transect from Wilsons Promontors to northeastern Tasmania ([2,20–22], this study). The species' ranges may have been wholly disjunct at glacial maxima, although this has not been definitely shown and there is a possibility that populations (maybe small) of one or both species persisted both east and west of Wilsons Promontory in a particular cycle. This would potentially allow the generation of phylogeographic structure within species.

There was little evidence of spatial separation of clades within the studied *Nerita*. Teske et al. [2] also found no regional phylogeographic structure in *N. atramentosa* (including unpublished COI data from Tasmania). *Nerita atramentosa* contains a large, robustly supported divergent clade but this did not show geographic localisation as it was found in all studies regions of the species distribution, including the Western Australian specimen studied by Williams and Ozawa [32]. It might however be speculated that the phylogenetic divergence of this clade from other *N. atramentosa* sequences was generated by haplotype loss during geographical isolation in an earlier climatic cycle. Teske et al. [44] highlighted the importance of local counter currents and over multiple generations, these may have eroded such geographic isolation. The probability of erosion would be increased if multiple climate cycles have occurred since the origin of the isolation.

Similar arguments could be advanced about the origins of the only multi-member bootstrap-supported clade in *N. melanotragus*, which was also not geographically localised. However, its support was comparatively low (72%), reflecting lesser divergence from other sequences in the species.

The timing of divergence for the haplotype groups in *N. atramentosa* might also be speculated. Assuming divergence rate of 2% per million years which is in the mid-range of the values suggested for COI in gastropods [45,46], the minimum 0.78% separation between members of the divergent clade and other members of species would suggest a most recent common ancestor about 390 kA, less if the rate were higher and more if it were lower, as has been suggested for Neritidae [19]. Nurnberg et al. [47] gave the dates of the glacial maxima occurring in the last 400,000 years as 10–26, 60–75, 130–190, 242–302 and 333–362. The fourth of these, which was accompanied by the largest fall in sea surface temperatures, and the fifth may be the events more probably associated the divergence in *N. atramentosa* than more recent cycles.

The broad but sporadic overlap of the divergent haplotype groups in *A. tasmanica* and the relatively large numbers of these makes it difficult to find a simple interpretation of phylogeographic patterns in this species based solely on climate cycles. Even if climate cycles have a role, other factors may have more influence on the evolution of this species. In this context, it is noteworthy that although the species is generally restricted to the coasts, a short-lived population was found at Dalhousie Springs in central Australia, presumably originating from propagule transport by birds [23]. Such transport would tend to make the distribution of variation more sporadic. The patterns of distribution in *A. tasmanica* are reminiscent of those in *Xenostrobus securis* (Lamarck, 1819), an estuarine mussel in which COI variation is highly sporadically distributed [48]. That species also shows very high divergence between nearby water bodies which may be reflected in the distribution of clades A and D in *A. tasmanica*. It is possible that micro- or meso-scale geographic factors, accompanied by occasional long-distance dispersal, are important in at least some southeastern Australian estuarine molluscs, including *A. tasmanica*.

It has previously been shown that the two divergent clades among the COI sequences of *Phallomedusa solida* have ranges that overlap almost completely [13]. It might be hypothesised that the clades resulted from geographic isolation during early climate cycles. However, ascribing these origins to a particular climatic cycle would generally be very difficult. There are low numbers of unobserved (lost) haplotypes in the COI parsimony network except in the path between the divergent clades (Supplementary Figure S4) with

correspondingly short branch lengths within the clades in phylogenetic analyses. This suggests that the divergence between the two main clades in this species is not predominantly due to stochastic processes. The divergence between the clades is 3.5%, which at a rate estimate of 2% per million years [45,46] would suggest an origin dated at more than one and a half million years ago. This period encompasses numerous climatic cycles [3], so that it would be difficult to identify those that had most influence in the evolution of this species.

In summary, the results of the present investigations suggest that there are clades within at least two species, *N. atramentosa* and *P. solida*, and possibly a third, *N. melanotragus*, that may have been influenced in their evolution by climate cycles before the most recent. It is unlikely that it will become possible to specify with certainty the cycles that have had most influence. However, improvements in the estimation of the rate of molecular evolution and developments in the evolutionary significance of the distribution of topological branch lengths [49] may show which cycles are most likely to have had most impact. The broad distribution of the divergent clades within the *Nerita* species and *P. solida* suggest that they do not have a high risk of extinction by contemporary climate change. This is more likely to occur among the divergent clades of *A. tasmanica*. Further study of this species is, by analogy with *X. securis* [48], likely to reveal divergent clades of very limited distribution which may be susceptible to either local or global disturbance.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/d15030346/s1>, Figure S1: *Nerita atramentosa* COI haplotype network, Figure S2: *Nerita melanotragus* COI haplotype network, Figure S3: *Ascorhis tasmanica* COI haplotype network, Figure S4: *Phallomedusa solida* COI haplotype network.

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Data Availability Statement: All sequences, including those newly collected here are available in GenBank. The sequence alignment matrices and maximum likelihood trees are available as grouped analyses in the Treebase project <http://purl.org/phylo/treebase/phyloids/study/TB2:S30128> (accessed on 23 February 2023).

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