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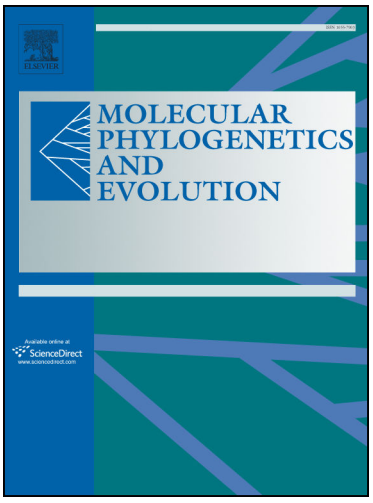

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Contrasting biogeographical patterns in *Margarella* (Gastropoda: Calliostomatidae: Margarellinae) across the Antarctic Polar Front

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Abstract

Members of the trochoidean genus *Margarella* (Calliostomatidae) are broadly distributed across Antarctic and sub-Antarctic ecosystems. Here we used novel mitochondrial and nuclear gene sequences to clarify species boundaries and phylogenetic relationships among seven nominal species distributed on either side of the Antarctic Polar Front (APF). Molecular reconstructions and species-delimitation analyses recognized only four species: *M. antarctica* (from the shores of the Antarctic Peninsula), *M. achilles* (endemic to South Georgia), *M. steineni* (South Georgia and Crozet Island) and the morphologically variable *M. violacea* (= *M. expansa*, *M. porcellana* and *M. pruinosa*), with populations in southern South America, the Falkland/Malvinas, Crozet and Kerguelen Islands. *Margarella violacea* and *M. achilles* are sister species, closely related to *M. steineni*, with *M. antarctica* sister to all these. This taxonomy reflects contrasting biogeographic patterns on either side of the APF in the Southern Ocean. Populations of *Margarella* north of the APF (*M. violacea*) showed significant genetic variation but with many shared haplotypes between geographically distant populations. By contrast, populations south of the APF (*M. antarctica*, *M. steineni* and *M. achilles*) exhibited fewer haplotypes and comprised three distinct species, each occurring across a separate geographical range. We hypothesize that the biogeographical differences may be the consequence of the presence north of the APF of buoyant kelps – potential long-distance dispersal vectors for these vetigastropods with benthic-protected development – and their near-absence to the south. Finally, we suggest that the low levels of genetic diversity within higher-latitude *Margarella* reflect the impact of Quaternary glacial cycles that exterminated local populations during their maxima.

Keywords Southern Ocean; Antarctic Polar Front, long-distance dispersal, benthic-protected development, rafting, *Margarella*.

Short running title: Biogeographical patterns in *Margarella* across the Antarctic Polar Front

1. Introduction

The genus *Margarella* Thiele, 1893 includes Antarctic and sub-Antarctic small to medium-sized vetigastropods that are abundant on hard substrate inter- and shallow sub-tidal shores (Zelaya, 2004). Near-shore species of the genus are frequently found in ecosystems dominated by macroalgae, on which they are commonly found grazing (Adami and Gordillo, 1999; Rosenfeld et al., 2011, 2017; Amsler et al., 2015). However, some species are restricted to deeper waters between 40 – 1080 m (Hain, 1990).

The taxonomy of *Margarella* has had a confused history and even currently, specific identifications and relationships among Magellanic and Antarctic species are poorly understood (Powell 1951; Forcelli 2000; Zelaya, 2004; Williams et al. 2010; Williams, 2012). Several species were originally described in the genus *Margarita* Leach, 1819 (not Leach, 1814) (= *Margarites* Gray, 1847), a group restricted to the Northern Hemisphere that is now placed in a separate family, Margaritidae Thiele, 1924. Zelaya (2004), in a detailed morphological revision of the group, confirmed that Antarctic, South American and South Georgian species, previously identified as *Margarites*, should be placed in *Margarella*. More recently, Williams et al. (2010) used multilocus phylogenies to show that *Margarella antarctica* (Strebel, 1908) was a calliostomatid. Moreover, Williams (2012) confirmed this placement for four further species and, in recognition; Williams (2013) established the new subfamily Margarellinae. Conversely, several species from New Zealand, previously assigned to *Margarella* have proved to belong to the genus *Cantharidus* Montfort, 1810 (Trochidae: Cantharidinae) (Williams et al., 2010; Willams, 2012; Donald and Spencer, 2016), within a distinct clade, the subgenus *Pseudomargarella* Donald and Spencer, 2016.

The World Register of Marine Species database (http://www.marinespecies.org) records 18 *Margarella* species distributed in Antarctic and sub-Antarctic areas. Five – *M. antarctica*, *M. refulgens* (E.A. Smith, 1907) *M. whiteana* Linse, 2002, *M. gunnerusensis* Numanami, 1996 and *M. crebrilirulata* (E.A. Smith, 1907) – occur around the Antarctic Peninsula, the Ross Sea and East Antarctica. Three species

are recognized along southern South America – *M. expansa* (Sowerby, 1838), *M. violacea* (King, 1832) and *M. pruinosa* (Rochebrune and Mabille, 1885) – and their shells differ primarily in external coloration and shape (Powell 1951; Forcelli 2000). The species *M. pruinosa* has a restricted distribution, from the Strait of Magellan to the Santa Cruz province in Argentina. Both, *M. expansa* and *M. violacea* are abundant in the Magellan province and are also found in the Falkland/Malvinas Islands where they overlap with the endemic *M. wacei* (Melvill and Standen, 1918). *Margarella expansa* also occurs eastward to reach geographically distant sub-Antarctic oceanic areas including Crozet and Kerguelen Islands (Cantera and Arnaud, 1984; Troncoso et al., 2001), but *M. violacea* apparently does not, the record of Troncoso et al. (2001) from the Kerguelen Islands being erroneous (see Rosenfeld et al. 2011). Although there are reports of *M. expansa* from several locations south of the Antarctic Polar Front (namely, South Georgia, the Antarctic Peninsula and Cape Adare, West Antarctica), Rosenfeld et al. (2011) reviewed these records (all of which are over 100 years old) and concluded that they were based on various misidentifications. Dell (1990) and Zelaya (2004) had reached the same conclusion about specific cases and, in further support, we note that there are no recent observations of *M. expansa* from any of these places nor did our sampling on the Antarctic Peninsula and South Georgia detect it either. The species *M. porcellana* Powell, 1951 has been recorded at Marion Island and the Kerguelen Islands (Cantera and Arnaud, 1984; Troncoso et al., 2001). South Georgia has at least six apparently endemic species – *M. achilles* (Strebel, 1908), *M. steineni* (Strebel, 1905), *M. jason* Powell, 1951, *M. tropidophoroides* (Strebel, 1908), *M. obsoleta* Powell, 1951, and *M. subantarctica* (Strebel, 1908). Finally, *M. bouvetia* Powell, 1951 and *M. macquariensis* Hedley, 1916 are narrow endemics from Bouvet and Macquarie Islands, respectively. Nevertheless, boundaries between these nominal species are still not clear. Moreover, the taxonomic separation of South American species is particularly difficult because, besides coloration, the shell-shape differences are subtle (Powell, 1951; Zelaya, 2004). Indeed, levels of intraspecific variation in some populations are sometimes greater than those reported among species (Powell, 1951; Forcelli, 2000).

Like many Southern Ocean invertebrates, *Margarella* species lack a free-swimming dispersive stage and exhibits protected benthic development where free-crawling juveniles emerge from mature egg capsules (Picken, 1979; Zelaya, 2004; Rosenfeld et al., 2011). This key life-history trait seems to have played a significant role in the biogeography of several marine groups by enhancing their respective speciation potential (Poulin and Féral, 1996, Pearse et al., 2012; Thatje, 2012). At the same time, the ecological association of some species with buoyant macroalgae gives them a high potential for rafting, which may explain their wide distributions across different sub-Antarctic provinces, in spite of their low dispersive potential (Helmuth et al., 1994; Nikula et al., 2010; Cumming et al., 2014; Moon et al., 2017; González-Wevar et al., 2018). Other species, lacking such associations are more narrowly distributed and indeed, molecular comparisons might detect a series of unrecognized cryptic species across the Southern Ocean, as has been the case in other groups of marine invertebrates (Hunter and Halanych, 2008; Leese et al., 2008; Wilson et al., 2009; Janosik et al., 2011; González-Wevar et al., 2019).

Here, we present a molecular-genetic study of seven nominal species collected at high-latitude *Margarella* populations from the Antarctic Peninsula, southern South America, the Falkland/Malvinas Islands, South Georgia, Crozet and Kerguelen Islands. We carried out phylogenetic reconstructions based on mitochondrial and nuclear markers, species delimitation analyses, mitochondrial divergence-time estimations, as well as population-based analyses. Through the integration of these methodologies and the first molecular investigation of the type species (*M. expansa*), we aim to further understand evolutionary relationships within *Margarella* in the Southern Ocean. Moreover, we elucidate the distribution of lineages of this higher latitude calliostomatid genus, as well as the taxonomic status of the various populations. Understanding these specific issues in *Margarella* provides new insights about the biogeography and evolution of the near-shore marine benthic biota in this important area of the world.

2. Material and Methods

2.1. Sample collection, DNA preparation and sequence editing

Margarella individuals were collected from inter- and sub-tidal ecosystems at different localities in the Southern Ocean including the southern portion of the Magellan Province (MP) in South America, the Antarctic Peninsula (AP), South Georgia (SG), the Falkland/Malvinas Islands (FI) Crozet Island (CZ), and Kerguelen Islands (KI) (Fig. 1; Table S1). Specimens were identified following Powell (1951), Troncoso et al. (2001), Zelaya (2004), and Rosenfeld et al. (2011).

Whole specimens were fixed in 95% ethanol and DNA was extracted from the head and foot using standard salting-out methodology described by Aljanabi and Martínez (1997) and/or the QIAGEN DNEasy Blood & Tissue kit (QIAGEN Inc.). Universal primers were used to amplify a partial fragment of the mitochondrial cytochrome-b gene (Cytb) (Merrit et al., 1998) and the nuclear rRNA gene 28S (Littlewood et al., 2000). The typically-used 'barcoding' gene of COI does not amplify in *Margarella* (see Williams et al., 2010; Williams, 2012, this study) and therefore could not be used. Forward and reverse sequences for each marker were assembled independently and edited using GENEIOUS R8 (http://www.geneious.com). Alignments and base composition of nucleotide sequences were analyzed for each marker independently in MEGA 7.0 (Kumar et al., 2016) using MUSCLE (Edgar, 2004) with standard settings. Mitochondrial codon usage was determined using the Effective Number of Codons (ENC) in DnaSP v5 (Librado and Rozas, 2009) following Wright (1990). New Antarctic and sub-Antarctic *Margarella* sequences will be deposited at GenBank under the following Accession Numbers: Cytb (MT763288 – MT763345) and 28S rRNA (MT764782 – MT764786). Similarly, molecular information concerning museological material from the Western Australian Museum (WAM) will also be available in Table S2.

2.2 Phylogenetic reconstructions, species delimitation analyses and divergence time estimates

Mitochondrial (Cytb) phylogenetic reconstructions included between 3 and 15 sequences of each nominal *Margarella* species, together with new sequences of South American calliostomatid genera *Calliostoma*, *Photinula*, and *Photinastoma*, as well as sequences of the tegulid outgroups *Tegula lividomaculata* and *T. brunnea*. In the case of the broadly distributed species such as *M. violacea* and *M. expansa*, we included sequences from individuals collected from different sub-Antarctic provinces (i.e. MP, FI, CZ and KI).

Nuclear (28S rRNA) phylogenetic reconstructions used new and previously published (Williams, 2012) *Margarella* sequences, notably *M. refulgens*, *M. biconica* and *M. crebrilirulata* all from the Weddell Sea and *M.* sp. from Bouvet Island. For comparative purposes we included sequences of different vetigastropods groups and outgroups following Williams et al. (2010) and Williams (2012). Our phylogenetic estimation used maximum parsimony (MP), maximum likelihood (ML) and Bayesian analyses (BA), performed in MEGA, PHYML (Guindon and Gascuel, 2003) and MrBayes v.3.1.2 (Huelsenbeck and Ronquist, 2001), respectively. The program JModelTest v.2.1.10 (Darriba et al., 2012) was used to select the HKY + I + G (Cytb) and the TN93 (28S rRNA) as the optimal substitution models for the ML and BA analyses. Nodal support for MP and ML analyses was inferred using non-parametric bootstrap (BS) with 1,000 pseudo-replicates (Felsenstein, 1981). Bayesian-inference posterior probabilities (BPP) were estimated using the Metropolis coupled Markov-chain Monte-Carlo algorithm (MCMC) running four chains for 50 x 10^6 generations, with trees sampled every 1,000 generations. Stationarity of the analyses was inferred when the average standard deviation of split frequencies was less than 0.01 (Huelsenbeck and Ronquist, 2001). The initial 10% of the parameter values were discarded (burn-in) and posterior probabilities were estimated as the fraction of trees showing a particular clade. Finally, posterior probability density was summarized as a maximum clade credibility tree using TreeAnnotator v.1.6.1

(http://beast.bio.ed.ac.uk/TreeAnnotator) and visualized using FigTree v.1.4.3 (http://tree.bio.ed.ac.uk/software/figtree).

Two different methods were used for the delimitation of species within *Margarella*: the Automatic Barcoding Gap Discovery (ABGD) (Puillandre et al., 2012) and the Generalized Mixed Yule Coalescent (GMYC) (Pons et al., 2006). The ABGD method employs genetic distances to detect barcoding gaps between candidate species based on genetic distance values, which can be observed whenever the divergence among organisms belonging to the same species is smaller than divergence among organisms from different species (Puillandre et al., 2012). ABGD analysis was performed on the online web-server (http://www.abi.snv.jussieu.fr/public/abgd) and was run using the default settings (Pmin = 0.001, Pmax = 0.1, Steps = 10, X (relative gap width) = 1.5, Nb bins = 20). The GMYC method was implemented in the R environment (R, version 2.4.1) (Ihaka and Gentleman, 1996) and attempts to detect the transition in the tree where the branching pattern switches from being attributed to speciation (one lineage per species) to when it can be attributed to the intra-species coalescent process (Pons et al. 2006).

A relaxed molecular clock analysis was implemented for mtDNA sequences using an uncorrelated-lognormal (ucln) model of molecular evolutionary rate heterogeneity and the HKY + I + G substitution model implemented in BEAST v.1.7.5 (Drummond and Rambaut, 2007; Drummond et al., 2012). A birth-death speciation prior was used for branching rates in the phylogeny. Four chains were run twice for 50 x 10^6 generations, and trees were sampled every 1,000 generations. Given an absence of suitable *Margarella* fossils to calibrate our analysis, we used a conservative phylogenetic mtDNA mutation rate (1%), following previous studies in vetigastropods (Williams et al., 2010; Williams, 2012). Convergence of model parameters was estimated by plotting the marginal posterior probabilities versus the generations in Tracer v.1.5 (http://beast.bio.ed.ac.uk/Tracer). Effective sample-size values were estimated for each parameter to ensure adequate mixing of the MCMC (ESSs > 1,000).

2.3 Genetic diversity and structure, genealogical relationships and morphological comparisons.

We determined levels of mtDNA polymorphism in *Margarella* populations using standard diversity indices such as the number of haplotypes (*k*), haplotype diversity (*H*), number of segregating sites (*S*), average number of pairwise differences (∏) and nucleotide diversity (π) for each species and localities using DnaSP. We also estimated levels of mtDNA population differentiation following Pons and Petit (1996) through the haplotype frequencies (G_{ST}) and mean pairwise differences (N_{ST}) in Arlequin v. 3 (Excoffier et al., 2005). The statistical significance of these analyses was determined using permutation tests (20,000 iterations).

Genealogical relationships in *Margarella* populations were reconstructed using maximum parsimony networks in MEGA and visualized in Hapview (Salzburger et al., 2011). For comparative purposes, we also constructed maximum parsimony networks based on 28S rRNA sequences including new and available *Margarella* sequences (Williams et al. 2010; Williams, 2012). Finally, in order to determine if shell coloration constitutes a suitable descriptor of specific status in *Margarella* we associated individuals' colors onto their respective mtDNA and nucDNA maximum parsimony networks.

3. Results

3.1 DNA polymorphism

The Cytochrome-b alignment of Antarctic and sub-Antarctic *Margarella* (n = 404) included a total of 396 nucleotide positions coding for 132 amino acids, with no indels or stop codons. Mitochondrial and nuclear sequences were not saturated at any position and no evidence for mtDNA codon bias was detected (ENC = 45.04). A total of four fixed substitutions (positions 48, 71, 86, and 123) were recorded in the mtDNA data set and all of them differed between Antarctic and sub-Antarctic lineages. A total of 69 variable positions (17.42%) were recorded of which 54 (78.26%) were parsimoniously informative. Mitochondrial sequences were A-T rich (65.6%). Nuclear 28S rRNA (n = 84) sequences included a total of 740 nucleotide positions and were highly conserved. Only 8 positions (1.08%) were variable of

which only 5 (62.5%) were parsimoniously informative. Nuclear sequences were G-C rich (58.7%).

3.2 Phylogeny and species delimitation analyses

The phylogenetic reconstructions clearly discriminated major taxonomic groupings of trochoideans with high bootstrap and posterior probabilities (Figs. 2 and 3). Similarly, the monophyly of the clades within *Margarella* was strongly supported by the different markers/methods and all the analyses resolved the relationships within the genus with minor topological inconsistencies (Figs. 2 and 3). Out of the seven nominal *Margarella* species we sequenced, however, phylogenetic reconstructions discriminated the presence of only four species-level clades. The first mtDNA clade (C1) comprised individuals of *M. antarctica* collected across the Antarctic Peninsula (Fig. 2). A second clade (C2) included individuals of *M. steineni* collected at Cumberland Bay, South Georgia and a single individual from Crozet Islands (Fig. 2). The third clade (C3) consisted of individuals identified as *M. achilles* collected at Cumberland and Grytviken bays, South Georgia (Fig. 2). Finally, the fourth clade (C4) comprised sub-Antarctic *Margarella* individuals collected from different provinces and identified as the nominal species *M. expansa* (SA, FI and KI), *M. violacea* (SA, FI CZ and KI), *M. pruinosa* (SA), and *M. porcellana* (KI) (Fig. 2).

The nucDNA phylogenetic reconstructions recognized the presence of the same four different species-level clades within *Margarella* (Fig. 3), with the single sample of *M. refulgens* from Williams (2012) as a sister species to all our clades. Nevertheless, nucDNA relationships among them differed from the ones recorded through mtDNA reconstructions. For instance, mtDNA placed *M. antarctica* as sister to the remaining three clades (albeit with limited statistical significance), whereas nucDNA reconstructions recovered it as sister to *M. steineni* (Fig. 3).

Both ABGD and GMYC species-delimitation methods corroborated the mtDNA and nucDNA reconstructions within *Margarella*, recovering the same four groups. Consequently, the specimens of *Margarella* that we sampled across the sub-Antarctic, including populations from South America, Falkland/Malvinas, Crozet

and Kerguelen Islands were treated as a single evolutionary unit under the senior name, *Margarella violacea*, with which we synonymize *M. expansa syn.* nov, *M. pruinosa syn.* nov and *M. porcellana syn.* nov.

3.3 Divergence-time estimates and diversification-rate variation analyses

Levels of mtDNA genetic divergence between the four *Margarella* species (uncorrected p-distances) varied between 9.8% (*M. antarctica* vs *M. achilles*) and 2.8% (*M. violacea* vs *M. achilles*) (Table 1). Divergence-time estimations within *Margarella* based on mtDNA sequences suggest that the origin of the analyzed species occurred at the end of the Miocene around 7 Ma (8 – 5 Ma) with the separation of *M. antarctica* from the clade of analyzed sub-Antarctic species (Fig. 4). Subsequently, the separation between *M. steineni* and the rest of the analyzed sub-Antarctic species (L3 and L4) may have occurred around 5 Ma (6.5 – 4 Ma). Finally, the separation between *M. achilles* from *M. violacea* was dated ~ 2 Ma (3 – 1.5 Ma) (Fig. 4).

3.4 Population-based analyses and genealogical relationships in Margarella lineages

Population-based analyses and genealogical reconstructions based on mtDNA sequences included a total of 305 individuals of *M. violacea* and 90 of *M. antarctica.* Because of the low number of individuals collected for *M. pruinosa* (n = 4), *M. porcellana* (n = 9), *M. steineni* (n = 11) and *M. achilles* (n = 10), these taxa were excluded from comparative population analyses.

Low levels of genetic diversity (mtDNA and nucDNA) and a complete absence of population structure characterized *M. antarctica* across its Antarctic Peninsula distribution (Table 2). For instance, levels of mtDNA polymorphism (*H* and ∏) varied between 0.065/0.065 (Doumer Island) and 0.259/0.271 (Fildes Bay) (Table 2). The more widespread sub-Antarctic species *M. violacea* showed higher levels of genetic diversity (Table 3) and a more complex pattern of genetic structure, with Puerto Williams (Beagle Channel), Possession Bay (eastern mouth of the Strait of Magellan), Hookers Point (FI) and Port (KI) the most differentiated populations. By

contrast, the rest of the South American populations showed no evidence of significant genetic structure (Table S3). Standard diversity indices (*H*/∏) in sub-Antarctic populations of *M. violacea* varied between 0.069/0.069 (Puerto Williams) and 0.868/3.696 (Possession Bay) (Table 3).

The maximum parsimony haplotype network for *M. antarctica* showed a star-like topology with a very short genealogy including six different haplotypes (Fig. 5a). The central dominant haplotype (H1) in *M. antarctica* was broadly distributed across the Antarctic Peninsula and present in 88.4% of the analyzed individuals (Fig. 5a). By contrast, the network for *M. violacea* included 45 haplotypes and a more expansive genealogy (Fig. 5a). The network for *M. violacea* showed a dominant haplotype (H2), four medium-frequency ones (H3 – H6), several at low-frequency (n = 8) and many unique (n = 33) haploypes (Figure 5a). The dominant (H2) haplotype in *M. violacea* was broadly distributed across southern South America, whereas two of the medium-frequency haplotypes (H3 and H4) were spread across the sub-Antarctic, from South America to Kerguelen Islands. The third medium-frequency haplotype (H5) was only recorded on Falkland/Malvinas Island and at Possession Bay, at the eastern mouth of the Strait of Magellan (Fig. 5a). Finally, a fourth medium-frequency haplotype (H6) was found in Crozet and Kerguelen Islands (Fig. 5a). Haplotype mtDNA networks for *Margarella* species at South Georgia exhibited two common haplotypes in *M. steineni* and several low-frequency ones in *M. achilles* (Fig. 5). A single individual collected at Possession Island (Crozet Islands) showed two substitutional steps from the dominant haplotype recorded from South Georgian *M. steineni*.

The 28S rRNA network included a single allele (HI) shared by all the analyzed individuals of *M. antarctica* (n = 16) (Fig. 5b). Similarly, *M. achilles* individuals from South Georgia shared a single allele (HII), while *M. steineni* populations from South Georgia and Crozet Islands also shared a single allele (HIII) (Fig 5b). The species *M. steineni* and *M. achilles* were separated from *M. antarctica* by two and three substitutional steps, respectively (Fig. 5b). Finally, a single allele (HIV) was recorded in all but one *M. violacea* individual. This singleton allele was separated by one substitutional step from the dominant sequence recorded in the species.

Available 28S rRNA sequences of the species *M. refulgens* and *Margarella* sp. (Williams et al., 2012) were separated by two and three substitutional steps, respectively, from the dominant haplotype recorded in *M. achilles*. Accordingly, and considering the levels of nuclear divergence between species of *Margarella* that were clearly discriminated through mtDNA (*M. antarctica*, *M. achilles* and *M. steineni*), both *M. refulgens* and *Margarella* sp. could be valid species. In contrast, nucDNA sequences of the nominal species *M. crebrilirulata* and *M. biconica* (Williams et al., 2012) were identical to those recorded in this study for *M. steineni*. Accordingly, the relationship among these three nominal taxa requires further studies; examining mtDNA sequences would make a good start.

Comparative morphological and genetic data within sub-Antarctic populations of *Margarella* showed a complete disassociation between shell coloration and mtDNA/nucDNA sequences (Fig. S1). Sub-Antarctic individuals identified as the nominal sub-Antarctic species *M. expansa*, *M. pruinosa*, *M. porcellana* and *M. violacea* were in most cases identical of their respective mtDNA and nucDNA sequences across their distributions in the different sub-Antarctic areas here analyzed (Fig. S1).

4. Discussion

4.1 Biogeography of Margarella

Our phylogenetic reconstructions and species-delimitation analyses in *Margarella* clearly support the critical role of the Antarctic Polar Front (APF) in the biogeography and evolution of this high-latitude trochoidean genus. Sub-Antarctic populations previously considered as four separate species from South America and Kerguelen Islands, both north of the APF (Rintoul, 2011; Park et al., 2014), formed a single clade with high levels of genetic homogeneity despite being separated by thousands of kilometers. We consider these populations to form just one biological species, *M. violacea* (= *M. expansa*, *M. pruinosa* and *M. porcellana*) exhibiting a wide range of colors that appear to be associated with different habitats. High levels of genetic homogeneity across thousands of kilometers have been reported in other sub-Antarctic shallow marine kelp-associated invertebrates

including pulmonates of the genus *Onchidella* (Cumming et al. 2014) and *Siphonaria* (González-Wevar et al. 2018), as well as in crustaceans (Leese et al., 2010; Nikula et al. 2010). Accordingl to molecular reconstructions, shell coloration and morphology does not represent effective descriptors of specific status in *Margarella*. In fact, field observations suggest that shell coloration in *Margarella* seems to be a better associated to habitat preferences considering that typical violet/purple individuals were largely associated with rocky ecosystems dominated by kelps and red macroalgae (i.e. *Lessonia* sp., *Macrocystis pyrifera*, *Iridaea cordata*, *Gigartina skottsbergii*) as recorded by Adami and Gordillo (1999) and Rosenfeld et al. (2011, 2015). In contrast, olivaceous and green individuals were more abundant in areas dominated by soft sediments (Rosenfeld et al. 2011), while sub-Antarctic white individuals were frequently found in the lower intertidal closely associated with encrusting coralline algae (Rosenfeld pers. comm.).

Conversely, *Margarella* populations south of the APF showed high levels of genetic divergence and the presence of at least three species-level clades. One species was restricted to the Antarctic Peninsula (*M. antarctica*) while another clade was endemic to South Georgia (*M. achilles*). Finally, *M. steineni* was found in South Georgia but we also recorded a single individual at Crozet Islands. Such results contrast with previous phylogeographic studies in near-shore marine species showing that South Georgia represents the northern limit of several Antarctic species including the limpet *Nacella concinna* (Hoffmann et al., 2011; González-Wevar et al., 2013), the crinoid *Promachocrinus kerguelensis* (Wilson et al., 2007; Hemery et al., 2012), the sea star *Odontaster validus* (Janosik et al. 2011), and fishes like *Gobionotothen gibberifrons* (Matschiner et al., 2009).

The contrasting biogeographical pattern in populations of *Margarella* north and south of the APF may be a consequence in part of the respective abundances of buoyant kelps, which are potential vectors for long-distance dispersal of these vetigastropods with low dispersal potential (Picken, 1979; Zelaya, 2004; Rosenfeld et al. 2011, 2017). According to this view, sub-Antarctic *Margarella* maintain connectivity through long-distance dispersal mediated by rafting. That these sub-Antarctic populations constitute a single species fits well with this hypothesis.

Contrarily, south of the APF, the absence of buoyant kelps in the Antarctic Peninsula seems to preclude the connectivity of Antarctic populations with those from South Georgia. The presence of the macroalgae *Durvillaea antarctica* and *Macrocystis pyrifera* on South Georgia might be held against our hypothesis, but we note that the prevailing winds and currents would likely keep algal wracks (and hence any rafting *Margarella*) south of the APF. The close relationship (and, indeed, possible synonymy) of *M. biconica* and *M. crebrilirulata* from the Weddell Sea and East Antarctica with *M. steineni* from South Georgia is congruent with our suggestions. The presence of buoyant kelps in South Georgia and the prevailing winds could also explain the close affinity recorded between the single individual from Crozet with the South Georgian species *M. steineni*.

The nuclear-gene based topology in *Margarella* was not consistent with the mitochondrial genetic splits (although the identity of the clades was). Similar patterns have been obtained in other groups of marine molluscs including *Atrina* (Liu et al. 2011) and *Monodonta* (Zhao et al. 2017). Such conflict between mtDNA and nucDNA topologies may be the consequence of either incomplete lineage sorting or post-glacial introgression due to secondary contact (Toews and Brelsford 2012), or a lack of discrimination power in the more conserved nuclear DNA.

Mitochondrial divergence-time estimates suggest that the most recent common ancestor (tmrca) of the analyzed *Margarella* lineages lived at about the Mio-Pliocene boundary, between 7 and 5.5 Ma, long after the isolation of the Antarctica and the formation of the APF. We suggest that three *Margarella* lineages (the ancestors of *M. antarctica*, *M. steineni*, and *M. violacea*) subsequently diversified almost simultaneously (based on uncorrected p-distances differences), following independent evolutionary pathways in the Antarctic Peninsula, South Georgia, and somewhere in the sub-Antarctic (probably southern South America), respectively. We also recorded a recent cladogenetic process around 2 Ma, when *M. violacea* separated from the South Georgian species *M. achilles*, the result of a rare trans-APF dispersal event (although the nuclear tree suggests a different topology). Finally, considering main oceanographic patterns in the Southern Ocean, we also found evidence of trans-APF long-distance dispersal event in which *M. steineni*

from South Georgia colonized Crozet, > 5000 km north-eastward. Such recent long-distance dispersal events across the APF have been hypothesized in other Southern Ocean marine invertebrates including the limpets *Nacella* (González-Wevar et al., 2017) and the isopod *Septemserolis* (Leese et al., 2010), as well as in macroalgae (Fraser et al., 2009).

Mitochondrial divergence-time estimates in *Margarella* are in agreement with recent molecular studies in Southern Ocean marine invertebrates and fishes. For instance, the most diverse groups of nothothenioid fishes radiated between 11.6 and 5.3 Ma, more than 10 Ma after their origins (Near et al., 2012). Similarly, levels of molecular divergence between Antarctic and sub-Antarctic populations of octocorals suggest a separation of lineages during the middle Miocene ~ 12-6 Ma (Dueñas et al., 2016). Moreover, the origin and diversification of *Nacella* around the Southern Ocean occurred approximately 12 Ma and 8 – 5.5 Ma (González-Wevar et al., 2017). Levels of mtDNA divergence between Antarctic and sub-Antarctic lineages of *Margarella* (~7.0%) are similar to those reported in bivalves (Page and Linse, 2002; González-Wevar et al., 2019) and echinoderms (Hunter and Halanych, 2008; Janosik et al., 2011) suggesting that their respective radiations occurred at the end of Miocene, no more than 10 Ma. The middle Miocene is considered as an important time of oceanographic and climatic changes in the Southern Ocean associated with the intensification of the Antarctic Circumpolar Current (ACC) and to the re-establishment of Antarctic continental ice sheets (Flower and Kennett, 1994; Zachos et al., 2001; Lawver and Gahagan, 2003). Major oceanic circulation shifts during this period are probably associated with the complete development of a deep ACC (Dalziel et al., 2013). As proposed for other Southern Ocean mollusks (González-Wevar et al., 2017, 2019), fluctuations in latitudinal positioning and strengthening of the ACC after the MMCT may have favored the colonization of geographical distant provinces of the Southern Ocean.

4.2 Sub-Antarctic connectivity

Dispersal ability has been often assumed as good predictor of connectivity between local populations in aquatic ecosystems (Jablonski, 1986, Palumbi, 1994; Hellberg et al., 2002; Shanks et al., 2003; Marko, 2004; Cowen and Sponaugle, 2007; Haye et al., 2014). Hence, species with broadcast spawning and pelagic larval development should exhibit higher levels of connectivity and less genetic structure across broad spatial scales than species with direct and/or benthic development (Ronce, 2007; Ayre et al., 2009; Gillespie et al., 2012). Nonetheless, many species display inconsistent patterns of genetic structure with those predicted solely from their developmental modes (Weersing and Toonen, 2009; Mercier et al., 2013; Segovia et al., 2017). The high levels of genetic identity recorded in *Margarella violacea*, a species with protected benthic development, across thousands of kilometers in the sub-Antarctic is noteworthy considering that several comparable studies in the Southern Ocean have recovered the presence of locally endemic invertebrate lineages (Leese et al., 2008; Strugnell et al., 2008; Janosik et al., 2011; González-Wevar et al., 2017; Moon et al., 2017). For instance, comparative genetic analyses of sub-Antarctic broadcast-spawners like *Aequiyoldia* (González-Wevar et al. 2019) and *Nacella* (González-Wevar et al. 2017) identified different evolutionary units in South America, the Falkland/Malvinas and Kerguelen Islands that have been separated for several millions of years. In contrast, phylogeographic and populations-based studies in other Southern Ocean species with benthic-protected development have demonstrated the major role of long-distance dispersal. Examples include gastropods (Nikula *et al*., 2010a; Cumming *et al*., 2014; González-Wevar et al., 2018), chitons (Nikula *et al*., 2011b) crustaceans (Nikula *et al*., 2010), as well as non-buoyant kelps (Fraser et al., 2013).

As we note above, the phylogeographical patterns recorded in *Margarella* across the sub-Antarctic closely resemble those found in the southern bull-kelp *D. antarctica* (Fraser et al., 2009), as well as in Southern Ocean kelp-associated invertebrates (Leese et al. 2008; Nikula et al., 2010; Cumming et al., 2014; González-Wevar et al., 2018). Our findings are yet more evidence supporting that

long-distance dispersal mediated by bull-kelp rafting is key to understanding much Southern Ocean biogeography, especially for species like *M. violacea* that use macroalgae as a spawning substrate (Zelaya 2004).

4.3 Quaternary genetics

Earth's climate has undergone recurring fluctuations during the Quaternary Ice Ages of the last 2.5 million years. Many studies have demonstrated the severe impact of Quaternary glacial cycles on population dynamics at higher latitudes of the Southern Ocean including Antarctica (Convey et al., 2009; Allcock and Strugnell, 2012; Fraser et al. 2012; González-Wevar et al., 2013; Crame, 2018; Halanych and Mahon, 2018) and the sub-Antarctic (for a review see Moon et al., 2017; Waters, 2007; Cumming et al., 2014; González-Wevar et al., 2018).

As general pattern, *Margarella antarctica* showed strong signals of postglacial population expansion with the presence of: a) low levels of genetic diversity, b) the presence of a broadly distributed dominant haplotype, c) a star-like and very short genealogies (Fig. 5; Table 2). Conversely, sub-Antarctic *M. violacea* showed higher levels of genetic diversity, evidence of spatial structure and a more expanded and complex genealogy (Fig. 5; Table 3). Genetic comparisons in higher latitude *Margarella* populations identified an inverse correlation between genetic diversity and latitude, as reported in other marine near-shore invertebrates (González-Wevar et al., 2016).

Fraser et al. (2009) showed that the southern bull-kelp *Durvillaea antarctica* recently recolonized the sub-Antarctic, evidenced by the striking genetic homogeneity at scales of thousands of kilometers from South America to Macquarie Island. Following these arguments, and as recently suggested for two sub-Antarctic *Siphonaria* species (González-Wevar et al., 2018), we hypothesize that any *Margarella* species became extinct in the Kerguelen Islands during the LGM, before the islands were colonized during the glacial-interglacial transition by rafting *M. violacea* from geographically distant refugia, such as southern South America, where glacial impact is known to have been lower, particularly on the southwestern Atlantic coast (Rabassa et al., 2005). Such a process could have

occurred several times during the Quaternary and generated a “demographic reset” during glacial maxima on the sub-Antarctic Islands followed by interglacial recolonization or “demographic reboots”.

5. Conclusions

Molecular-based studies of shallow marine invertebrates are helping identify and define the distribution of evolutionary lineages in the Southern Ocean and aid our understanding of the underlying evolutionary processes (Waters, 2007; Strugnell et al., 2008; Fraser et al. 2009, 2012; Allcock and Strugnell, 2012; Near et al. 2012; Poulin et al. 2014; González-Wevar et al. 2017, 2018, 2019; Moon et al. 2017; Crame, 2018; Halanych and Mahon 2018). As previously shown in several sub-Antarctic invertebrates with protected benthic development, *Margarella* populations form a single broadly distributed evolutionary unit over large geographic distance, suggesting the existence of long-distance dispersal, at least between South America and the Kerguelen Islands. This pattern is in contrast to broadcast-spawning molluscs like *Nacella* (González-Wevar et al. 2017) and *Aequiyoldia* (González-Wevar et al. 2019), which are characterized by the presence of different lineages in these provinces, showing limited larval dispersal at such geographic scale. In the sub-Antarctic, the combination of protected benthic development with rafting is more effective at long-distance dispersal than free-living larval stages. However, in contrast to the sub-Antarctic populations, *Margarella* south of the APF constitutes several species-level genetic lineages endemic to South Georgia (with the exception of a single *M. steineni* specimen found in Crozet Islands) and the Antarctic Peninsula. South of the APF, Margarella species showed low levels of genetic diversity reflecting the impact of Quaternary glacial cycles that we suggest exterminated local populations during their maxima. Finally, we propose here that the geographic distribution of genetic lineages in *Margarella* across the Southern Ocean is closely related to the ecology of the species including their respective association with buoyant kelps, as well as its spawning behavior over macroalgae.

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Figure Legends

Fig. 1. Nominal species of *Margarella* analyzed and their respective distributions in the Southern Ocean including South America, the Antarctic Peninsula and sub-Antarctic Islands (Falkland/Malvinas, South Georgia, Crozet and Kerguelen).

Fig. 2. Bayesian maximum clade credibility tree of *Margarella* relationships based on mtDNA (Cytb) sequences. Bootstrap support values (MP and ML) and BPP are shown above the nodes (in that order). Species delimitation analyses based on the Automatic Barcoding Gap Discovery (ABGD) and Generalized Mixed Yule Coalescence (GMYC) methods are also shown. The colour of the nodes indicate the association of individuals to the recorded clades where red includes sub-Antarctic specimens belonging to the nominal species *M. violacea*, *M. pruinosa*, *M. expansa* and *M. porcellana*; dark blue = *Margarella antarctica*; blue = *Margarella achilles*; light blue = *Margarella steineni*.

Fig. 3. Bayesian maximum clade credibility tree of *Margarella* relationships based on nucDNA (28S rRNA) sequences. Bayesian posterior probability support values are shown above the nodes. Species delimitation analyses in *Margarella* based on the Automatic Barcoding Gap Discovery (ABGD) and Generalized Mixed Yule Coalescence (GMYC) methods are also shown. The colour of the nodes indicate the association of individuals to the recorded lineages where red includes sub-Antarctic specimens belonging to the nominal species *M. violacea*, *M. pruinosa*, *M. expansa* and *M. porcellana*; dark blue = *Margarella antarctica*; blue = *Margarella achilles*; light blue = *Margarella steineni*.

Fig. 4. Bayesian maximum clade credibility tree based on mtDNA (Cytb), showing divergence time estimates within trochoidean gastropods. Posterior probabilities are shown above the clades. Grey shaded regions depict 95% bayesian credible intervals (BCIs) for relative divergence times.

Fig. 5. A) mtDNA (Cytb) maximum parsimony networks including *Margarella* species collected at different localities in the Southern Ocean. B) nucDNA (28S rRNA) maximum parsimony network including new *Margarella* sequences from different localities in the Southern Ocean, as well as available ones from previous studies (Williams et al., 2010, Williams, 2012). A coloured circle represents each haplotype and indicate the main area where it was collected. The size of the circle is proportional to its frequency in the whole sampling effort.

Fig. S1. Compared morphologies/shell coloration and haplotypes of individuals of the sub-Antarctic, nominal species *Margarella expansa*, *Margarella violacea*, *Margarella porcellana*, and *Margarella pruinosa* collected from South America (SA), the Falkland/Malvinas Islands (FI), Crozet (CZ), and Kerguelen Islands (KI). A) maximum parsimony networks constructed based on mtDNA (Cytb) data. B) maximum parsimony network constructed based on nucDNA (28S rRNA) sequences.

Table 1. Pairwise levels (%) of genetic mtDNA (Cytb) divergence (uncorrected p-distances) between the recorded species of *Margarella*.

	M. antarctica	*M. steineni*	*M. achilles*	*M. violacea*
M. antarctica	****			
M. steineni	9.2	****		
M. achilles	9.8	7.4	****	
M. violacea	7.3	6.4	2.8	****

Table 2. Mitochondrial (Cytb) diversity indices and neutrality tests in *Margarella antarctica* across its distribution in the Antarctic Peninsula.

Locality	***n***	***k***	***H***	***S***	Π	π	**Tajima´s D**	**Fu´s FS**
Fildes Bay	29	4	0.259	3	0.271	0.0006		
Doumer Island	30	2	0.065	1	0.065	0.0001		
Ryder Bay	31	4	0.251	3	0.262	0.0006		
Total	90	6	0.190	5	0.198	0.0005	-1.73	-6.203**

Where: *n* = number of analyzed individuals; *k* = number of haplotypes; *S* = polymorphic sites; *H* = haplotype diversity; Π = average number of pairwise differences; π = nucleotide diversity. *p<0.05, **p<0.01, *** p<0.001.

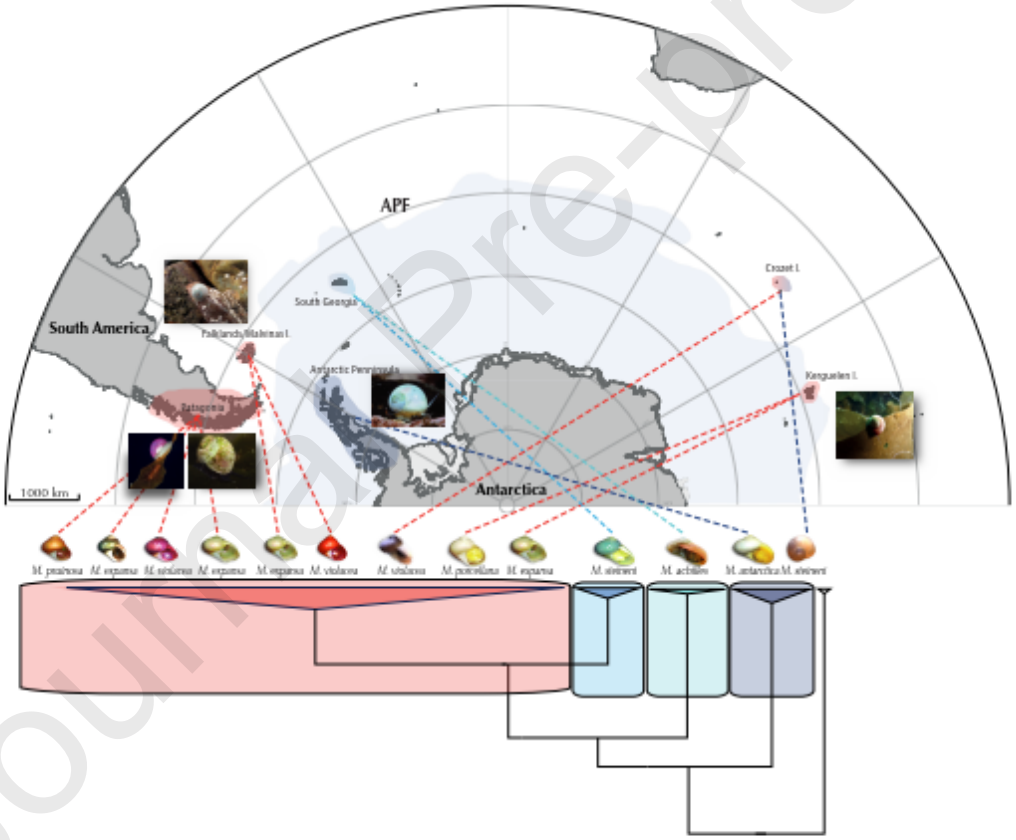
Table 3. Mitochondrial (Cytb) diversity indices and neutrality tests in *Margarella violacea* across its distribution in the sub-Antarctic where SA = South America, FI = Falkland/Malvinas Islands, CZ = Possession Island, Crozet and KI = Kerguelen Islands.

Locality	*n*	*k*	*H*	*S*	Π	π	Tajima´s D	Fu´s FS
Carlos III Island (SA)	16	4	0.575	3	0.708	0.0017		
San Isidro (SA)	26	7	0.415	7	0.674	0.0016		
Port Famine (SA)	33	4	0.229	3	0.239	0.0005		
Tierra del Fuego (SA)	44	8	0.434	8	0.635	0.0015		
Possession Bay (SA)	42	14	0.868	14	3.696	0.0091		
Yendegaia Bay (SA)	34	7	0.704	6	1.050	0.0025		
Puerto Williams (SA)	29	2	0.069	1	0.069	0.0001		
Hookers Point (FI)	39	10	0.487	10	0.659	0.0016		
Possession Island (CZ)	14	4	0.396	3	0.429	0.0010		
Port-Aux Francais (KI)	41	6	0.490	5	0.556	0.0013		
Total	319	48	0.762	35	2.613	0.0066	-1.60	-39.65***

Where: *n* = number of analyzed individuals; *k* = number of haplotypes; *S* = polymorphic sites; *H* = haplotype diversity; Π = average number of pairwise differences; π = nucleotide diversity. *p<0.05, **p<0.01, *** p<0.001.

Higlights

- Molecular analyses in *Margarella* contradict the current taxonomic knowledge in the group
- The Antarctic Polar Front (APF) represents an effective biogeographic barrier
- North of the APF *Margarella* includes a single and broadly distributed species
- South of the APF *Margarella* includes at least three different lineages
- The presence/absence of buoyant kelps plays a key role in *Margarella*'s biogeography



GRAPHICAL ABSTRACT: Biogeographic relationships among Antarctic and sub-Antarctic populations of the Southern Ocean calliostomatid genus *Margarella*. The Antarctic Polar Front (APF) represents an effective barrier between Antarctic and sub-Antarctic populations of *Margarella*. North of the APF Margarella includes a single broadly distributed species-level clade. South of the APF Margarella includes at least three different species in a much narrower geographical distribution. Contrasting biogeographical patterns recorded in Margarella are probably associated to the presence/absence of buoyant kelps in Antarctic and sub-Antarctic ecosystems.