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An Antarctic flock under the Thorson's rule: Diversity and larval development of Antarctic Velutinidae (Mollusca: Gastropoda)

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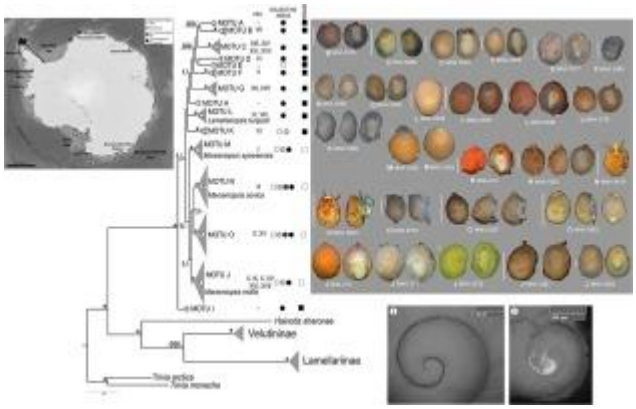
Abstract :

In most marine gastropods, the duration of the larval phase is a key feature, strongly influencing species distribution and persistence. Antarctic lineages, in agreement with Thorson's rule, generally show a short pelagic developmental phase (or lack it completely), with very few exceptions. Among them is the ascidian-feeding gastropod family Velutinidae, a quite understudied group. Based on a multilocus (COI, 16S, 28S and ITS2) dataset for 182 specimens collected in Antarctica and other regions worldwide, we investigated the actual Antarctic velutinid diversity, inferred their larval development, tested species genetic connectivity and produced a first phylogenetic framework of the family. We identified 15 Antarctic Molecular Operational Taxonomic Units (MOTUs), some of which represented undescribed species, which show two different types of larval shell, indicating different duration of the Pelagic Larval Phase (PLD). Antarctic velutinids stand as an independent lineage, sister to the rest of the family, with extensive hidden diversity likely produced by rapid radiation. Our phylogenetic framework indicates that this Antarctic flock underwent repeated events of pelagic phase shortening, in agreement with Thorson's rule, yielding species with restricted geographic ranges.

Graphical abstract



Highlights

► Velutinidae is a gastropod family retaining a planktotrophic larva in Antarctica. ► Diversity of Antarctic Velutinidae was analysed by species delimitation methods. ► 15 species were recovered, showing two different pelagic larval phase lengths. ► Antarctic velutinids are an independent lineage, sister to the rest of the family. ► A rapid radiation and the shortening of the pelagic phase shaped their diversity.

Keywords : Thorson's rule, Larval ecology, Integrative taxonomy, Antarctica, Gastropoda, Velutinidae

1. Introduction

Understanding the interplay of animal life-history trait variation, natural selection and environmental conditions, has always been a hot topic in science (e.g. Roff, 2002; Stearns, 1992). Relative benefits and trade-offs of given traits have been investigated in a variety of taxa in the attempt of understanding the underlying evolutionary mechanisms. Reproductive and developmental traits, such as the size and number of offspring and the larval type, represent crucial drivers of species ecological success and spatial distribution, with consequences at the community level and, in turn, on biodiversity patterns and dynamics (e.g. Kinlan & Gaines, 2003). In particular, the type of larval development is a key feature for benthic organisms, since it deeply influences individual dispersion, population connectivity and species resilience to disturbance (Becker et al., 2007).

The most debated theory assuming a geographical pattern of larval development diversity was formulated by Mileikovsky (1971) who, inspired by Gunnar Thorson's pioneer studies on larval development of marine invertebrates (e.g. Thorson, 1936, 1946, 1950), proposed the so-called 'Thorson's rule': a decrease in the number of species with pelagic development,

paralleled by an increase of the number of brooders towards the poles. Today, this paradigm is not considered as valid for all taxa (Arnaud & Hain, 1992; Pearse, 1994) and all habitats (Gallardo & Penchaszadeh, 2001). Factors other than geographic distribution, such as seawater temperature and productivity, have been demonstrated to be equally relevant in influencing the type of larval development (Marshall et al., 2012). However, meta-analyses performed at global scale suggested that in several cases, Thorson's rule still holds valid. For instance, it has been demonstrated that the proportion of marine invertebrates with pelagic larvae decreases moving pole-ward, along with the proportion of actively feeding larvae (planktotrophic) in comparison with non-feeding ones (lecithotrophic) (Marshall et al., 2012). This trend was stronger in some groups, such as molluscs, and in the southern hemisphere (Clarke, 1992; Marshall et al., 2012). Additionally, low temperature was associated with lower proportions of pelagic developers, especially in low productivity areas, whereas the proportion of feeding larvae increased with temperature but not with productivity (Marshall et al., 2012)

Life-history traits strongly affect the ecological dynamics of marine species, and this is especially true among benthic species, for which dispersal is mostly achieved during the larval phase. Several studies have explicitly linked the duration of the larval phase with species' dispersal ability, and estimates based on neutral genetic markers showed that species having longer lasting pelagic larval phases also have a higher rate of genetic connectivity (e.g. Collin, 2001; Modica et al., 2017). Because of the influence of larval development on population dynamics and, therefore, on their ability to respond to disturbance, this represents a key species trait to take into account for the management of marine protected areas (Kinlan & Gaines, 2003).

89 Pelagic development is adopted by the majority (~60-70%) of marine invertebrate species
90 and is generally considered as the ancestral state in gastropod molluscs (Marshall et al.,
91 2012). In gastropods, the type of development can be inferred by comparing the morphology
92 of the larval shell (protoconch), usually retained at the top of the adult shell (teleoconch).
93 Species with lecithotrophic or intracapsular development produce eggs with comparatively
94 higher quantity of yolk and, therefore, possess protoconchs with a bigger nucleus (i.e. the
95 initial portion built by the embryo, during the intracapsular life) and fewer whorls. On the
96 contrary, species with planktotrophic development have a protoconch with a smaller nucleus
97 and more whorls (Thorson, 1950; Lima & Lutz, 1990).
98 Very few studies describing pelagic phases of invertebrates are available for the Southern
99 Ocean (e.g. Stanwell-Smith & Barnes, 1997) but there is a general consensus that the number
100 of marine benthic invertebrates with a planktotrophic larva is not high (Hain & Arnaud,
101 1992).
102 Among Antarctic gastropods, the families Capulidae J. Fleming, 1822 and Velutinidae
103 Gray, 1840 represent model taxa to study the evolution of larval ecological traits, given the
104 completely opposite trends shown. In fact, while 90% of the Antarctic capulid species
105 undergo lecithotrophic development (Hain & Arnaud 1992; Schiaparelli et al., 2000; Fassio et
106 al., 2015), all Antarctic velutinid species have long lasting planktotrophic larvae (Hain &
107 Arnaud, 1992). Velutinid larval ecology is indeed intriguing for the exceptionally long
108 pelagic life reported for the Antarctic species (Hain & Arnaud, 1992; Bandel et al., 1993;
109 Peck et al., 2006), which is in general contrast with Thorson's rule. In this group, a peculiar
110 larva called "limacosphaera" is equipped with a rounded and soft muscular mantle
111 (deutoconcha) that surrounds the larval shell (Simroth, 1914; Lebour, 1937; Hain, 1990; Hain

& Arnaud, 1992; Bandel et al, 1993) and has been shown to remain in the pelagic phase up to 1.5 years in aquarium condition (Peck et al., 2006).

The nine Antarctic species of Velutinidae currently recognised are classified into two endemic genera: *Marseniopsis* Bergh, 1886 with 7 species and *Lamellariopsis* Vayssiére, 1906 with two species. According to the current systematics (Bouchet et al., 2005, Bouchet et al., 2017), this family comprises two subfamilies: Lamellariinae d'Orbigny, 1841 with six genera, and Velutininae Gray, 1840 with ten genera, plus a few genera *incertae sedis* (Gofas, 2009). Like the rest of the family (Beesley et al, 1998), Antarctic species rely on ascidians for feeding and for incubating eggs in the tunicate's cuticle (Numanami & Okutani 1991; Peck et al., 2006). Their shell is thin, fragile (Beesley et al., 1998) and in the majority of the cases also completely enclosed by the almost non-retractile mantle (Beesley et al., 1998). Mantle shape, texture and colour are highly variable (Behrens, 1980), usually mimicking the ascidian, sometimes with a remarkable match (Beesley et al., 1998; Behrens et al., 2014). Taxonomic studies of Velutinidae are particularly challenging due to the absence of diagnostic shell features and the high degree of convergence in mantle shape and colour patterns among different species. For these reasons, only a few works have attempted to revise the systematics of this family (e.g.: Behrens, 1980; Gulbin & Golikov, 1997, 1998, 1999, 2000, 2001). This is mirrored by the low number of available DNA sequences that correspond to 7 specimens only (GenBank, accessed on 01/06/2018) (Behrens et al., 2014; Heimeier et al., 2010; Barco et al., 2015).

The aims of the present study are to: (i) assess the Antarctic velutinid biodiversity based on a large number of specimens from a variety of sites;, (ii) infer the larval development of Antarctic velutinids, using protoconch morphology as a proxy, and discuss observed patterns in the framework of Thorson's rule; (iii) test the hypothesis that velutinid species with higher

dispersal capacities display higher genetic connectivity; and (iv) provide a molecular phylogenetic framework for the Antarctic velutinids.

2. Materials and methods

2.1. Taxon sampling

The dataset consisted of 182 specimens. Of these, 134 were obtained from the material collected during several Antarctic scientific expeditions (Fig. 1): i) the R/V Tangaroa "BioRoss" (2004) and "IPY-CAML" (2008) expeditions to the Ross Sea (New Zealand National Institute of Water and Atmospheric Research, NIWA); ii) the Italian National Antarctic Program (PNRA) expeditions from 2009-2014 to Terra Nova Bay (Ross Sea); iii) the expeditions "REVOLTA" (2014) and "CEAMARC" (2008) to the Dumont d'Urville Sea (Institut Polaire Français Paul-Emile Victor, IPEV and Muséum National d'Histoire Naturelle, MNHN, France); iv) the R/V Polarstern "PS81" (2013) expedition, "ANT XXIX" to the tip of the Antarctic Peninsula (Alfred Wegener Institute, AWI, Germany); v) the R/V Polarstern "PS65" (2003-2004) expedition to the Georg Von Neumayer base area. All specimens studied were adults, except for a limacosphaera larva (Italian National Antarctic Museum, MNA, MNA 6150) and two egg capsules (NIWA 36790.1 and NIWA 36893.2) collected from broods laid in ascidians tunics. All specimens were preserved in 96%-100% ethanol.

One additional sequence from an Antarctic velutinid larva, erroneously identified as "cf. *Niveria* sp." (a genus of the related family Triviidae), was retrieved from GenBank.

Samples of Velutinidae from temperate and tropical areas were obtained from the MNHN, NIWA and CASIZ (California Academy of Science Invertebrate Zoology Collection): 17

specimens were collected during the MNHN expeditions "PANGLAO 2004" (Philippine, 2004), "ATIMO VATAE" (Madagascar, 2010) and "BIOPAPUA" (Papua New Guinea, 2010), 19 specimens from New Zealand, one specimen of *Lamellaria latens* (O. F. Müller, 1776) from Brittany (France) and one of *Hainotis sharonae* (Willett, 1939) from Monterey (California, USA).

For 27 of the above listed specimens, sequences were kindly provided by Nicolas Puillandre (MNHN). Seven additional velutinid sequences were retrieved from GenBank. Sequences from two species of Triviidae Troschel, 1863, *Trivia arctica* (Pulteney, 1799) and *Trivia monacha* (da Costa, 1778), were used as outgroup (Colgan et al., 2007). See Fig. 1 and Table S1 for voucher ID, collecting localities, sequences details and GenBank accession numbers.

2.2. Molecular analyses

DNA was isolated from foot tissue of adult animals, and from the entire specimen of larvae and egg capsules, following a proteinase K/phenol–chloroform extraction protocol (Oliverio & Mariottini, 2001). Two mitochondrial and two nuclear gene fragments were amplified: the ~658 bp barcode region of the cytochrome oxidase I gene (COI); a ~700 bp region of the 16S rDNA gene; a ~700 bp region of the 28S rDNA gene; and a ~450 bp region of the ITS2 rDNA (see Table 1 for primer sequence and PCR conditions). Amplicons purified using Exosap-IT (USB Corporation) were sequenced by Macrogen Inc. (Spain).

2.3. Sequences editing and alignment

Forward and reverse sequences were assembled and edited with Geneious Pro v.11 (Kearse et al., 2012). COI sequences were manually aligned and checked for stop codons. 16S and ITS2 sequences were aligned with MAFFT 7 (Katoh et al., 2002). We used the Q-INS-i algorithm (Katoh & Toh, 2008), which accounts for secondary structures, for the ITS2, and the E-INS-i algorithm (Katoh et al., 2002), which accounts for multiple conserved domains and long gaps, for the 16S. The 28S sequences were aligned using the CLUSTAW algorithm (Thompson et al., 1994) implemented in Geneious.

2.4. Species delimitation

An Integrative Taxonomy approach, where species are regarded as hypotheses undergoing a process of falsification by subsequent tests (Samadi & Barberousse, 2006; De Queiroz, 2007), was used to delimit species boundaries (Modica et al., 2014; Puillandre et al., 2014). First, Preliminary Species Hypotheses (PSH, with Roman numerals) were defined based on mantle texture and colour pattern (traditionally employed in velutinid taxonomy) as observed in 51 live specimens, sampled and photographed during the BIOROSS, TAN0802 and PS81 expeditions. Then, morphological PSHs were compared with Molecular Operational Taxonomic Units (MOTUs) (Blaxter et al., 2005), based on the COI sequence alignment collapsed into haplotypes by the Alignment Transformation EnviRonment (ALTER) (Glez-Peña et al., 2010). MOTUs were formulated using three different methods: the Automatic Barcode Gap Discovery (ABGD) (Puillandre et al., 2012a; Puillandre et al., 2012b), the Generalized Mixed Yule Coalescent (GMYC) model (Pons et al., 2006) and the Bayesian implementation of the Poisson Tree Processes (bPTP) model (Zhang et al., 2013).

We retained as final species hypotheses the MOTUs that were represented in the majority of the partitions retrieved by the three species delimitation methods and that showed reciprocal monophyly (Knowlton, 2000; Reid et al., 2006) in a multilocus phylogenetic analysis of the molecular data (see below). The retained MOTUs were finally compared with the morphology-based PSHs.

A detailed description of the methods is reported in the Supplementary Material.

2.5. Phylogenetic reconstruction based on primary sequence information

Phylogenetic analyses were performed using Maximum Likelihood (ML) and Bayesian inference (BA) methods on each single-gene dataset (COI, 16S, 28S, ITS2) and on three combined datasets: (i) including all sequences (ALL), (ii) including only specimens from which sequences of all genes were available (COM), and (iii) including specimens with sequences for at least three genes (3/4).

In addition to the concatenation approach (combined datasets), multi-locus analyses were performed using the species tree approach. This method takes into account the stochastic sorting of lineages in the estimation of species trees from the gene trees, and recent research showed that it may outperform the sequence concatenation approach in estimating species phylogeny (Kubatko & Degnan, 2007; Heled & Drummond, 2010). To infer the species tree we used the multi-species coalescent model implemented in the *BEAST extension (Heled & Drummond, 2010) of the BEAST package.

2.6. Phylogenetic reconstruction based on ITS2 secondary structure information

ITS2 has proven to be a valuable marker for mollusc phylogenetics and taxonomy (e.g. Oliverio et al., 2002; Puillandre et al., 2011), especially when the information from both the

sequence and the secondary structure are exploited (Salvi et al., 2010; Salvi et al., 2014; Salvi & Mariottini, 2012; 2017). Including RNA secondary structures improves accuracy and robustness in reconstructing phylogenetic trees (e.g. Keller et al., 2010). Therefore, we performed an additional phylogenetic analysis based on ITS2 sequences and secondary structures using a combined model of sequence-structure evolution.

The secondary structure was predicted for each ITS2 sequence of a subset of 52 specimens on a thermodynamic basis using the software package RNA Structure 5.5 (Mathews et al., 1999; available on the Turner Lab Homepage http://rna.chem.rochester.edu). Candidate folding models were contrasted against secondary structure models proposed for molluscs in previous studies (Oliverio et al., 2002; Salvi et al., 2010; Salvi & Mariottini 2012).

A detailed description of the ITS2 secondary structure phylogenetic methods used on sequence-structure alignments is reported in the Supplementary Material.

2.7. Phylogeography and genetic connectivity analyses

Relationships between haplotypes were investigated for each species using the Median Joining (MJ) network approach (Bandelt et al., 1999) as implemented in PopART (popart.otago.ac.nz). MJ combines minimum spanning trees within a single network and uses a parsimony criterion to add to the network median vectors that could be interpreted as unsampled genotypes or extinct ancestral intermediates.

To assess if a planktotrophic larval development resulted in a high level of genetic connectivity among distant populations, two methods were applied to species for which at least five COI sequences were available. First, the correlation (r) between genetic distances and geographical distances was estimated using a non-parametric Mantel's test, with both log-

250 transformed and non-log-transformed pairwise distance matrices, using the Isolation by
251 Distance web service (Jensen et al., 2005; available at: ibdws.sdsu.edu).
252 The utility of the widely used Mantel's test has recently been questioned as it does not
253 explicitly take into account the existence of spatial autocorrelation, potentially leading to
254 biased results (e.g. Meirmans, 2012). Therefore, a spatial principal component analysis
255 (sPCA) was also used, as implemented in the R (https://cran.r-project.org) package 'adegenet',
256 version 2.0.0 (Jombart et al., 2008). This approach takes into account the variance between
257 the studied entities (in this case individuals) and also their spatial autocorrelation (Jombart et
258 al., 2008). The resulting score maps allow a visual assessment of the spatial genetic structures
259 that can be classified as either global or local (*sensu* Thioulouse et al., 1995): a global
260 structure may be related to patches, clines or isolation-by-distance patterns; a local structure
261 yields stronger genetic differences among neighbours than among random pairs of entities. A
262 detailed description of these methods is reported in the Supplementary Material.
263

264 *2.8. Larval shell morphology and development*

265 Protoconchs were measured using scaled camera lucida hand-drawings and photographs
266 from a Leica/Leo Stereoscan S440 Scanning Electron Microscope (SEM). For SEM,
267 specimens were dehydrated in solutions with increasing ethanol concentrations and a final
268 passage in HMDS (hexamethyldisilazane) (Nation, 1983).
269 Presence/absence of characters such as granulose sculptures on protoconch I (embryonic
270 shell), longitudinal marked ribs on protoconch II (larval shell) and subsutural stripes, were
271 recorded. Quantitative characters, such as nucleus diameter and maximum width, half whorl
272 and one whorl diameter, protoconch I and protoconch I+II number of whorls and maximum

diameter were taken following the protocol proposed by Verduin (1977). Length of protoconch II was calculated as the difference between protoconch I+II and protoconch I.

Measurements were taken from 28 Antarctic and four non-Antarctic specimens. In addition, measurements were retrieved from protoconch photographs and drawings of *Coriocella nigra* Blainville, 1824 from Australia (Riedel, 2000: pl. 8 fig. 9), *Hainotis sharonae* (Willett, 1939) from California, USA (Riedel, 2000: fig. 28b), *Marsenina rhombica* (Dall, 1871) from North Pacific (Riedel, 2000: fig. 28a), *Calyptoconcha pellucida* (A. E. Verrill, 1880) from West Sahara (Bouchet & Warén, 1993: figs 1766-1767) and *Marseniopsis* cf. *mollis* from the east coast of the Weddell Sea (Bandel et al., 1993: fig. 9).

To explore protoconchs data in search of discrete groups, a cluster analysis was performed using the UPGMA (Sokal & Michener, 1958) hierarchical bottom-up clustering method, which allows finding the most appropriate number of clusters, instead of providing it a priori. Node support was assessed by 100 bootstrap replicates. The Pearson coefficient was used to assess linear correlation among distribution range (estimated as the distance between the two farthest collection points) and the average nucleus diameter, and a two-tailed t-test was used to assess the significance. A moderate correlation was assumed for $0.7 > r < 0.85$ and a high correlation for $r \geq 0.85$ (significant for $p < 0.05$). All analyses and graphics were done with Past 3.14 (Hammer et al., 2001).

3. Results

3.1. Species delimitation

Specimens for which photos were taken *in vivo* (51), were partitioned into 17 morphological PSHs (I-XVII) (Fig. 2 and Table S2). A nominal taxon was associated to four PSHs (I-IV), out of the total 17, as described below.

PSH I had the same colour pattern (orange spots and light background) and mantle texture (thick, wrinkled and jelly-like) as the holotype of *Marseniopsis syowaensis* Numanami & Okutani, 1991, collected in Langhovde (near Syowa Research Station, Eastern Antarctica). This species was also reported from Peter I Island (Bellingshausen Sea) (Aldea et al., 2009), that is near the area where our specimens were sampled (tip of the Antarctic Peninsula).

PSH II corresponded, for the lime-yellow mantle colour and the smooth and elliptical dorsum shape, to *M. mollis*, whose type locality is Cape Adare (Ross Sea). Numanami (1996) reported for this species a circum-Antarctic distribution, including record of larvae collected at the East side of the Antarctic Peninsula (Hain, 1990; Hain & Arnaud, 1992), near our sampling locality (tip of the Antarctic Peninsula).

PSH III was identified for the polygonal dorsum shape, the mantle texture and the colour pattern, as *M. conica*. Cape Adare (Ross Sea) is *M. conica* type locality, while our specimens were collected at the tip of the Antarctic Peninsula. However, Numanami (1996) reported a wide Antarctic distribution range for this species, including the Weddel Sea, where larvae of this species had been collected (Hain 1990, 1992).

PSH IV corresponded, in shape and colour, to *Lamellariopsis turqueti* Vayssière, 1906, whose type locality is Anvers Island (west side of the Antarctic Peninsula) not far from where our specimens were collected (tip of the Antarctic Peninsula).

316 Molecular species delimitation methods identified several partitions of the dataset
317 consisting of a number of MOTUs ranging between 12 and 21. Only MOTUs present in the
318 majority of the partitions and comprising a supported monophyletic clade were retained (Fig.
319 2). This workflow identified 15 MOTUs, named A to O. Five of them (MOTUs A, D, E, H
320 and I) were represented by a single, highly divergent, specimen.
321 All MOTUs were compared with the morphology-based PSHs (Table S2). MOTUs A, H, I
322 and D were lacking PSH assignation because no observations of live specimens were
323 available. MOTUs B, E, F, K, M and N corresponded to one PSH each, while MOTU L
324 comprised specimens ascribed to two PSHs. For MOTUs C, G, J and O there was no
325 congruence with PSH.
326 A detailed description of the results is reported in the Supplementary Material.
327

328 *3.2. Molecular phylogeny*

329 The Bayesian analysis based on the ALL combined dataset (Figs 2 and 3) produced a tree
330 with higher support at internal nodes for Antarctic MOTUs and a more resolved topology at
331 subfamily level, compared to single gene analyses (Figs S5-S12). In this tree, the family
332 Velutinidae resulted monophyletic and, within this family, four major lineages were identified
333 (Figs 2 and 3). One supported clade comprised all the Antarctic species and was the sister
334 group to the rest of the velutinids. Two clades included genera ascribed to the subfamilies
335 Velutininae and Lamellariinae, respectively. Two discrepancies with current systematics were
336 detected: the Antarctic genera, supposed to be part of the subfamily Velutininae, were
337 recovered, instead, as a distinct lineage; the species *Hainotis sharonae* (CASIZ181317)
338 supposed to belong to the subfamily Lamellariinae, was retrieved as a fourth independent
339 lineage. The internal topology of the Antarctic clade was not fully resolved in most

phylogenetic reconstructions. Only five single-gene trees identified one MOTU (MOTU B or I) as sister taxon to the rest of this clade.

ITS2 trees based on sequence-structure analyses (Fig. S13) retrieved the Antarctic clade and the subfamily Lamellariinae as monophyletic (B = 98% and 100%). Congruently with ITS2 tree based on primary sequence only, MOTU I was identified as the sister clade to all the other Antarctic specimens, but without significant support.

In the species tree (Fig. S14) MOTUs J, O, N and M formed a well-supported clade (PP = 0.96) while internal nodes were not supported.

3.3. Genetic connectivity

Haplotype networks of 8 Antarctic MOTUs and of *Lamellaria* sp. from New Zealand were obtained from Median-joining network analyses (Fig. 4). Networks of MOTUs distributed over multiple localities showed a lack of geographic structure in the haplotype distribution and some of them also a star-like pattern.

Isolation by Distance analyses were conducted on MOTUs C, G, J, L, M, N, O and *Lamellaria* sp. Only MOTUs O and J showed a significant (p-value: 0.02-0.04) albeit extremely weak (r=0.12-0.24) correlation between geographic and genetic distances (Fig. S15)

The sPCA carried out on the same MOTUs did not find any significant genetic spatial structure, either global or local (p-values>0.05; Fig. S16).

3.4. Protoconch morphology

Measurements of protoconchs are reported in Table S3. An abrupt transition between protoconch I and II, or between protoconch II and teleoconch (the adult shell), was detected for most but not for all the specimens. For the single-specimen MOTUs D and H it was not possible to take measurements because the protoconch was broken.

Two discrete protoconch morphologies were observed, here referred to as “type 1” and “type 2” (Fig. 5). All “type 1” protoconchs had marked longitudinal ribs on protoconch II while “type 2” can occasionally have ribs on protoconch II (33%) or granular sculptures on protoconch I (20%). “Type 1” had a smaller nucleus (54–300 µm) compared to “type 2” (383–875 µm). “Type 2” protoconchs showed a peculiar 'flattened and globular' protoconch I, with clear-cut protoconch I-II boundary, detectable in the vast majority of the specimens. “Type 1” protoconchs showed smaller nucleus maximum width and diameter of half and one protoconch whorl, smaller protoconch I and I+II diameters, but more whorls compared with “type 2”. However, only in 40% of “type 2” specimens an unquestionable protoconch-teleoconch transition was identified, and only in 27% of them the number of whorls of protoconch II was scored. Marked axial subsutural stripes were observed only in MNA 5375 (MOTU L), MNA 5337 (MOTU G), *Coriocella nigra* and the two *Lamellaria* sp. specimens.

The cluster analysis split specimens into two groups (B = 83%) (Fig. S17). One cluster comprised specimens from Antarctic MOTUs with wider distributions across both Weddell and Ross Seas (MOTU J, O, M and N) plus MOTU E that was represented by a single specimen from the tip of the Antarctic Peninsula, along with all non-Antarctic specimens. This group included specimens with “type 1” protoconchs (more whorls, smaller nucleus and smaller maximum diameter). The other cluster comprised Antarctic specimens collected only at the tip of the Antarctic Peninsula (MOTU A, B, C, F, G, I and L) or in the Ross Sea and

Dumont d'Urville area (MOTU K), with "type 2" protoconchs (fewer whorls and larger nucleus and maximum diameter). Two specimens showed slightly deviating morphology patterns. The MOTU I specimen (from the tip of the Antarctic Peninsula), clustered with "type 2" specimens, but did not present the characteristic protoconch I shape of "type 2" (flattened and globular) and detectable protoconch boundaries. Instead it showed longitudinal rib sculptures, present in all "type 1" protoconchs and in only another "type 2" specimen (MNA 5373 - MOTU L). However, protoconch morphometrics were in the range of "type 2" protoconchs. MOTU E (tip of the Antarctic Peninsula) clustered with "type 1" Antarctic MOTUs with wide geographic ranges and with non-Antarctic species; for this MOTU, protoconch I was not measured since a clear discontinuity mark was lacking.

We observed a high correlation between distribution range and average nucleus diameter ($r=-0.89$, $p=0.0006$) (Fig. 6).

Molecular barcoding assigned the two brood samples to MOTU J - *M. mollis* (NIWA 36790.1) and to MOTU O (NIWA 36893.2), respectively. In these broods, like in those described by Peck et al. (2006) as *M. mollis*, eggs were grouped in 'batches' of capsules and each brood was composed of several of them (5-8 in Peck et al., 2006, 12 in NIWA 36893.2 and 13 in NIWA 36790.1) (Fig. 7). Sample NIWA 36893.2 shared with that of Peck et al. (2006) broods separated by strips of ascidian cuticle and batches with a diameter smaller than that of the ascidian cuticle encircling them. In sample NIWA 36790.1 all batches were laid together near the surface of the ascidian body and were not separated by cuticle strips.

4. Discussion

4.1. Hidden diversity and phylogenetic patterns

The samples analysed in this study included velutinid species that can be ascribed to at least 8 different genera, corresponding to ~40% of those currently reported by WoRMS (Horton et al., 2018), and originating from four major biogeographical regions (i.e. the Southern Ocean, the North Atlantic, the Indo-Pacific and the North Pacific).

The Integrative Taxonomy approach was effective in assessing species delimitation. Nine MOTUs (A, B, D, E, F, G, H, I and L) were consistently identified by all methods employed. For the six remaining MOTUs (MOTUs C, J, K, M, N and O), the integration of the different criteria in our workflow allowed to converge to biologically plausible interspecific boundaries. The result was a final partition more robust than it could have been obtained by using a single-method approach.

For the Southern Ocean, 9 velutinid nominal species are currently accepted (Bouchet, 2012; Gofas, 2009; Marshall & Bouchet, 2016): two *Lamellariopsis* and seven *Marseniopsis.* Four of these nominal taxa, showing distinctive morphological features, matched one of the identified MOTUs (*M. mollis* = MOTU J, *M. conica* = MOTU N, *M. syowaensis* = MOTU M, and *L. turqueti* = MOTU L). Morphological descriptions of velutinid Antarctic species are mainly based upon characters, such as dorsal colour and shape, which we found to have a high intraspecific variability and extensive interspecific convergence. Therefore, it was not possible to confidently assign the remaining MOTUs to described taxa. Nevertheless, even after employing all available names for distinct MOTUs, there would still be at least six Antarctic MOTUs for which new names are necessary.

The two mitochondrial and two nuclear molecular markers used in this study allowed identifying phylogenetic relationships between Antarctic and non-Antarctic species but did

not fully resolve the relationships within the Antarctic clade. Overall, Antarctic velutinids emerged as a highly supported independent lineage (Fig. 3) that underwent a considerable diversification. We recovered this lineage as the sister to the rest of the family, congruently with a general trends observed in other mollusc families and other marine groups in Antarctica, that radiated as flocks in the Southern Ocean (e.g. Wilson et al., 2009; Barco et al., 2012; Chenuil et al., 2017). The distant relationships between Antarctic and New Zealand taxa are congruent with results obtained for other taxa: the benthic fauna of Antarctica has been shown to have a higher similarity with the fauna of South America than with that of New Zealand (Griffiths et al., 2009; Linse, 2002). This finding suggests searching the sister taxon of Antarctic velutinids among Southern American species.

In our analyses, the clade representing the subfamily Velutininae (Fig. 3) comprised genera traditionally ascribed to this subfamily (*Marsenina* Gray, 1850, *Onchidiopsis* Bergh, 1853 and *Velutina* Fleming, 1820), but not the Antarctic genera *Marseniopsis* and *Lamellariopsis.* Likewise, the genera ascribed to the subfamily Lamellariinae (*Coriocella* Blainville, 1824 and *Lamellaria* Montagu, 1816), with the exception of *Hainotis sharonae*, formed a clade. If confirmed for a wider taxonomic coverage, the partitioning obtained in the present study suggests that a new subfamily will be necessary to accommodate the genera *Marseniopsis* and *Lamellariopsis*.

The specimen CASIZ 181317 from Monterey, California (USA) was morphologically identified as *Hainotis sharonae.* The assayed specimen, however, had no relationship with the *Marseniopsis-Lamellariopsis* clade, and its placement as an independent lineage is also worth of further investigation.

In contrast with the good phylogenetic resolution at the subfamily level, the relationships among the Antarctic species were not completely resolved despite the use of several methods,

455 suggesting that the lack of phylogenetic resolution might be related to the speciation pattern
456 behind the diversification of the Antarctic clade. Antarctic velutinids, in fact, might represent
457 a flock, i.e. the result of a rapid radiation which is notoriously difficult to resolve in
458 phylogenetic analyses (e.g. Cummins & McInerney, 2011). Phylogenetic trees based on
459 combined datasets revealed some relationships between species. *M. mollis* (MOTU J), *M.*
460 *conica* (N), *M. syowaensis* (M) and MOTU O represented a monophyletic group. MOTUs A,
461 B, C and E also made a monophyletic group. MOTU B or MOTU I were proposed as the
462 sister taxon to the rest of Antarctic species in distinct analyses, but further study would be
463 necessary to validate either hypothesis.

464 Colour and shape patterns of Antarctic specimens were not generally congruent with their
465 assignation to MOTUs. Except for some species showing unique combinations of colours and
466 shape (i.e. *M. syowaensis*, *M. conica,* MOTU B, E and F), the rest of MOTUs showed
467 overlapping morphologies among different taxa (e.g. MOTUs C, G and *M. mollis*) as well as a
468 marked intraspecific variability (e.g. *M. mollis* and MOTU O). Therefore, the use of external
469 morphology alone for species identification would mostly lead to incorrect assignations.

470 Colour variation patterns in Velutinidae can be related to host specificity: velutinid
471 morphology has been often shown to be cryptic, mimicking the ascidians on which they live
472 and lay eggs, suggesting that colours may originate from ascidian pigments incorporated
473 during feeding (Dias & Delboni, 2008; Lambert, 1980). Such a trophic homochromy, well
474 known in the related gastropod families Triviidae and Ovulidae (Liltved, 1989; Schiaparelli et
475 al., 2005), could ascribe the intraspecific colour variation to different sets of exploited
476 ascidian species. Interestingly, the presence of intraspecific colour variability in monophagous
477 species may parallel an intraspecific colour variation in the ascidian host. For example *M.*
478 *mollis* feeds on *Cnemidocarpa verrucosa* (Lesson, 1830) (Peck et al., 2006) which is highly

479 variable in shape and colour (Tatian et al., 1998). Noteworthy, the spectre of colour variability
480 reported for Antarctic ascidians (transparent, yellow, orange, red, brown and black) (Tatian et
481 al., 1998) completely overlaps the colour range of Antarctic velutinids.
482

483 *4.2. Planktotrophic larval development and high genetic connectivity*

484 All velutinid protoconchs studied in this work, compared with others of the same family,
485 strongly indicate a planktotrophic development, sharing a short protoconch I (max 0.84
486 whorls) and the presence of a protoconch II (up to 1.96 whorls) (Behrens et al., 2014; Gulbin
487 & Golikov, 2000). The large nucleus diameter (up to 875 µm) and protoconch I maximum
488 diameter (up to 1333 µm) of "type 2" protoconchs were still compatible with a planktotrophic
489 development. Moreover, the limacosphaera muscular mantle (deutoconcha) is able to
490 compensate the loss of buoyancy due to larger and/or heavier embryonic and larval shells, as
491 those detected in "type 2" protoconchs (Bandel et al., 1993).

492 Our work clearly captured a general larval development trend in Southern Ocean
493 velutinids. The two groups in which Antarctic specimens were divided showed two distinct
494 patterns. "Type 1" group, with smaller nucleus diameter (indicating smaller amount of yolk),
495 and higher protoconch I and I+II number of whorls (suggestive of long planktonic larval life),
496 included all the assayed non-Antarctic species (5 genera) from various biogeographical
497 regions, plus all Antarctic species with a wide geographic range and one species (MOTU E)
498 represented by a single specimen. "Type 2" group comprised exclusively Antarctic species,
499 with geographic ranges restricted either to the tip of the Antarctic Peninsula or to the Ross
500 Sea. These species showed bigger protoconch nucleus and fewer protoconch whorls
501 (indicating both a greater amount of yolk and a purportedly shorter planktonic larval life).

502 Despite the lack of detailed data about the ecology of these species, some hypotheses can be
503 formulated to explain their developmental strategy.

504 The general trade-off between two different larval development strategies is well known
505 among marine benthic invertebrates: smaller eggs, planktotrophic larvae and high female
506 fertility *v.* larger eggs, lecithotrophic larvae and lower female fertility (Thorson, 1950; Todd
507 & Doyle, 1981). The larval development dichotomy has been also explained in a comparative
508 sense (Pianka, 1970). It can be visualised as an r-K continuum along which organisms with
509 lecitotrophic larvae are considered as K-strategists (characterized by slow growth, deferred
510 maturity, greater longevity, iteroparity, low fecundity, large yolky eggs), and those with
511 planktotrophic larvae as r-strategists (characterized by fast growth, shorter longevity,
512 semelparity, high fecundity, small eggs) (Pianka, 1970). “Type 2” protoconch species (with
513 larger nucleus and bigger larvae) may therefore be suggestive of a trend of some Antarctic
514 velutinid lineages to rely more on yolk as energy resource for their larvae. In this case, the
515 group may have been positively selected because of the advantages of being closer to a K-
516 strategy in this environment, due to i) the short length of the summer phytoplankton bloom,
517 which may not provide the necessary amount of energy/food for the larvae, and ii) a possibly
518 wide and homogeneous distribution of their ascidian preys.

519 Conversely, “type 1” protoconch species (r-strategists relying on active larval feeding)
520 probably represent the ancestral development condition of the family, shared with all non-
521 Antarctic species considered in this dataset, in agreement with literature data describing this
522 family as possessing long lasting planktotrophic larvae (Beesley et al., 1998; Gulbin, 2005).
523 The retention of this ancestral condition in some Antarctic velutinid species might be due to a
524 more scattered distribution of their ascidian preys, although present data do not allow
525 verifying this hypothesis. Moreover, the inclusion in this group of the two largest Antarctic

526 velutinid species (*M. mollis* and *M. syowaensis*: attaining 7 and 11.5 cm respectively)
527 (Numanami & Okutani, 1991) may result from a positive selection on size imposed by
528 planktotrophy (since bigger size allows to produce more offspring), rather than represent a
529 case of polar gigantism (Chapelle & Peck, 1999), a debated topic despite some evidences in
530 Mollusca and other taxonomic groups (Moran & Woods, 2012).

531 The intuitive correlation between pelagic larval duration (PLD) and propagules dispersion
532 distance has already been demonstrated (Shanks, 2009), implying that PLD is a good
533 indicator of dispersal potential with a crucial role played by larval behaviour in dispersal
534 ability. Protoconch number of whorls indicated that "type 1" species have longer PLD (and
535 thus higher dispersal capacity) than "type 2". Lester et al. (2007), working on a large-scale
536 dataset of several marine taxa from tropical and temperate ecosystems worldwide, showed
537 that the dispersal ability of a species is not always the principal determinant of the range size.
538 However, at a smaller scale (e.g. within regions), a positive correlation of dispersal ability and
539 range size has been demonstrated in many cases, for example in Indo-Pacific molluscs
540 (Perron & Kohn, 1985) and tropical reef fishes (Lester & Ruttenberg, 2005).

541 Our data on Antarctic velutinids showed an inverse correlation between geographic range
542 and nucleus diameter (Fig. 6), suggestive of a relation between longer PLD (as inferred from
543 the nucleus diameter) and wider geographic ranges, although other ecological factors, such as
544 distribution of the ascidian hosts, may have also played an important role in shaping species'
545 ranges. The four most abundant Antarctic species (*M. mollis*, *M. conica*, *M. syowaensis* and
546 MOTU O) are also those with potentially longer PLD. This is congruent with the notion that
547 shallow waters in Antarctica are dominated by a large number of individuals belonging to few
548 species with planktotrophic development (Poulin et al., 2002). Considering the planktotrophic
549 larval development described for the family Velutinidae (Lebour, 1937; Hain & Arnaud,

1992; Bandel et al., 1993; Beesley et al., 1998; Peck et al., 2006) and our inference from protoconch morphology of long PLD, a high genetic connectivity was expected in the Antarctic species (Kinlan & Gaines, 2003). In fact, our analyses rejected any isolation-by-distance patterns and genetic-spatial structures for the Antarctic *M. mollis*, *M. conica*, *M. syowaensis* and MOTU C, G, L, O, and for *Lamellaria* sp. from New Zealand. This was also confirmed by the star-like shape of haplotype networks, with several instances of haplotypes shared by specimens collected at remarkably distant sites, including Weddell-Ross Sea sharing (~4000 km), and Georg Von Neumayer-Dumont d'Urville sharing (~7000 km). Genetic connectivity analyses did not show a significant difference between "type 1" and "type 2" MOTUs, although this result may have been biased by the restricted distribution of all "type 2" specimens that were all collected in a small area (~180 km wide) at the tip of the Antarctic Peninsula.

Several additional patterns emerged through the integration of phylogenetic, protoconch morphology and distribution data.

MOTU I was found only at the tip of the Antarctic Peninsula and may represent the sister taxon to the rest of the Antarctic species (a hypothesis to be tested on larger dataset). This lineage showed a protoconch similar to "type 2" but with some unique features that may characterise a third type, if confirmed with more specimens.

Among the other Antarctic species, one monophyletic group of species (*M. mollis*, *M. conica*, *M. syowaensis* and MOTU O) retained what can probably be considered as the ancestral protoconch state (type 1) corresponding to longer PLD, and this may have allowed them to colonize distant areas and maintain wider ranges. This group includes the most common (*M. mollis*) and the largest (*M. syowaensis*) species. MOTU E (type 1) shared a common ancestor with four other MOTUs. The three of them with a known protoconch

morphology (MOTUs A, B and C) produce eggs with larger amount of yolk (type 2) and were collected at the tip of the Antarctic Peninsula. The other six MOTUs produce eggs with larger amount of yolk and are restricted either to the tip of the Antarctic Peninsula (F, G, H, L) or to the Ross Sea (D and K). The switch to the production of this type of eggs in several lineages may thus represent a trend of Antarctic velutinids towards a larval development relying more on yolk as energy source (and probably yielding a shorter PLD), considered advantageous in the Southern Ocean, where the phytoplankton bloom is strongly seasonal and short in time (Picken, 1980).

Flock-like radiations have occurred repeatedly in the Southern Ocean, where long-term isolation and unique environmental conditions played a major role in prompting these events. Congruently, Antarctic velutinids emerged as an independent lineage from the rest of the family and underwent a considerable radiation. What distinguishes them from the majority of Antarctic molluscs is their ability to maintain a planktotrophic larva in an ecosystem that usually counter-selects this developmental mode. However, several Antarctic velutinids produce eggs with a larger amount of yolk, larvae with shorter PLD and have smaller geographic ranges. Therefore, in this primarily planktotrophic family, a trend emerged within the Antarctic radiation towards a shortening of the actively feeding planktonic larval phase, in perfect agreement with Thorson's rule.

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625

626 **Appendix A. Supplementary material**

627 Supplementary data can be found online at XXXXXX.

628 Genetic sequences are deposited in GenBank (accession numbers: MK047747 - MK048104).

629

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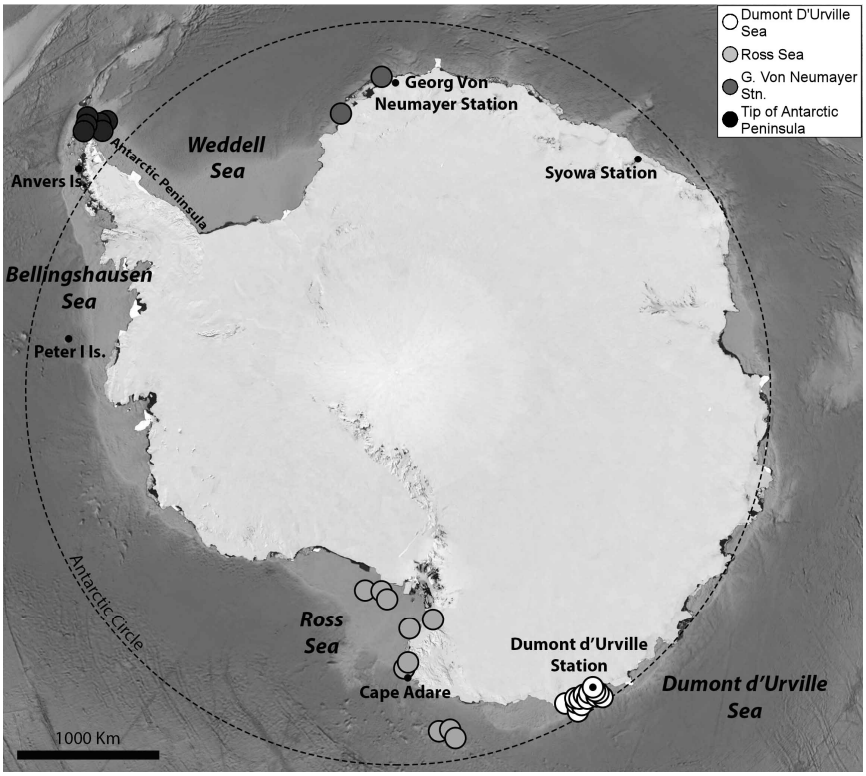


Fig. 1 Map of the Antarctic sample collecting localities.

Table 1 Gene fragments employed, primer pairs used for amplification with references and substitution models used in phylogenetic analysis. PCR conditions: initial denaturation (94°C/4'); 35 cycles of denaturation (94°C/30''), annealing (48-51°C for COI, 52°C for 16S, 58-60°C for 28S and ITS2/40'') and extension (94°C/60''); final extension (72°C/10'). N: number of sequences in the single-gene alignment (in parentheses those newly produced in this study); bp: length of the trimmed alignment.

Gene fragment	Size	Primer	Reference	N	bp	Substitution model
Cytochrome oxidase I (COI)	658 bp	LCO1490 HCO2198	Folmer et al. 1994	182 (174)	612	COI-I: GTR+I+G COI-II: F81+G COI-III: GTR+I+G
16S rDNA	~700 bp	16SA CGLeuR 16SH	Palumbi 1996 Hayashi 2003 Espiritu et al. 2001	70 (65)	761	GTR+I+G
28S rDNA	~700 bp	C1 D2	Jovelin & Justine 2001	66 (66)	692	GTR+G
Second internal transcribed spacer (ITS2)	~450 bp	ITS-3d ITS-4r	Oliverio & Mariottini 2001	53 (53)	486	HKY+G

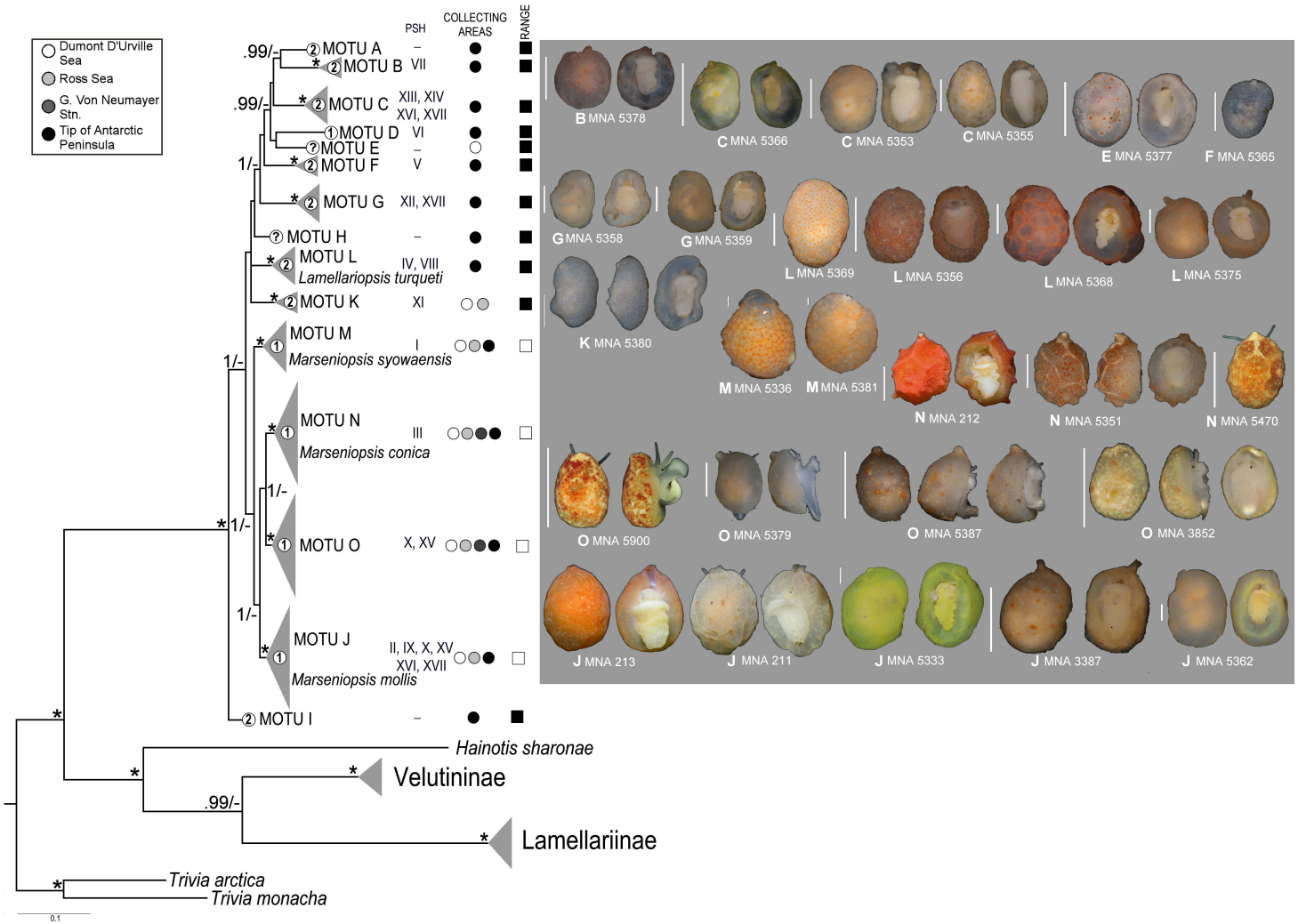


Fig. 2 Bayesian tree based on the ALL combined dataset (COI, 16S rDNA, 28S rDNA and ITS2) with photos from alive animals. Numbers at nodes indicate only high support values (PP≥.98; B ≥90). Asterisks indicate highly supported nodes in both ML and BA analysis. Numbers inside circles indicate protoconch type (1 or 2) or missing information (?). For each MOTU: roman numbers indicate Preliminary Specie Hypothesis (PSH), circles indicate collecting areas and squares indicate the distribution range (black=restricted, white=wide).

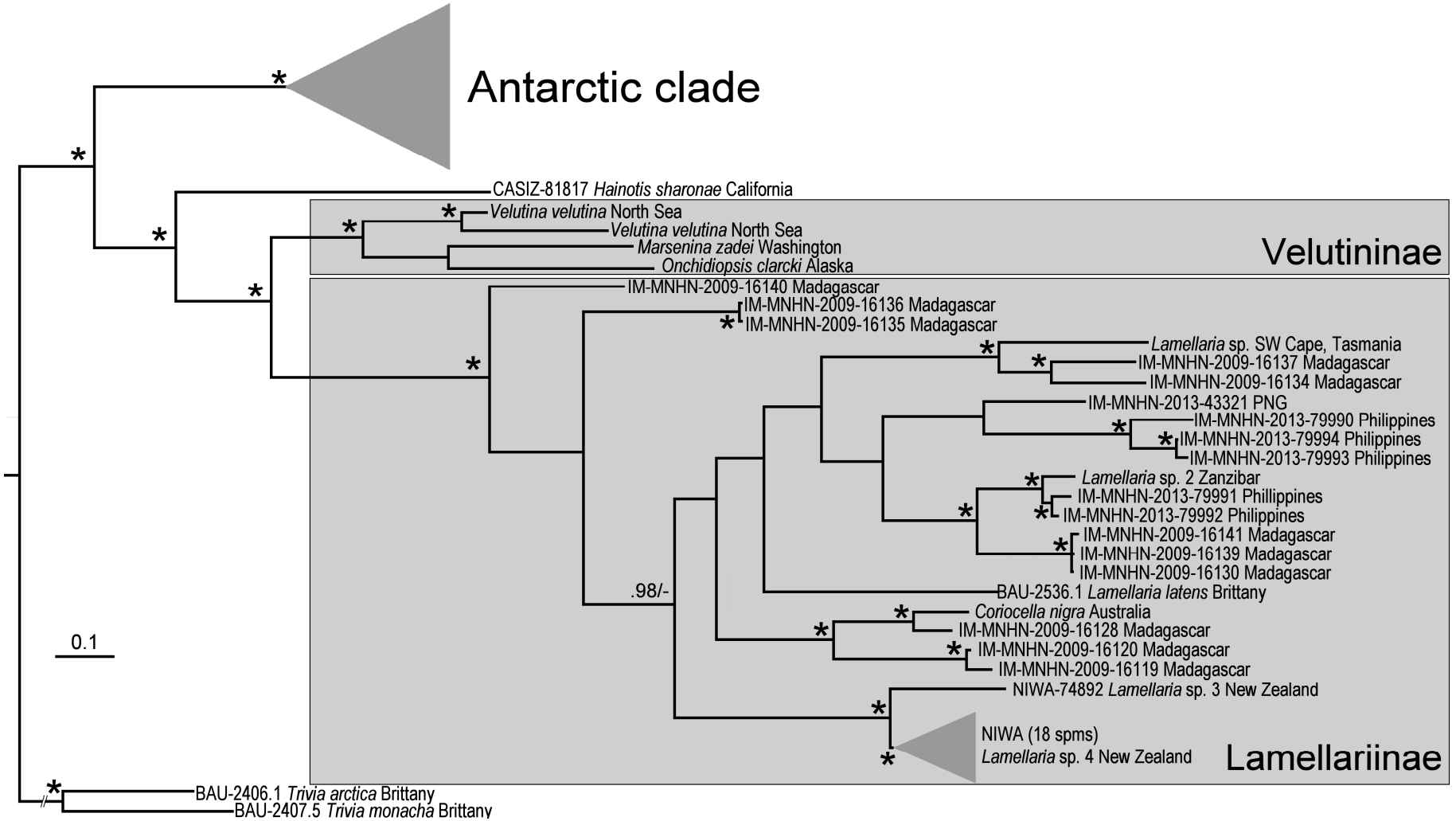


Fig. 3 Bayesian tree based on the ALL combined dataset (COI, 16S rDNA, 28S rDNA and ITS2. Numbers at nodes indicate only high support values (PP≥.98; B ≥90). Asterisks indicate highly supported nodes in both ML and BA analysis.

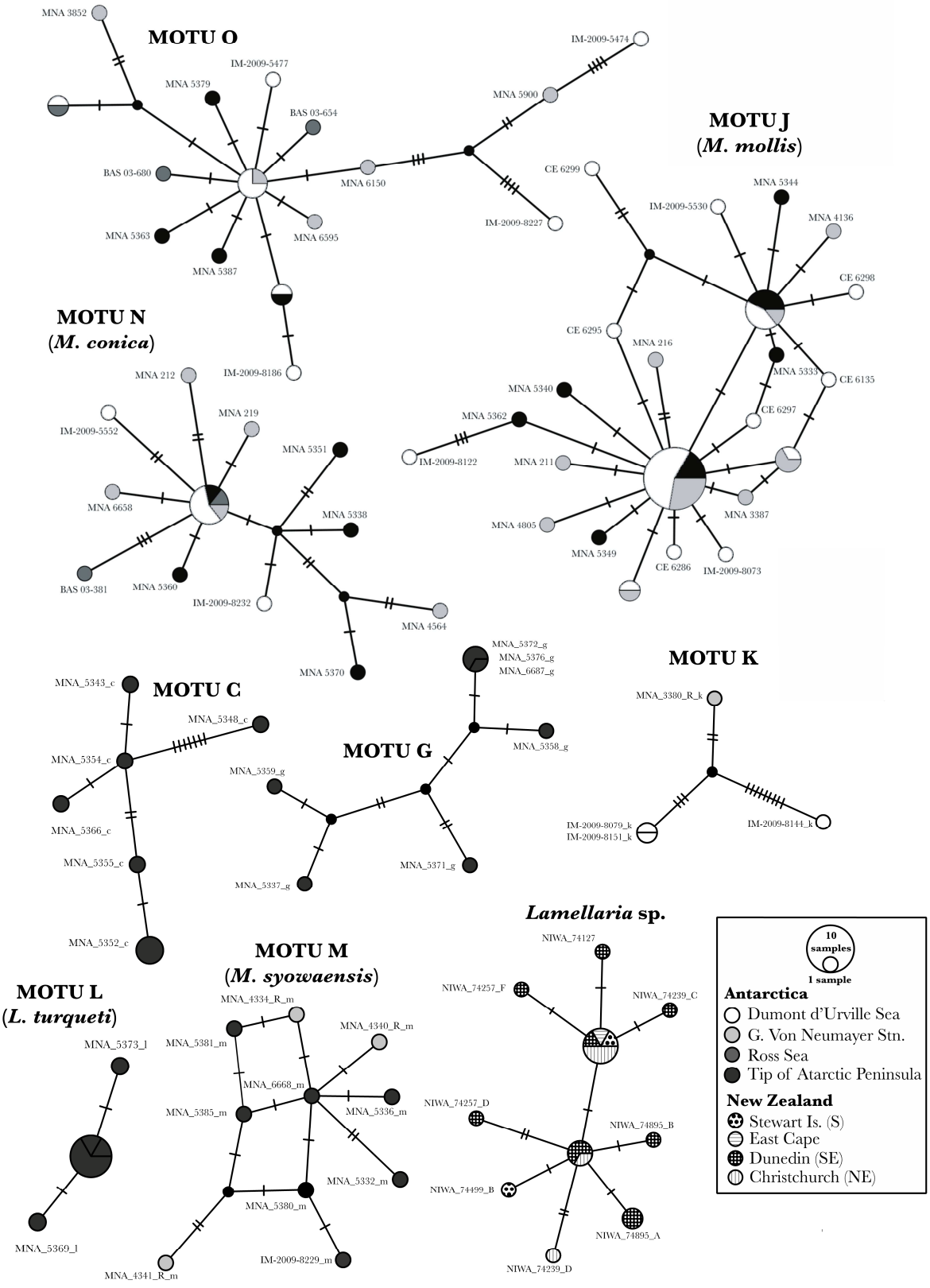


Fig. 4 Median joining networks of COI sequences of MOTUs O, N (*M. conica*), J (*M. mollis*), C, G, K, L (*L. turqueti*), M (*M. syowaensis*) and *Lamellaria* sp.

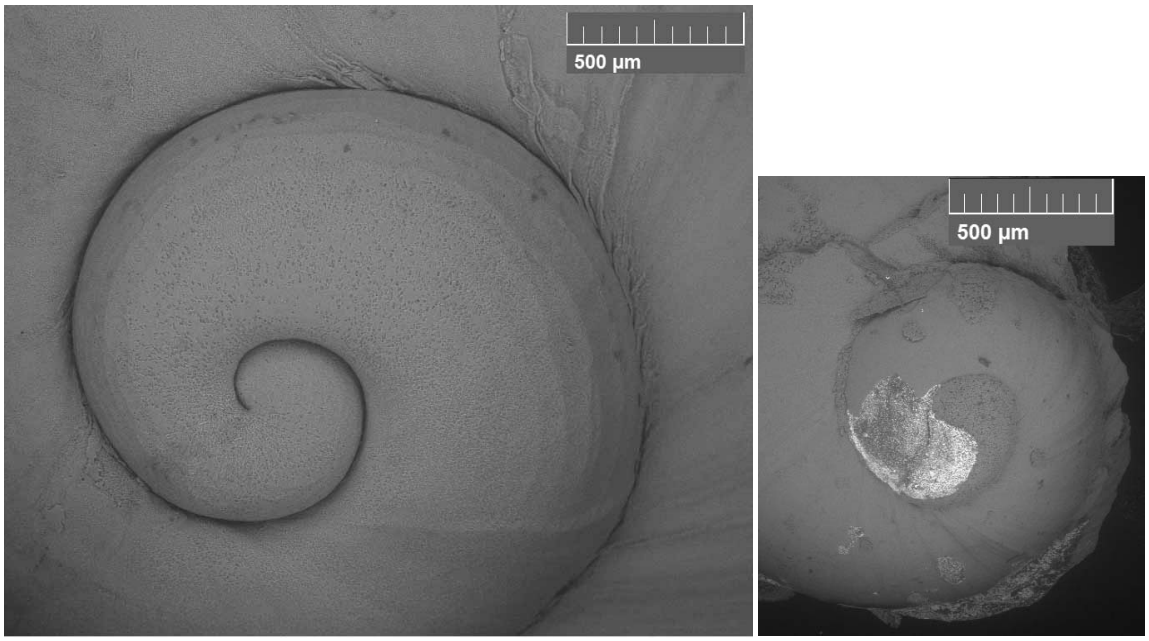


Fig. 5 SEM photographs of protoconch type 1 (right) and 2 (left). In “type 2” visible longitudinal ribs and end of protoconch II. In “type 2” visible peculiar 'flattened and globular' protoconch I shape and end of protoconch II.

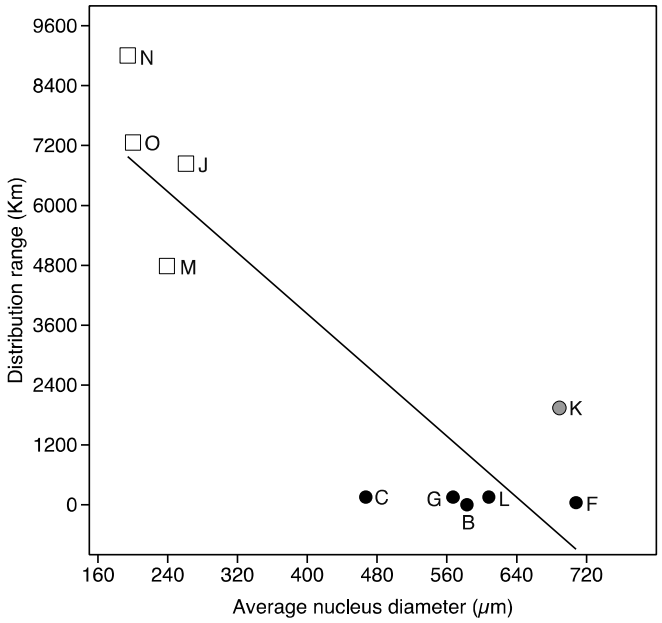


Fig. 6 Plot of average nucleus diameter (µm) vs MOTU distribution range (Km). Colours indicate MOTU sampling localities: white=wide distribution, black=only at the tip of the Antarctic Peninsula, grey=only in the Ross Sea. Shapes indicate MOTU protoconch type: square=type 1, circle=type 2.

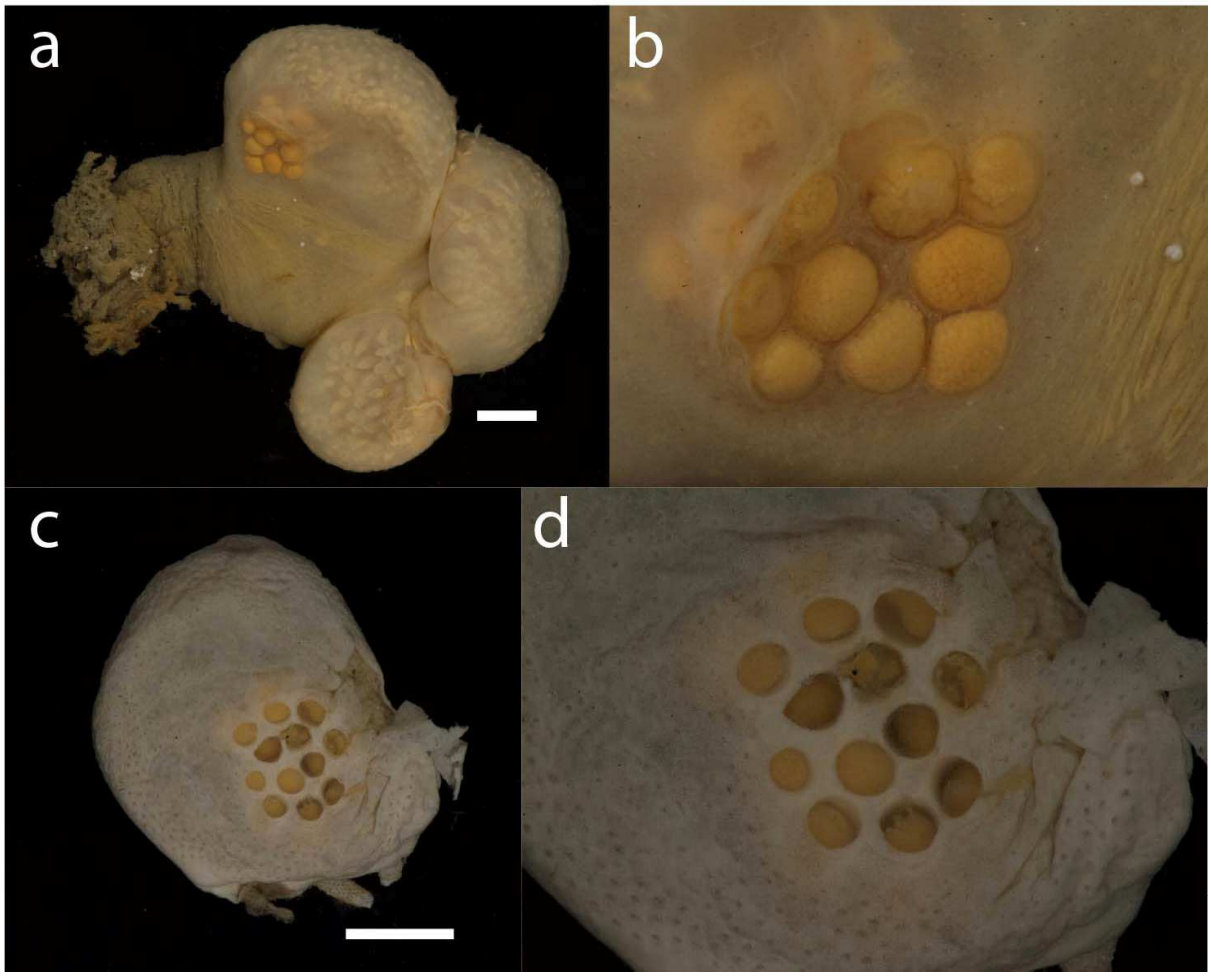
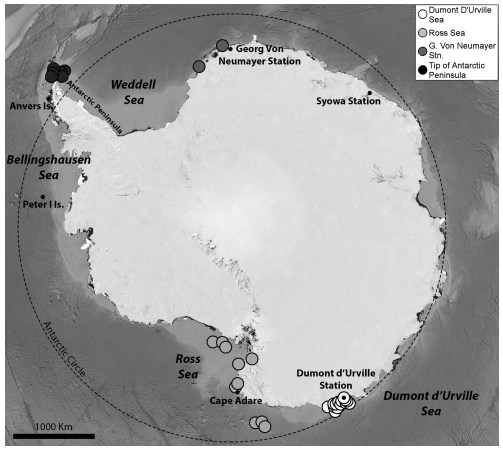


Fig. 7 Velutinid broods on ascidian from the Ross Sea. NIWA 36893.2 - MOTU O (a-b); NIWA 36790.1 - MOTU P - *M. mollis* (c-d). Scale bar = 1 cm.


Dumont D'Urville Sea
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Georg Von Neumayer Station
Weddell Sea
Syowa Station
Anvers Is.
Antarctic Peninsula
Bellingshausen Sea
Peter I Is.
Antarctic Circle
Ross Sea
Dumont d'Urville Station
Cape Adare
Dumont d'Urville Sea
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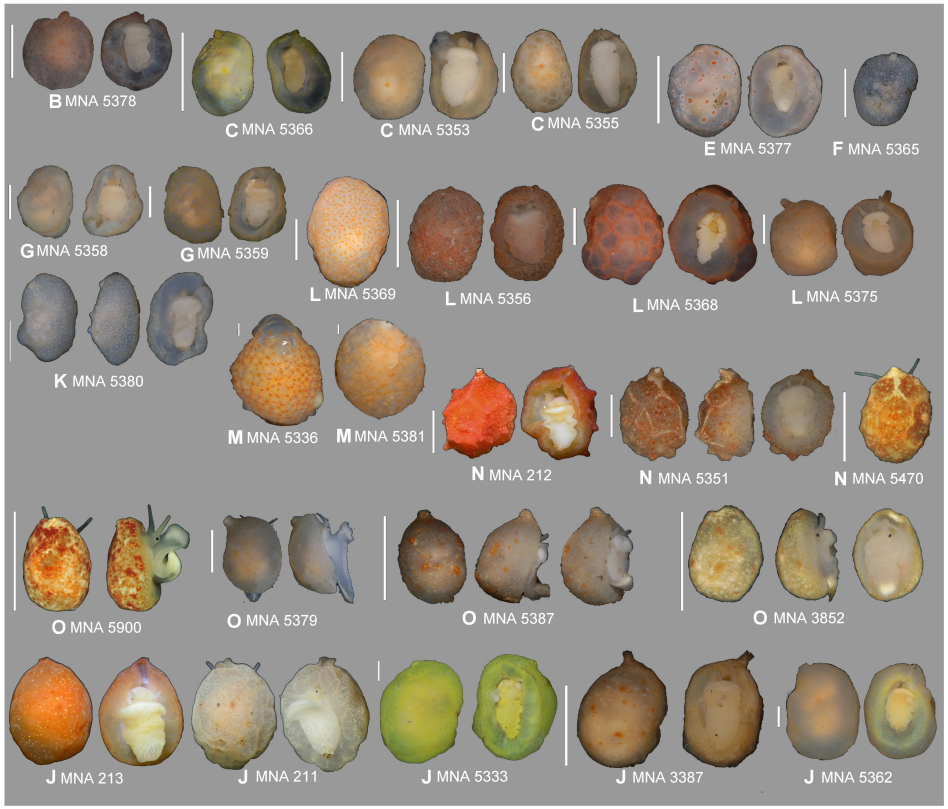
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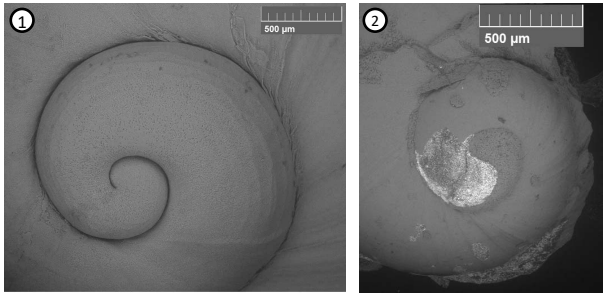
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