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ORIGINAL PAPER

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More evidence of speciation and dispersal across the Antarctic Polar Front through molecular systematics of Southern Ocean *Limatula* (Bivalvia: Limidae)

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Abstract The previously unclear taxonomic status of the high-Antarctic bivalve *Limatula ovalis* Thiele, 1912 and the sub-Antarctic *L. pygmaea* (Philippi, 1845) was investigated using molecular techniques (18S rDNA, 16S rDNA, ITS-1). *L. ovalis* and *L. pygmaea* were recovered as sister taxa, and *L. hodgsoni* (Smith, 1907) as their sister, supporting the subgenus *Antarctolima* Habe, 1977. Various different molecular clock calculations placed the timing of the *L. ovalis*/*pygmaea* divergence (1.36–8.03 MYA with 16S rDNA, 6.81–19.12 MYA with 18S rDNA, 0.24–2.87 MYA with ITS-1) well after the formation of the Antarctic Polar Front (23.5 MYA, APF), indicating a more recent speciation process. The vicariance hypothesis that the APF is a barrier for geneflow favouring speciation processes in the Southern Ocean has to be questioned.

Introduction

The bivalve family Limidae is represented in the Antarctic by seven species of the genus *Limatula* Searles-Wood, 1839. Their vertical distribution ranges from the sublittoral to the deep sea, but highest densities are found between 27 and 125 m for *Limatula hodgsoni* (Smith, 1907) with 25 inds./m^2 (Mühlenhardt-Siegel 1989). The preferred habitat is bedrock or any softbottom with hardbottom-like components such as gravel or sponges (Nicol 1966; Hain 1990). *Limatula* build cobweb-like nests (Hain 1990; own dive observations).

The focus of this study is the phylogenetic relationship between the taxa *Limatula ovalis* Thiele, 1912 and *Limatula pygmaea* (Philippi, 1845) and its implications for Antarctic biogeography. These particular taxa were chosen because of their differing distributions but presumed close systematic relationship. *Limatula ovalis* is present only in the high Antarctic, where water temperatures are below 0°C for most or all of the year. *Limatula pygmaea* is present in the sub-Antarctic and Magellanic Region, which has a more temperate, marine climate (>0°C year round). If these taxa are shown to be closely related, this disjunct distribution will have important implications for the evolutionary history and formation of the Antarctic and sub-Antarctic biota.

The similarity in shell morphology and difference in ranges between these two species has been noted previously (Fleming 1978; Dell 1990; Hain 1990). Fleming (1978) produced a large review of the genus *Limatula*, but without considering phylogeny closely. He noted the highly conservative nature of *Limatula* shell morphology, thus making shell characters difficult to use for phylogeny reconstruction. Molecular data may provide more useful characters in a phylogenetic sense. Such techniques may be able to probe beneath morphological convergence since molecular characters can be less affected by environmental selective pressures (Steiner and Müller 1996). Molecular techniques can be especially helpful in describing relationships in marine taxa, since geographical barriers are often less obvious in an apparently homogenous marine environment like the Southern Ocean (Geller 1998; Bargelloni et al. 2000b).

Molecular techniques have been employed previously in studying Antarctic marine populations (Bargelloni et al. 2000b; Gaffney 2000), though rarely on bivalves (but see Canapa et al. 1999, 2000). These studies have confirmed both the important role of historic geological processes and physical barriers in the formation of the biota of the high Antarctic and sub-Antarctic, particularly the

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Antarctic Polar Front (APF) (Medlin et al. 1994; Crame 1999; Bargelloni et al. 2000b). The APF formed after the opening of a deep-water passage (Drake Passage) between South America and Antarctica approximately 23.5 million years ago (MYA) and led to the formation of the circum-Antarctic current and Antarctica's increased isolation (Lawver et al. 1992; Crame 1999). These studies have also highlighted the complexities of phylogeographic relationships caused by dispersals and differing life histories (Stankovic et al. 2002).

For this study, 3 different DNA regions were chosen (18S nuclear ribosomal rDNA, 16S mitochondrial rDNA, ITS-1 nuclear DNA) to provide resolution at different systematic levels. This range of genes can give a picture of not only how these two congeneric taxa are related, but also higher level (superfamily) relationships dating from the Ordovician (500 MYA) and lower-level population differences within the same species.

It is when the systematic relationships provided by molecular systematics are overlaid with the biogeographic distributions of the taxa considered that phylogeographic conclusions can be reached. ''Biogeography is completely reliant on an accurate understanding of phylogenetic relationships'' (Bargelloni et al. 2000b, citing Futuyma 1998).

Materials and methods

The specimens (Table 1) were collected by scuba diving (Falkland Islands and Antarctic Peninsula), Agassiz-trawl (Antarctic Peninsula) or TV grab (all other specimens), at depths of between 8 and 360 m. They were preserved in 70–80% pre-cooled ethanol. The shells are archived at the British Antarctic Survey. Sequences of 18S and 16S ribosomal DNA (rDNA) from other taxa were downloaded from EMBL/GenBank (Table 2) to provide systematic context and outgroups.

Genomic DNA was extracted using a modified version of CTAB-chloroform extraction from Stothard et al. (1996), diverging from the protocol in the digestion phase, when approximately 100 mm^3 of washed, crushed, mantle tissue was digested in 0.6 ml lysis buffer and 60 µl proteinase K (1 mg/ml) and incubated at 55°C for 12 h.

The 18S rDNA sequence [~1,940 base pairs (bp)] required two steps, firstly tagged primers 18S F (5′-CCG AAT TCG TCG ACA ACC TGG TTG ATC CTG-3′) and 18S R (5′-CCC GGG ATC CAA GCT TGA TCC TTC TGC AGG TTC ACC TAC-3′) (Steiner and Müller 1996) were used in a hot start PCR reaction on a Biometra Uno PCR machine in a 25-µl reaction of 25 ng of template DNA, dH_2O, one Amersham Pharmacia Biotech Ready-To-Go PCR bead and 1 µl of primers 18S F and R mixed (25 pmol of each), with an amplification profile of 5 cycles of 40 s at 94°C, 40 s at 50°C and 2 min at 72°C, followed by 35 cycles of 40 s at 94°C, 40 s at 61°C and 2 min at 72°C, with a final extension of 8 min at 72°C. Then nested primer sets were used to amplify two sections of the fragment, the first using primer 18S F*-T3 (5′-att aac cct cac taa agC AAC CTG GTT GAT CCT G-3′) and R2-ST12 (5′-cga tga aga acg cag cgA GAA CTR CGA CGG TAT C-3′). The second fragment was amplified with primer 18S F2-ST12 (5′-cga tga aga acg cag cgT CAG AGG YTC GAA GAC G-3′) and 18S R*-T3 (5′-att aac cct cac taa agC CTT CTG CAG GTT CAC CTA C-3′) (Steiner and Hammer 2000). Upper-case letters represent the template binding sequence for the nested PCR (F*, R2, F2, R*). The lower-case tags (T3, ST12) are for cycle sequencing. The reaction mix was exactly as for the previous PCR, with a profile of 35 cycles of 40 s at 94°C, 40 s at 52°C and 1 min at 72°C, with a final extension of 10 min at 72°C.

The 16S rDNA sequence (~525 bp) was amplified using primers 16Sar (5′-CGC CTG TTT ATC AAA AAC AT-3′) and 16Sbr (5′-CCG GTC TGA ACT CAG ATC ACG T-3′) (Palumbi et al. 1991) as above, with 40 cycles of 30 s at 94°C, 30 s at 52°C and 1 min at 72°C, with a final extension of 7 min at 72°C.

The ITS (Internal Transcribed Spacer) region was amplified using primers ETTS 1 (5′-TGC TTA AGT TCA GCG GGT-3′) and ETTS 2 (5′-TAA CAA GGT TTC CGT AGG TGA A-3′) (Kane and Rollinson 1994) as above, with 30 cycles of 30 s at 95°C, 30 s at 60°C and 90 s at 72°C, with a final extension of 3.5 min at 72°C.

PCR products were purified with either Qiagen QIAquick PCR Purification or Gel Extraction Kits. The cleaned PCR products were eluted in 30 µl dH_2O and stored at –20°C. An ABI Prism Big Dye Terminator Cycle Sequencing Kit was used on a Perkin Elmer GeneAmp 2400 PCR machine. Sequences were produced on a Perkin Elmer ABI Prism 377 or 373 DNA Sequencer at the DNA sequencing facility at the Natural History Museum. Sequences were assembled from chromatogram files in Sequencer 4.1.1.b1 (GeneCodes 2000). The ITS sequences were cut down to ~410 bp to include as many specimens as possible, since this section was of the best quality amongst the largest number of specimens.

The three datasets (18S, 16S, ITS-1) were aligned separately in ClustalX (Thompson et al. 1997), with a variety of gap-opening and gap-extension penalties. The different alignments were optimised based on the fit of dataset to derived cladograms (Giribet and Wheeler 1999) as judged by Bremer Total Support Index (ti)

Table 1. Antarctic limid specimens from this study and PCR success/EMBL accession numbers

BAS ref.	Taxon Family Limidae	Collection area	Lat. (S)	Long.	18S EMBL access. no.	16S EMBL access. no.	ITS-1 EMBL access. no.
L.8a	*Limatula ovalis*	Antarctic Peninsula	64°36′	62°54′W	=AJ422060	N	N
L.7a	*L. ovalis*	Antarctic Peninsula	67°35′	68°20′W	=AJ422060	AJ422068	AJ422069
L.22a	*L. ovalis*	Antarctic Peninsula	67°34′	68°07′W	AJ422060	=AJ422068	AJ422070
L.6a	*L. ovalis*	Weddell Sea	71°07′	11°28′W	N	N	AJ422071
L.10a	*L. pygmaea*	Marion Island	46°54′	37°45′E	AJ422061	=AJ422067	AJ422074
L.12a	*L. pygmaea*	Marion Island	46°54′	37°45′E	=AJ422061	N	AJ422075
L.12c	*L. pygmaea*	Marion Island	46°54′	37°45′E	=AJ422061	AJ422067	N
L.14a	*L. pygmaea*	Marion Island	46°54′	37°45′E	N	N	AJ422076
L.18a	*L. pygmaea*	Marion Island	46°54′	37°45′E	=AJ422061	=AJ422067	N
L.9a	*L.* cf. *pygmaea*	Falkland Islands	51°42′	57°51′W	=AJ422061	AJ422066	AJ422073
L.21a	*L. hodgsoni*	Weddell Sea	70°51′	10°34′W	AJ422062	AJ422065	AJ422072
L.15a	*L. simillima*	Marion Island	46°54′	37°45′E	AJ422063	AJ422064	N/A
L.19,20	*Escalima goughensis*	Marion Island	46°54′	37°45′E	N	N	N/A

Table 2. 18S and 16S rDNA sequences downloaded from EMBL

Subclass	Superfamily	Family	Genus and species	18S EMBL accession no.	16S EMBL accession no.
Anomalodesmata	Pandoroidea	Lyonsiidae	*Lyonsia hyalina*	AF120540	N/A
		Pandoridae	*Pandora arenosa*	AF120539	N/A
	Poromyoidea	Cuspidariidae	*Myonera* sp.	AF120544	N/A
		Poromyidae	*Tropidomya abbreviata*	AJ389657	N/A
Heterodonta	Cardioidea	Tridacnidae	*Hippopus porcellanus*	D84661	AF122974
			Tridacna derasa	D84658	AF122976
	Veneroidea	Veneridae	*Mercenaria mercenaria*	AF106073	N/A
			Venus verrucosa	AJ007614	N/A
Pteriomorphia	Arcoidea	Arcidae	*Arca noae*	X90960	N/A
			Arca plicata	AJ389630	N/A
			Barbatia virescens	X91974	N/A
		Glycymerididae	*Glycymeris pedunculata*	AJ389631	N/A
	Limoidea	Limidae	*Ctenoides annulata*	AJ389653	N/A
			Lima lima	AJ389652	N/A
			Limaria hians	AF120534	N/A
	Limopsoidea	Limopsidae	*Limopsis enderbyensis*[a]	AJ422057	N/A
			Limopsis marionensis[b]	AJ422058	AJ422059
	Mytiloidea	Mytilidae	*Mytilus californianus*	L33449	AF317057
			Mytilus edulis	L33448	AF317055
			Mytilus galloprovincialis	L33452	AF317543
			Mytilus trossulus	L33454	AF023594
	Ostreoidea	Gryphaeidae	*Hyotissa hyotis*	AJ389632	N/A
			Hyotissa numisma	AJ389633	N/A
		Ostreidae	*Lopha cristagalli*	AJ389635	AF052066
			Ostrea edulis	U88709	AF052068
	Pectinoidea	Pectinidae	*Adamussium colbecki*[c]	AJ242534	AJ243882
			Chlamys islandica	L11232	AJ243573
			Pecten jacobaeus	N/A	AJ245394
	Pinnoidea	Pinnidae	*Atrina pectinata*	X90961	N/A
			Pinna muricata	AJ389636	N/A
	Plicatuloidea	Plicatulidae	*Plicatula australis*	AF229626	N/A
			Plicatula plicata	AJ389651	N/A
	Pterioidea	Pteriidae	*Electroma alacorvi*	AJ389641	N/A
			Pteria macroptera	AJ389637	N/A

[a]Antarctic specimen from Weddell Sea (70°51′S, 10°34′W) (Page 2001)
[b]Southern Ocean specimen from Marion Island (46°54′S, 37°45′E) (Page 2001)
[c]Antarctic specimen from Terra Nova Bay, Ross Sea (74°04′S, 164°07′E) (Canapa et al. 1999, 2000)

and ensemble character indices (Kitching et al. 1998). For the 18S and 16S datasets, a gap opening to extension ratio (pairwise and multiple alignment) of 15-1 was preferred, whereas 15-15 was preferred for the ITS-1. These alignments are available from the authors.

Maximum-parsimony and maximum-likelihood (HKY85 model) analyses were carried out separately on the optimised ClustalX alignments using PAUP* 4.0b5 (Swofford 2001) (parsimony search: heuristic with 1,000 random replicates for 18S, Branch and Bound for all others; TBR tree bisection and reconnection) from all three datasets. Gaps were treated as both a "new state" and as "missing data". All indices exclude uninformative characters. A successive weighting analysis based on rescaled consistency index (rc) was performed (Adamkewicz et al. 1997). The resulting topologies were then assessed and compared with bootstrap analysis (10,000 replications) (Felsenstein 1985) and Bremer support indices (Bremer 1994) calculated with TreeRot version 2 (Sorenson 1999). Pairwise base divergences were calculated in PAUP.

Molecular clock estimates for 16S rDNA limid/pectinid bivalve sequences were calculated by taking the first appearance in the fossil record of the Limidae and Pectinidae (Harland et al. 1989; Benton 1993), with upper and lower limits set as the younger and older of the pairwise first appearances. This was calibrated with the pairwise percent divergence (uncorrected *p*-distances) (all substitutions and tranversions only) of 16S sequences from this study (*L. simillima* and *Adamussium colbecki*). 18S rDNA limid molecular clock estimates were calculated as above by comparing the first appearance of three limid genera (*Ctenoides*, *Lima*, *Limatula*) (Cox et al. 1969) and the relevant 18S sequences (*L. simillima*, *Lima lima*, *Ctenoides annulata*). ITS-1 *L. ovalis*/*pygmaea* molecular clock estimates were calculated by calibrating the ML analysis-derived sequence divergences (see below) between *Limatula pygmaea* populations on Marion Island and the Falklands against the known geological age of volcanic Marion Island (Branch et al. 1991).

Results

Phylogenetic analyses

The 18S dataset was the largest (2,286 aligned base pairs) and had the greatest taxonomic diversity and depth. With gaps treated as "missing", 3 most parsimonious cladograms (MPCs) of 2,381 steps were recovered (ti = 0.227, RC = 0.420, CI = 0.519, RI = 0.714) and with gaps as a "new state", 4 cladograms of 3,382 steps (Fig. 1) were found (ti = 0.235, RC = 0.441, CI = 0.551, RI = 0.721). Further tree-building techniques were employed with gaps treated as a new state, due to the superior fit of these cladograms (Giribet and Wheeler 1999). A successive weighting analysis based on

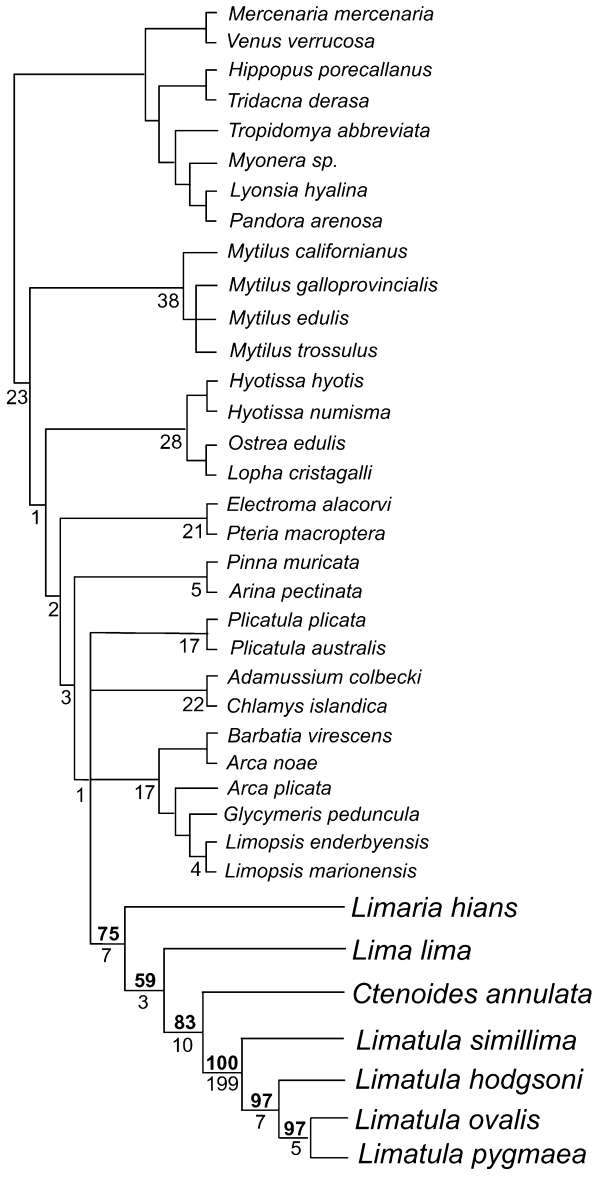

Fig. 1. Strict consensus of 18S "gaps as new state" parsimony search, with detailed view of limid topology (bootstrap values above, Bremer values below) (4 MPCs, 3,382 steps, ti = 0.235, RC = 0.441, CI = 0.551, RI = 0.721)

rescaled consistency index (rc) resulted in two MPCs after two reweightings, with exactly the same topology as two of the MPCs from the unweighted "gaps as new state" search above. A maximum-likelihood (ML) analysis was performed (HKY85 model, ti/tv = 2, assumed nucleotide frequencies = empirical, assumed proportion of invariable sites = none, distribution of rates at variable sites = equal) with a –log likelihood value of the single topology of 15,699.288. Loading this topology into an unweighted parsimony search cost 3,385 steps (ti = 0.234, RC = 0.440, CI = 0.550, RI = 0.721), and is only slightly suboptimal in parsimony terms. All analyses found the same underlying limid topology (Fig. 1). The Pectinoidea was recovered as a sister taxon in the weighted parsimony and ML topologies, two of the three gaps as "missing" MPCs and half of the "new state" MPCs.

For the 16S dataset (622 aligned bp) parsimony search, gaps were treated as a "new state" as above. Two MPCs of 1,392 steps (Fig. 2) were found (ti = 0.207, RC = 0.574, CI = 0.695, RI = 0.803). A successive weighting search recovered one of the two MPCs from the unweighted analysis. The ML analysis (parameters as above) recovered 1 cladogram (–log likelihood of 4,906.853) with a slightly different topology amongst *Limatula* (this topology in an unweighted parsimony search: 1,420 steps, ti = 0.187, RC = 0.553, CI = 0.681, RI = 0.789). All 16S topologies recovered the Pectinoidea as the sister to the Limoidea.

The ITS-1 dataset consisted solely of new *Limatula* sequences from this study (3 *Limatula ovalis*, 4 *Limatula pygmaea* and 1 *Limatula hodgsoni* as the outgroup) and contained 438 aligned bases. Two branch-and-bound parsimony searches were performed with gaps treated as "missing" (1 MPC, 109 steps, ti = 0.330, RC = 0.961, CI = 0.971, RI = 0.979) (Fig. 3) and as a "new state" (1 MPC, 160 steps, ti = 0.319, RC = 0.901, CI = 0.929, RI = 0.949). Further analyses treat gaps as "missing". Three different parsimony-weighted searches were then run: (1) gaps costing double base changes, (2) transversions costing double transitions, (3) successive weighting based on rc. Two ML analyses were performed (one as above, the other: HKY85 + G + I model, ti/tv = estimated, assumed nucleotide frequencies = empirical, assumed proportion of invariable sites = estimated, distribution of rates at variable sites = gamma, shape parameter = estimated) and recovered a single topology

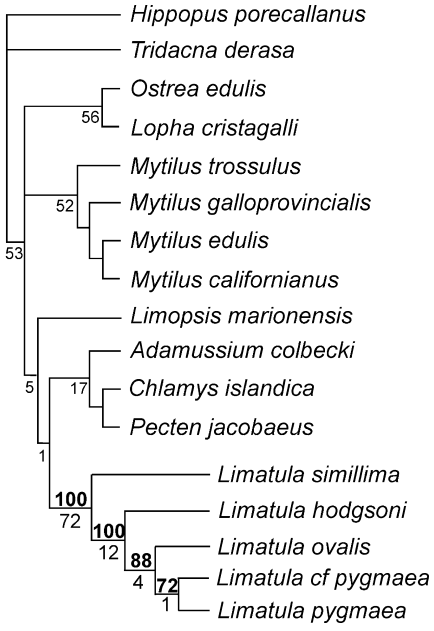

Fig. 2. Strict consensus of 16S "gaps as new state" parsimony search, with detailed view of *Limatula* topology (bootstrap values above, Bremer values below) (2 MPCs, 1,392 steps, ti = 0.207, RC = 0.574, CI = 0.695, RI = 0.803)

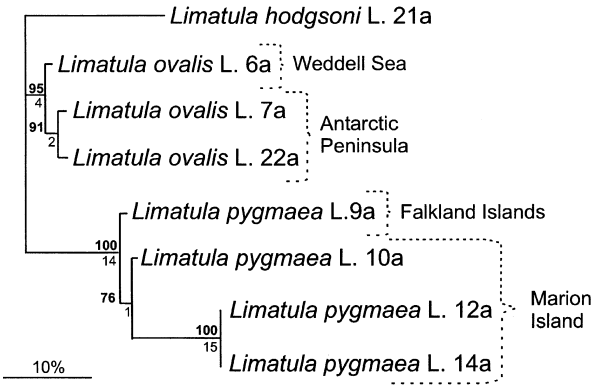

Fig. 3. Area phylogram of ITS-1 ML search (and "gaps as missing" parsimony MPC topology) (bootstrap values above, Bremer values below) (ML model = HKY85 + G + I, –log likelihood = 1,139.993, 1 MPC, 109 steps, ti = 0.330, RC = 0.961, CI = 0.971, RI = 0.979)

with a –log likelihood of 1,139.993 (HKY85 + G + I). The ML (Fig. 3) and all weighted parsimony analyses were identical to the single unweighted parsimony topology.

Sequence variation

The 18S *Limatula* sequences proved to vary little, as the *Limatula ovalis* sequences were identical, even though coming from two areas (Weddell Sea and Antarctic Peninsula). The five *Limatula pygmaea* and *Limatula* cf. *pygmaea* sequences were also all identical, despite coming from widely situated localities (Marion Island and the Falkland Islands). There were two differences between the *Limatula ovalis* and *Limatula pygmaea* sequences. The 16S sequences showed slightly more variation between geographic areas and taxa. The *Limatula* cf. *pygmaea* specimen from the Falkland Islands differed by two bases from the three Marion Island *Limatula pygmaea* sequences, even though their 18S sequences were exactly the same. The two *Limatula ovalis* sequences (both from the Antarctic Peninsula) were identical to each other, but differed by 8 bases from the *Limatula pygmaea* sequences.

The ITS-1 sequence percent divergences (derived from ITS-1 ML HKY85 + G + I analysis above, Fig. 3) may also betray population structure (Table 3). The Antarctic Peninsula *Limatula ovalis* specimens (L.7a, L.22a) diverge by less than 2% from the Weddell Sea *Limatula ovalis* specimen (L.6a). All the *Limatula ovalis* taxa together diverge from two of the *Limatula pygmaea* (L.9a, L.10a) by approximately 10%. These two *Limatula pygmaea* specimens are from different areas (Falkland Islands, Marion Island). Interestingly, the Falkland Islands *Limatula pygmaea* (L.9a) is as different from the other two Marion Island *Limatula pygmaea* (L.12a, L.14a) as it is from the three *Limatula ovalis* specimens. The Marion Island *Limatula pygmaea* (L.10a) diverges by over 8% from the other two Marion Island specimens, but less than 2% from the *Limatula pygmaea* from the Falkland Islands.

The geologically calibrated molecular clock estimates of limid/pectinid divergence rates for 16S rDNA were 0.1184–0.1641% per MY (all substitutions) and 0.0667–0.0925% (transversions only). Limid genera rates for 18S rDNA were 0.0411–0.0837% per MY (all substitutions) and 0.0217–0.0444% (transversions only). *Limatula ovalis/pygmaea* divergence rates for ITS-1 were 6.60–37.88% per MY (all substitutions), depending on the population of *Limatula pygmaea* used, and thus betray both the complicated population differences in *Limatula pygmaea* referred to above and the high rate of ITS-1 sequence change through time.

Discussion

Antarctic biogeographic implications

Well-supported results derived from taxonomic data (Fig. 4) can be potentially revealing in a biogeographic sense. This study strongly supports both the close relationship between *Limatula ovalis* and *Limatula pygmaea* (in all analyses) and the differentiation between them (Fig. 5). Therefore, they appear to represent two closely related, but distinct, lineages. *Limatula pygmaea* is found widely throughout the Magellanic region and sub-Antarctic islands, while *Limatula ovalis* is restricted to the high Antarctic (Antarctic Peninsula and Weddell Sea). Given their proven close systematic relationship, this could represent a classic case of passive vicariance through the barrier of the APF. *Limatula pygmaea* may

Table 3. ITS-1 sequence percent divergences (derived from ITS-1 ML HKY85 + G + I analysis) amongst *Limatula ovalis/pygmaea* (% divergence below *diagonal*, number of base differences above *diagonal*)

	L.ovalis 6a	*L.ovalis* 7a	*L.ovalis* 22a	*L.*cf *pygmaea* 9a	*L.pygmaea* 10a	*L.pygmaea* 12a	*L.pygmaea* 14a
L.ovalis 6a	X	7	7	31	32	51	53
L.ovalis 7a	1.98%	X	3	34	34	53	55
L.ovalis 22a	1.80%	0.80%	X	33	33	50	52
*L.*cf *pygmaea* 9a	9.21%	9.98%	9.79%	X	5	32	35
L.pygmaea 10a	10.32%	11.09%	10.90%	1.65%	X	17	19
L.pygmaea 12a	18.14%	18.92%	18.73%	9.47%	8.45%	X	0
L.pygmaea 14a	18.14%	18.92%	18.73%	9.47%	8.45%	0.00%	X

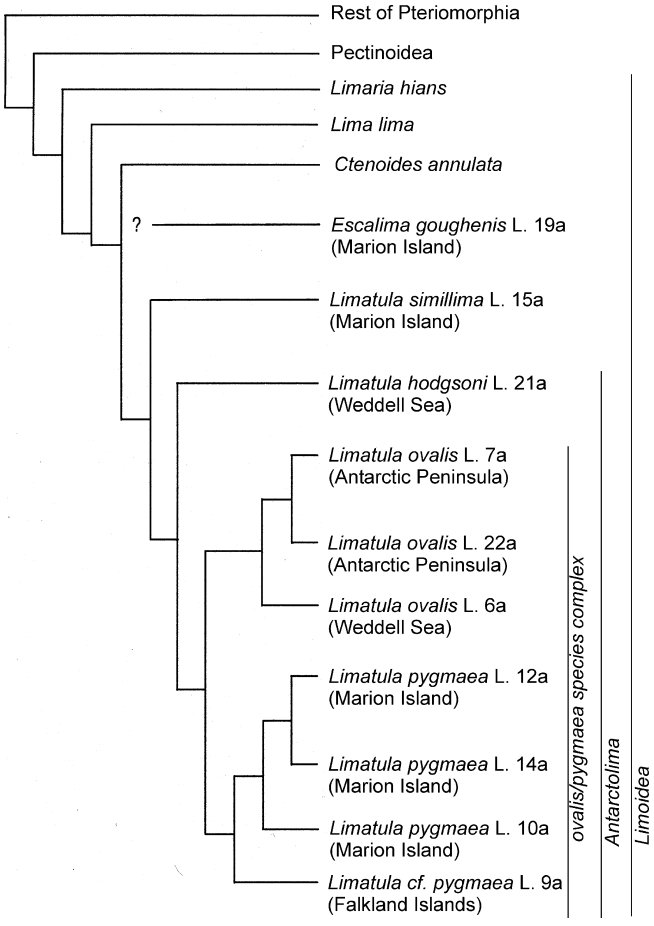

Fig. 4. Summary of limid phylogeny hypotheses presented in this study

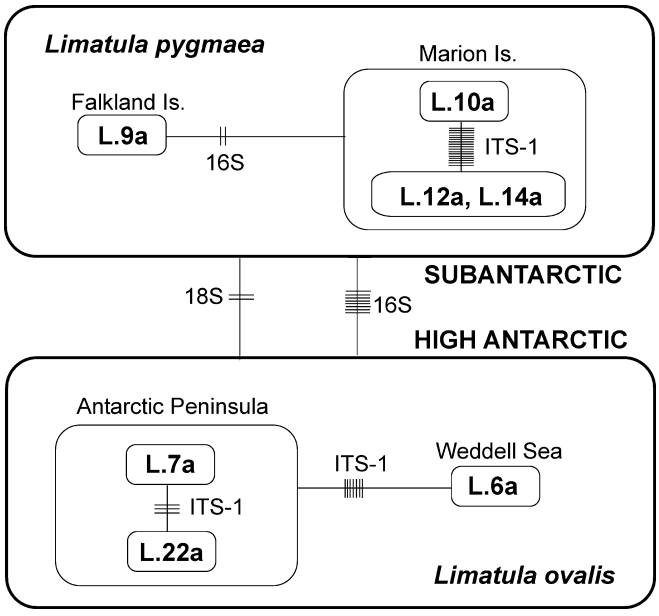

Fig. 5. Phylogeographic pattern of *Limtula pygmaea/ovalis* species complex at three systematic levels (*cross-hatches* represent number of base differences between taxa)

have remained on the South American mainland and was dispersed by the circum-Antarctic current to colonise the islands of the sub-Antarctic. *Limatula ovalis* may have been isolated in the high Antarctic, with limited dispersal possible between the Weddell Sea and Antarctic Peninsula. While this is certainly possible, it also could be that *Limatula ovalis* is the result of a one-off "jump" dispersal across an already existing APF (Bargelloni et al. 2000b), possibly along the Scotia Arc, and then subsequent isolation led to its divergence.

These different hypotheses require the addition of the element of time to choose between them. If securely dated *Limatula* fossils were to be found on either side of the Drake Passage, this could potentially provide the required evidence. Given this has not been the case, molecular clock estimates can be used to estimate the time of cladogenesis and then be compared against important geologically dated events, in this case the opening of the Drake Passage (23.5 MYA; Crame 1999). Calculations based on 16S rDNA rates derived above give estimated dates of divergence between *Limatula ovalis* and *Limatula pygmaea* of 5.79–8.03 MYA (all substitutions), 2.06–2.85 MYA (transversions only), 1.36–2.57 MYA (rates from Held 2001, transversions only and all substitutions) and 1.73 MYA (rate from Bargelloni et al. 2000a, transversions only). 18S rDNA rates give estimated divergence dates of 6.81–13.87 MYA (all substitutions) and 9.33–19.12 MYA (transversions only). ITS-1 rates give estimated divergence dates of 0.24–2.87 MYA (all substitutions).

This implies that molecular clock estimates can vary depending on the method chosen. The geological calibrations used here for 16S and 18S rDNA (first appearance in the fossil record) are very ancient and not guaranteed to be a true date of cladogenesis. The rates from Held (2001) and Bargelloni et al. (2000a) are calibrated against much more recent events (opening of the Drake Passage ~23.5 MYA and separation of Antarctica and Australia ~38 MYA), and so are more likely to be accurate, but they were derived from phylogenetically distant serolid isopods and notothenioid fish and so may be inappropriate for these taxa. Further complicating factors are varying rates of change between bivalve lineages (Rice et al. 1993). Steiner and Hammer (2000) found the Limidae had the highest substitution rates of 18S rDNA amongst pteriomorph bivalves, which could therefore confound rates derived from a comparison with the Pectinidae. Variations over time within a lineage can also violate clock-like assumptions (Hillis et al. 1996).

Despite the various caveats, all the different estimates place the *Limatula ovalis/pygmaea* split as being relatively recent (1.36–8.03 MYA, 6.81–19.12 MYA or 0.24–2.87 MYA) and so, importantly, well after the formation of the APF; therefore, dispersal across an already existing APF is the likely explanation. Fleming (1978) noted that no *Limatula* had been found in the Miocene deposits of the Ross Sea. This could be explained if they had only penetrated the high Antarctic

well after the formation of the APF, although it must be noted that these deposits are very fragmentary. This timing of the *Limatula ovalis*/*pygmaea* split is congruent with both high-Antarctic/sub-Antarctic splits between the notothenioid fish *Patagonotothen* and *Lepidonotothen* (9 MYA in Bargelloni et al. 2000a, 6.6–7.1 MYA in Stankovic et al. 2002) and the krill *Euphausia vallentini* and *E. frigida* (~7 MYA in Bargelloni et al. 2000b). Thus the APF does not appear to be a rigid barrier, as "high-Antarctic" notothenioid fish are occasionally encountered in the sub-Antarctic (Stankovic et al. 2002). It must also be remembered that while distribution comparisons with other taxa are interesting, biogeographic regions are likely to differ depending on the group being studied.

Limatula ovalis/*pygmaea*'s sister taxon, *Limatula hodgsoni*, has a present distribution covering the Magellan region, sub-Antarctic (South Georgia) and high Antarctic (Weddell Sea). Given this, the split between *Limatula hodgsoni* and *Limatula ovalis*/*pygmaea* also does not appear to be due to vicariance. One would expect different geographic distributions, but *Limatula hodgsoni* is present in both the regions where *Limatula ovalis* and *Limatula pygmaea* are found. It is of course possible that vicariance was the original cause of speciation, followed by later dispersal in both directions across the APF by *Limatula hodgsoni* and one of the other two taxa. It would be a mistake to assume that present distributions necessarily reflect ancient ones, especially as the many cooling/warming episodes in Antarctic geological history would have led to changes in the ranges of Antarctic marine taxa (Clarke and Crame 1997).

At the higher systematic level, an important question in terms of Antarctic biogeography is whether the limids in this study represent a single Southern Ocean lineage, or whether they are the result of numerous "incursions" into the Southern Ocean from the World Ocean by related, but distinct, limid lineages. Limids have a long history in the Antarctic, having been present there since at least the Cretaceous (Crame 1996; Stillwell and Gazdzicki 1998), and so in situ evolution would certainly be a possibility. The bivalve fauna of the late Eocene (33–42 MYA) from the La Meseta formation (Seymour Island, Antarctic Peninsula) is related to current Antarctic fauna (Crame 1996, 1999). Despite this, it seems likely that the different genera of limids present in the Southern Ocean (*Acesta*, *Escalima*, *Limatula*) do not form a clade with limids found elsewhere. The different genera of limids, e.g. *Limatula*, are spread throughout the globe. At the family level, all Antarctic bivalves are cold-tolerant members of cosmopolitan families, rather than endemics (Egorova 1985), thus reflecting the long-standing links between the fauna of the Antarctic and the rest of the world (Crame 1996).

The restriction of the limid subgenus *Antarctolima* Habe, 1977 (including *Limatula hodgsoni*, *Limatula ovalis*, *Limatula pygmaea*) to the Southern Ocean, though, may reflect the increasing isolation of the Antarctic fauna after the formation of the circum-Antarctic current (i.e. possibly since late Oligocene, 23.5 MYA) (Clarke and Crame 1989; Crame 1999). A further question is the geographic origin of *Antarctolima*. Fleming (1978) mentioned that the fossil species *Limatula crebresquamata* found in late Oligocene (~26 MYA) deposits in Victoria, Australia, shows a similar pyriform shape and structure, and may be the ancestral stock of *Limatula hodgsoni*. Having said this, Fleming (1978) went on to say that it is actually much more likely that the *Antarctolima* entered the Southern Ocean from South America, "like the majority of circumpolar Antarctic benthic organisms". This seems logical given the presence of multiple extant *Limatula* species in South America, including *Limatula hodgsoni* and *Limatula pygmaea* (Linse 2001).

Systematics implications

At the higher systematic level, the Pectinoidea were recovered as the sister taxon to the Limoidea (Fig. 4) in both the 18S (weakly) and 16S analyses, agreeing with the molecular findings of Steiner and Hammer (2000) and some morphological studies (e.g. Carter 1990; Starobogatov 1992; Carter et al. 2000). Within the Limoidea, the topology from this study agrees with Steiner and Hammer (2000). *Ctenoides* appears to be the most likely candidate as sister to *Limatula*, though this is based on a very limited taxonomic sampling. The relationships of limid genera are in need of a systematic revision (Scarabino 1998).

Both Habe (1977) and Fleming (1978) described a new *Limatula* subgenus with *Limatula hodgsoni* as the type. Habe's name, *Antarctolima*, is the correct one due to its publication precedence. Fleming's *Squamilima* (= *Antarctolima*) constituted a new subgenus of broadly pyriform shell outline and Southern Ocean distribution, and also included *Limatula pygmaea* and *Limatula ovalis*. He placed *Limatula simillima* in the subgenus *Limatula s.s.* Fleming (1978) implied that the *Antarctolima* of the Southern Ocean formed a monophyletic clade, describing them as a "chain of species". He also raised the possibility of parallel evolution, which could have occurred if the pyriform shell outline was a result of an adaptive response to a common Antarctic environmental factor. This explanation seems less likely given that closely related *Limatula* species with tall, elongated shells (*Limatula simillima* and *Limatula* cf. *subauriculata* Montagu, 1808) are also found in the same Southern Ocean waters (Fleming 1978). More recent morphological work based on shell characters has confirmed the *Antarctolima* as a single clade (Dell 1990; F. Scarabino, personal communication).

The split between *Limatula s.s.* and *Antarctolima* is confirmed in this study by the molecular results of 18S and 16S sequences, in that *Limatula simillima* (representing *Limatula s.s.*) is the most basal of the Antarctic limids and that *Antarctolima* (represented here by

Limatula hodgsoni, *Limatula ovalis*, *Limatula pygmaea*) forms a strong clade. This study found *Limatula hodgsoni* to be the most basal of the *Antarctolima* and constituted a sister group to the *Limatula ovalis*/*pygmaea* clade.

The main question within the Antarctic *Limatula* is whether *Antarctolima* and *Limatula s.s.* (*Limatula simillima*) are likely to form an Antarctic clade, as they do in this study. This seems unlikely given that representatives of *Limatula s.s.* can be found in all oceans, while *Antarctolima* is restricted to the Southern Ocean (Fleming 1978).

Intraspecific genetic variation

The majority of the *Limatula pygmaea* from this study come from Marion Island, over 6,400 km from the Falkland Islands, whence comes the other specimen. All the *Limatula pygmaea* have an identical 18S sequence. At the 16S level though, there is a potentially interesting difference in that the *Limatula pygmaea* from the Falklands differs from the three Marion Island specimens in two base changes. This may represent geographical structured populations.

The ITS-1 sequences also provide some interesting structure within the Marion Island population (Figs. 3, 5). Two specimens (L.12a, L.14a) which, although sharing the same 16S sequence with the other Marion Island specimen (L.10a) to the exclusion of the Falkland Islands specimen, also show a variation of over 8% in the ITS-1 sequence from the sympatric L.10a. The different 16S sequences still separate these two specimens (and areas), but the implication is that two lineages of *Limatula pygmaea* may exist at Marion Island.

This population structure could indicate that the Marion Island population has become isolated after colonisation from the Magellanic region. The Falkland Islands can be considered as part of the Magellanic region, since many species are shared between them; this is unsurprising given the shallow shelf between them (Linse 2001).

Furthermore, this could imply secondary contact between the lineages, with one a more recent immigrant to Marion Island. Despite this possibility, both parsimony and maximum likelihood analysis of the ITS-1 (and 16S) placed the three Marion Island specimens in a clade (76% bootstrap, Fig. 3) to the exclusion of the Falkland Islands specimen, irrespective of the phenetic similarity of the ITS-1 sequences of L.10a and L.9a.

Conclusions

The high-Antarctic *Limatula ovalis* and sub-Antarctic *Limatula pygmaea* are shown to be sister taxa in all analyses, with *Limatula hodgsoni* as their sister taxon, thus supporting the subgenus *Antarctolima*. The likely explanation of the *Limatula ovalis*/*pygmaea* relationship is dispersal across the Antarctic Polar Front, and thus it may not be as impervious a barrier to immigration and emigration as previously thought. This may imply that the process of speciation within the Antarctic, responsible for the present diversity, continues today. Potential conclusions from this study are limited by the sample size, but further molecular, shell microstructure and basic alpha taxonomic (F. Scarabino, personal communication) studies of these and other taxa from wider geographic areas will likely further refine these conclusions.

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