RESEARCH ARTICLE

Seven snail species hidden in one: Biogeographic diversity in an apparently widespread periwinkle in the Southern Ocean

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Abstract

Aim: The Antarctic Circumpolar Current imparts significant structure to the Southern Ocean biota. The Antarctic Polar Front is a major barrier to dispersal, with separate species (or sometimes intraspecific clades) normally occurring either side of this feature. We examined the biogeographic structure of an apparent exception to this rule in a widespread genus of the Southern Ocean, the periwinkle snail, *Laevilitorina*.

Location: Southern Ocean.

Taxon: Littorinidae, Laevilitorininae, *Laevilitorina*.

Methods: Using 750 specimens from 16 Southern Ocean *Laevilitorina* populations across >8000 km, we analysed mitochondrial COI and nuclear 28S sequences to uncover the evolutionary history of these marine near-shore snails. We utilized multi-locus phylogenetic reconstructions, species-delimitation analyses, divergence-time estimations and geometric morphometrics.

Results: Molecular data revealed that the widespread nominal species *L. caliginosa* comprises seven species-level clades, all supported by morphological data, whereas the Antarctic nominal species *L. antarctica*, *L. claviformis* and *L. umbilicata* are conspecific. Six "*caliginosa*" clades are restricted to southern South America, but one lineage extends from Antarctica to distant sub-Antarctic islands on both sides of the APF.

Geometric morphometrics also identified significant differences among these clades, but uncoupled from genetic differentiation.

Main conclusions: The apparent trans-APF distribution of the poorly dispersing *Laevilitorina caliginosa* is largely illusory: this taxon consists of at least seven discrete species, only one of which has a trans-APF distribution. Similar to most *Laevilitorina* species, the remaining six "*caliginosa*" clades are narrow endemics. Biogeographical patterns in *Laevilitorina* reflect the role of vicariance associated with geological processes together with recent long-distance dispersal events. *Laevilitorina* originated near the Eocene/Oligocene boundary and diversified during the Miocene and the Pliocene. *Laevilitorina* is not a cryptic-species complex: speciation was accompanied by hitherto unrecognized morphological differentiation. This study represents the most detailed molecular work on Southern-Ocean littorinids and reveals unforeseen diversity across this globally important region.

KEYWORDS
Antarctic, cryptic species, dispersal, Littorinidae, species-delimitation analyses, sub-Antarctic, vicariance

1 | INTRODUCTION

The Southern Ocean occupies ~35 $\times 10^6$ km^2 around the Antarctic continent and is a major driver of global oceanic circulation (Rintoul, 2011). The composition, abundance and distribution of the biota in this vast region has been shaped by geologic, oceanographic and climatic processes since the fragmentation of the Gondwanan continental landmasses (Aronson et al., 2007; Halanych & Mahon, 2018; Koubbi et al., 2014). The Antarctic Circumpolar Current (ACC) flows clockwise from west to east and is delimited by two main fronts: the sub-Antarctic Front (SAF) and the southern ACC Front (sACCF) (Rintoul, 2011) (Figure 1). In between these fronts is the Antarctic Polar Front (APF), which constitutes an area where cold northward-flowing Antarctic waters meet relatively warmer sub-Antarctic currents (Gille, 2014). The position of the APF has major biogeographic consequences for the marine benthic life in the Southern Ocean (Crame, 2018; Halanych & Mahon, 2018; Koubbi et al., 2014; Poulin et al., 2014) because of the strong thermal boundaries and the deep-reaching flow of the ACC, which create barriers to poleward heat transport (Dufour et al., 2015). Hence, the APF impedes the dispersal of sub-Antarctic organisms into the Antarctic, and vice versa (Aronson et al., 2007; Clarke et al., 2005; Fraser et al., 2018). Accordingly, marine benthic communities in the Southern Ocean are highly endemic, strongly bio-regionalized (Figure 1) and exhibit major adaptations to cold, which makes them particularly vulnerable to global warming (Griffiths et al., 2017; Morley et al., 2020).

Comparative molecular analyses of co-distributed Antarctic and sub-Antarctic species of marine invertebrates (González-Wevar et al., 2017, 2019; Poulin et al., 2014; Thornhill et al., 2008), macroalgae (Billard et al., 2015) and even penguins (Frugone et al., 2019; Pertierra et al., 2020) have demonstrated the presence of different species-level clades on both sides of the APF. Moreover, evidence from a growing number of studies indicates that the origin and diversification

of several near-shore marine organisms occurred no more than 10 Ma, long after the separation of continental landmasses and the initiation of the ACC (González-Wevar et al., 2017, 2018, 2019, 2021; Near et al., 2012; Poulin et al., 2014). Thus, although occasional dispersal must have occurred over long (evolutionary) timescales, there is little ongoing gene flow across the APF (Moon et al., 2017). So far, molecular data suggest that there is almost no evidence of successful biological dispersal into the Antarctic from lower latitudes since the last glacial period (Chenuil et al., 2018; Fraser et al., 2018; González-Wevar et al., 2012; Halanych & Mahon, 2018; Poulin et al., 2014).

Nevertheless, in spite of the dynamic oceanographic and tectonic settings that operated in the region during the last 50 Ma and its marked bio-regionalization, there are numerous examples of seemingly broadly distributed near-shore marine taxa. Molecular studies of most of them, however, have demonstrated that they consist of different evolutionary units in the Antarctic and sub-Antarctic (Billard et al., 2015; González-Wevar et al., 2019; Poulin et al., 2014; Thornhill et al., 2008), which in several cases include cryptic species complexes, common in Southern-Ocean marine invertebrates (Allcock et al., 2011; Baird et al., 2011; Chenuil et al., 2018; Janosik & Halanych, 2010; Wilson et al., 2009), or undescribed species reflecting overly conservative taxonomy (Arango et al., 2011). Although some studies have confirmed the presence of broadly distributed taxa, these cases are restricted to either the Antarctic continent (Díaz et al., 2018; Raupach et al., 2010) or around the sub-Antarctic (Fraser et al., 2009; González-Wevar et al., 2018, 2021; Güller et al., 2020). In summary, the ACC plays a major role connecting populations around the Antarctic continent and, separately, the sub-Antarctic, while the APF isolates antarctic and subantarctic biotas (Crame, 2018; Halanych & Mahon, 2018; Poulin et al., 2014).

Littorinid snails of the genus *Laevilitorina* comprise 21 nominal shallow-benthic periwinkle species (http://marinespecies.org), which live around the Southern Ocean including southern South America, the Antarctic Peninsula, and sub-Antarctic islands. *Laevilitorina*

FIGURE 1 Schematic representation of the Southern Ocean (light blue) and the general benthic biogeographic provinces described for the region (modified from Koubbi et al., 2014). The Antarctic Circumpolar Current (ACC) is delimited by two main fronts: the sub-Antarctic Front (SAF) and southern ACC Front (sACCF). The Antarctic Polar Front (APF) lies between these main fronts. Recognized sub-Antarctic areas include Magellan province in southern South America (red), sub-Antarctic Islands of the Indian Ocean (green), Macquarie Island (yellow) and sub-Antarctic New Zealand islands (pink). A single Antarctic province (dark) includes the Antarctic continent and some islands located south of the APF (e.g. South Georgia, Bouvet).
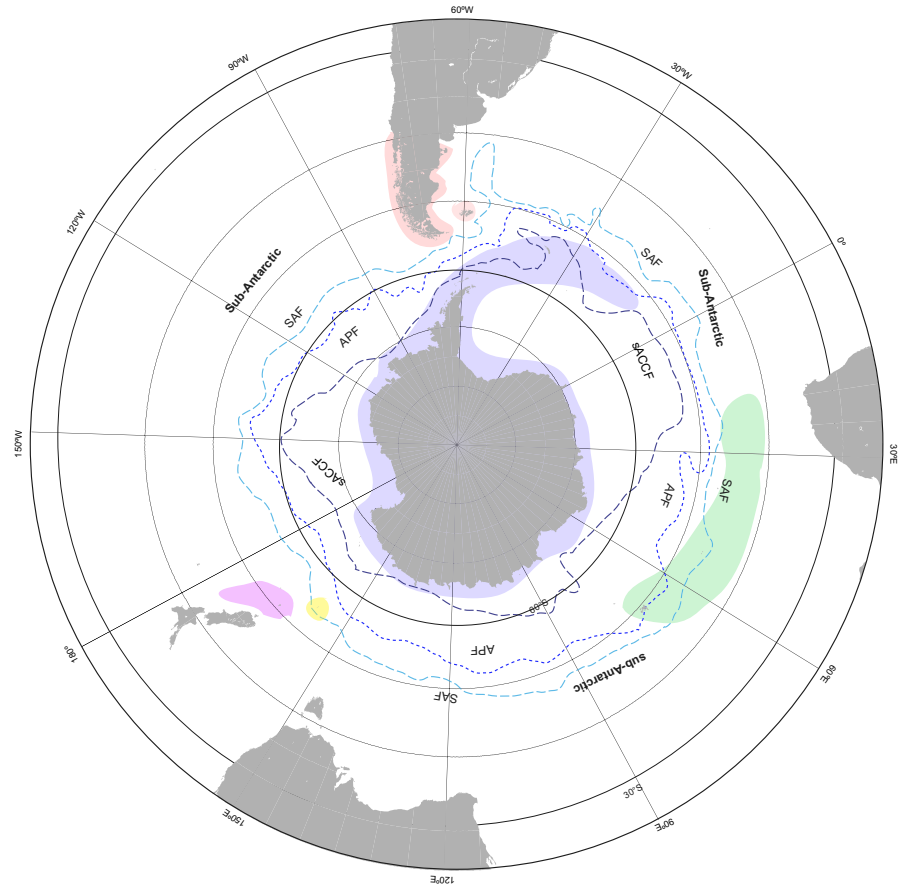
species lack a free-swimming dispersive stage and exhibit protected benthic development with crawling juveniles emerging directly from egg masses (Simpson & Harrington, 1985). Accordingly, *Laevilitorina* species exhibit low autonomous vagility capacity and are poor dispersers, properties reflected by the fact that most of the species are narrow endemics. The single exception, *Laevilitorina caliginosa* (Gould, 1849), apparently shows a broad distribution with populations in southern South America, the Antarctic Peninsula and sub-Antarctic islands such as the Falkland/Malvinas, South Georgia, Marion, Crozet, Kerguelen and Macquarie (Figure 2a,b) (Griffiths & Waller, 2016; Reid, 1989; Simpson & Harrington, 1985).

The ostensible existence of a broadly distributed species with low dispersal potential is paradoxical and three hypotheses may be offered to explain it: (a) anthropogenic activities, (b) passive long-distance dispersal (LDD) mediated by floating objects (i.e. rafting), or (c) the presence of hidden diversity in the form of an unrecognized cryptic-species complexes. There is no evidence supporting the role of anthropogenic activities in the dispersal of marine organisms at the scale of the whole Southern Ocean. *Laevilitorina caliginosa* lives closely associated with buoyant macroalgae and LDD mediated by rafting could constitutes a reasonable explanation for its broad distribution (Griffiths & Waller, 2016). Additionally, as recorded in several Southern Ocean marine near-shore invertebrates (Baird et al., 2011; González-Wevar et al., 2017, 2019; Wilson et al., 2009), *L. caliginosa* could harbour genetically close, morphologically indistinguishable (i.e., cryptic) species.

In order to understand the evolutionary history of *Laevilitorina* in the Southern Ocean, we performed multi-locus molecular-phylogenetic

reconstructions, mitochondrial and nuclear species-delimitation analyses, mitochondrial divergence-time estimations and geometric morphometric analyses to compare populations of *L. caliginosa* from different regions (South America, the Antarctic Peninsula and sub-Antarctic islands), as well as the Antarctic endemics, *L. antarctica*, *L. claviformis* and *L. umbilicata*. We aimed to reveal the phylogeographic structure within *Laevilitorina* and hence evaluate the potential role of vicariance and long-distance dispersal in the biogeography of the genus. We also used our data to shed light on the origin and diversification of *Laevilitorina* around the Southern Ocean. Moreover, geometric morphometric analyses allowed us to evaluate the relevance of cryptic speciation in the diversification of the group. Through addressing these issues, we provide new insights on evolutionary relationships, biogeographical and actual diversity patterns of a widespread element of the Southern Ocean, an area of the planet that has been relatively neglected in spite of its global importance.

2 | MATERIALS AND METHODS

2.1 | Sample collection, DNA preparation and sequence editing

Individuals of *Laevilitorina* were sampled from inter- and sub-tidal ecosystems across the Southern Ocean including *L. caliginosa* populations from southern South America, the Antarctic Peninsula and sub-Antarctic islands (Falkland/Malvinas, South Georgia, Marion,

(a)

(b)

(c)
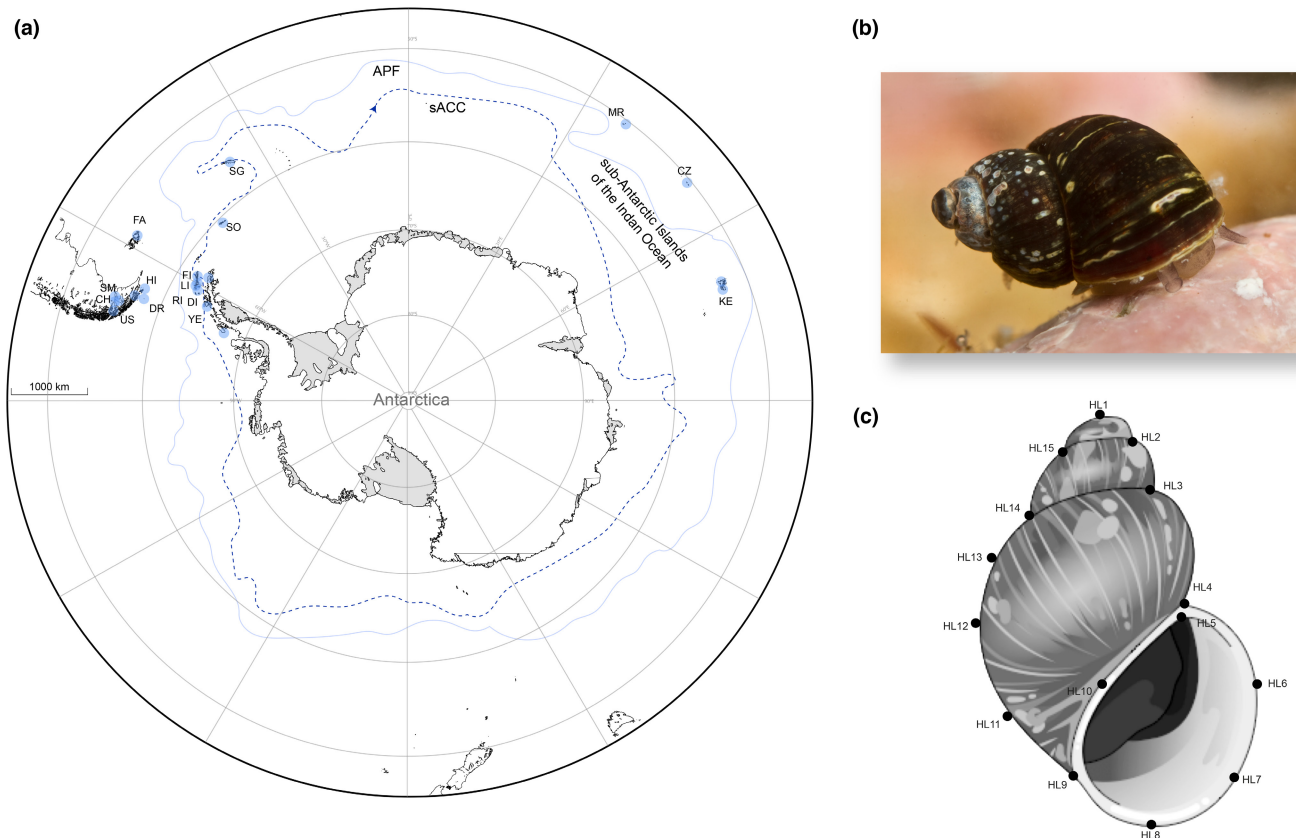
FIGURE 2 (a) Sampling of *Laevilitorina caliginosa* populations across its distribution in the Southern Ocean including populations from southern South America (SA), Antarctic Peninsula (AP) and sub-Antarctic islands of the Indian Ocean (sAIO). AV, Avian Island; CH, Cape Horn; CZ, Crozet Island; DI, Deception Island; DR, Diego Ramírez Island; FA, Falkland/Malvinas Islands; FI, Fildes Bay; HI, Hornos Island; KE, Kerguelen Islands; LI, Livingston Island; MR, Marion Island; RI, Robert Island; SG, South Georgia; SM, Strait of Magellan; SO, South Orkney Islands; YE, Doumer Island. APF and sACCF indicate the relative positions of the Antarctic Polar Front and the southern ACC Front, respectively. (b) Adult individual of *L. caliginosa* in its natural habitat in the Beagle Channel (photograph courtesy of Gonzalo Arriagada). (c) Ventral view of a shell of *L. caliginosa* showing the position of the 15 analysed landmarks (HL1 to HL15). The number of mtDNA/nucDNA *L. caliginosa*'s sequences per main area are SA ($n = 332/110$); AP ($n = 180/60$); SG ($n = 90/40$); MR ($n = 10/10$); CZ ($n = 50/20$); and KI ($n = 90/40$).

Crozet and Kerguelen) (Figure 2a; Table S1 and S2), as well as three endemic Antarctic-Peninsula nominal species (*L. antarctica*, *L. claviformis*, and *L. umbilicata*). Specimens were fixed in ethanol (95%) and taxonomic identification was based on the original descriptions and subsequent revisions (Engl, 2012; Gould, 1849; Preston, 1912, 1916; Reid, 1989; Smith, 1902; von Martens & von Pfeffer, 1886). Nucleic acids were prepared using a standard salting-out method (Aljanabi & Martinez, 1997) and the QIAGEN Dneasy Blood & Tissue Kit (QIAGEN Inc.). Universal primers were used to amplify a partial fragment of the mitochondrial cytochrome c oxidase subunit I (Folmer et al., 1994), and the nuclear rRNA 28S (Littlewood et al., 2000) genes. The nuclear fragment here analysed expands between the end of the ITS2 and D1 region and is suitable to infer phylogenetic relationships in littorinids (Reid et al., 2012). Forward and reverse sequences were assembled and edited independently using GENEIOUS 5.1.7 (Kearse et al., 2012). Alignments and base composition of nucleotide sequences were analysed separately in MUSCLE (Edgar, 2004) and MEGA X (Kumar et al., 2018), respectively. Mitochondrial codon usage was estimated using the effective number of codon value (ENC) (Wright, 1990) in DnaSP v.5

(Librado & Rozas, 2009). New *Laevilitorina* sequences have been deposited in GenBank under the following Accession Numbers: COI (MZ321820–MZ321864) and 28S rRNA (MZ322329–MZ322390 and MZ381414–MZ381445). Molecular information concerning the analysed specimens and museum material from the Western Australian Museum (WAM) is available in Table S1.

2.2 | Phylogenetic reconstructions

Mitochondrial (COI) and nuclear (28S rRNA) phylogenetic reconstructions included 5–10 individuals of *Laevilitorina caliginosa* per locality across the species distribution (Figure 2a), as well as at least 5 specimens of the Antarctic species. For comparative purposes, we also used 10 individuals of the Antarctic littorinid *Laevilacunaria antarctica*, a member of the sister genus. In addition, we included sequences of littorinid genera such as *Echinolittorina*, *Austrolittorina*, *Afrolittorina* and outgroups (*Lacuna pallidula* and *Bembicium auratum*) following Williams et al. (2003) and Reid et al. (2012). Phylogenetic relationships were estimated using maximum parsimony (MP),

maximum likelihood (ML) and Bayesian analyses (BA). MP and ML analyses were performed in MEGA X, while BA were done using MrBayes v.3.1.2 (Huelsenbeck & Ronquist, 2001). Nucleotide substitution models for ML and BA were estimated for each marker independently and for the concatenated dataset using, respectively, the Akaike information criterion (AIC) and the Bayesian information criterion (BIC) in JmodelTest v.2.1.10 (Darriba et al., 2012). The models TN93+I+ G|HKY+I+ G (COI), HKY+I+ G|T92+I+ G (28S rRNA) and GTR+I+ G (COI+28S rRNA) were selected as optimal for ML|BA analyses. Nodal supports for MP and ML analyses were inferred using non-parametric bootstrap (BS) with 1000 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 100×10^6 generations and trees were sampled every 1000 generations. Stationarity of the analyses was inferred when the average standard deviation of split frequencies was less than 0.01 (Huelsenbeck & Ronquist, 2001). The initial 10% of the parameter values were discarded and posterior probabilities were estimated as the fraction of trees showing a particular node. Posterior-probability densities were 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).

2.3 | Species-delimitation analyses and divergence-time estimations

Mitochondrial (COI) and nuclear (28S rRNA) species delimitation analyses in *Laevilitorina* were carried out independently using three different methods: the automatic barcoding gap discovery (ABGD) (Puillandre et al., 2012), the generalized mixed yule Coalescent (GMYC) (Pons et al., 2006) and the multi-rate Poisson Tree Processes (mPTP) (Zhang et al., 2013). Species-delimitation analyses using ABGD and mPTP were performed on their respective on the web-servers (http://www.abi.snv-jussieu.fr/public.abgd) and (https://species.h-its.org), respectively. The GMYC method (Ihaka & Gentleman, 1996) was performed in the R environment (R, version 2.4.1) using the package "splits" (Pons et al., 2006).

To estimate divergence times, a relaxed molecular-clock analysis was implemented for mtDNA sequences using an uncorrelated-lognormal (ucln) model of molecular evolutionary rate heterogeneity and the GTR+I+ G substitution model implemented in BEAST v.1.7.5 (Drummond et al., 2012; Drummond & Rambaut, 2007). A birth-death speciation prior was used to estimate branching rates in the phylogeny. Four chains were run twice for 200×10^6 generations and trees were sampled every 1000 generations. We used several calibration points previously estimated for the time of the most recent common ancestors (TMRCA) of the littorinid genera *Echinolittorina*, *Littorina*, *Littoraria*, *Austrolittorina*, *Afrolittorina* and *Tectarius* (Reid et al., 2012). The convergence of model parameters was estimated by plotting the marginal posterior probabilities versus the generations in Tracer (Rambaut

et al., 2018). Effective sample-size values were estimated for each parameter to ensure the adequate mixing of the MCMC (ESSs >500).

2.4 | Geometric morphometric analyses

A total of 105 adult shells of *L. caliginosa* were photographed with a Leica EZ4W stereoscopic microscope. Shells were placed on a grid of 1 cm squares and stabilized using foam to standardize planar orientation. Shells were carefully oriented in ventral position with the aperture parallel to the grid facing the camera. Two lights were pointed at 45° angle above the shells and colour images were captured, measured and digitalized using the software Leica LAZ ES (Leica Microsystems). For comparative geometric morphometric analyses, specimens were grouped using a priori information based on molecular data and species-delimitation analyses.

Images were transformed into thin-plate splines (TPS) using tpsUtil (Rohlf, 2009) and landmarks were used to capture two-dimension Cartesian coordinates (*x*, *y*) in tpsDig 2.12 (Rohlf, 2009). Scaling was performed using tpsDig to correct for the size of the individual and to describe the shape of the object in terms of the spatial relationship between its parts rather than its dimensions. A total of 15 homologous landmark points (HL) (Figure 2c) were captured for each individual, which allowed us to decompose shell morphology under the criteria of homology, repeatability, coplanarity and shell-shape coverage following Bookstein (1991). Among the selected landmarks HL1–HL5, HL9 and HL12 – HL15 are Type I landmarks, representing direct juxtapositions of tissue types or probable homologies (Bookstein, 1991). Homologous landmark 1 (HL1) is the apex of the shell, whereas HL2–HL5 are upper and lower sutures of succeeding whorls. HL12, HL13 and HL15 are duplications of HL5, HL4 and HL2, respectively, on the left profile. The other landmarks were selected based on overall form of aperture and the last major whorl. Landmark coordinates for all specimens were analysed using MorphoJ v.1.06b (Klingenberg, 2011).

Landmarks were superimposed using Procrustes Fit, which maps the landmark configuration of each specimen in a dataset onto each other so that corresponding points are as close as possible. This process allows us to scale specimens to comparable sizes based on centroid size and minimizes the sum of squared distances between corresponding points (Rohlf, 1999). A principal component analysis (PCA) of shape was performed to determine the linear combination of variables that accounts for most of the variation in the data. The scores on the first two principal components were used in a canonical variate analysis (CVA) to discriminate among the analysed a priori groups. Finally, we estimated the percentage of correctly reassigned specimens through a discriminant function analysis (DFA) in MorphoJ.

Finally, to assess the relationship between molecular (uncorrected p-distances) and morphological (Procrustes distances) data in *Laevilitorina*, we performed a test of congruence among distance matrices (CADM) (Legendre & Lapointe, 2004). To do so, we used

molecular and morphological distances with the CADM global function through the Kendall coefficient matrix concordance (W) implemented in the APE package (Paradis & Schliep, 2019). High and significant Kendall coefficients suggest a high concordance and therefore correlation between genetic and morphological matrices.

3 | RESULTS

3.1 | DNA polymorphism

The cytochrome c oxidase subunit I data set consisted of 250 individuals and 620 nucleotide positions coding for 206 amino acids. No insertions/deletions (indels) or stop codons were detected among the analysed individuals. A total of 253 variable positions (40.8%) were found in the COI data set and 239 of them were parsimoniously informative (94.4%). Mitochondrial sequences were A–T rich (62%). The final alignment of the nuclear gene 28S rRNA included 150 individuals and a fragment of 721 nucleotide positions. A total of 162 positions (22.4%) were variable in the 28S rRNA data set and 149 of them (91.9%) were parsimoniously informative. Nuclear sequences were G–C rich (54.5%). Mitochondrial and nuclear sequences were not saturated and we found no evidence for mtDNA codon bias (ENC = 39.28).

3.2 | Phylogenetic reconstructions

Multi-locus phylogenetic reconstructions using different methods clearly discriminated major taxonomic littorinid groupings, notably a monophyletic *Laevilitorina*, with high bootstrap and posterior probabilities (Figure 3). Within *Laevilitorina*, no topological inconsistencies were found between mtDNA and nucDNA reconstructions (Figure S1 and S2). Nevertheless, phylogenetic relationships within the genus were unexpected as none of the nominal species were monophyletic. On the one hand, all the methods and molecular markers distinguished seven species-level clades (=lineages) within the nominal *L. caliginosa* (Figure 3), with levels of mtDNA genetic divergence (uncorrected p-distances) between 21.4% and 3.0% (Table S3). On the other hand, phylogenetic reconstructions failed to distinguish the Antarctic nominal species *L. antarctica*, *L. umbilicata* and *L. claviformis* (Figure 3), and they shared haplotypes and alleles. Moreover, this Antarctic clade (*L. antarctica/L. claviformis/L. umbilicata*) falls within the "*caliginosa*" lineages, rendering the latter paraphyletic (Figure 3). Accordingly, the diversity in *L. caliginosa* fell into two non-sister clades with high levels of divergence (average > 15%) between them (Figure 3). The first group included (Figure 3 red clade) four lineages, three sympatric and, indeed, syntopic, from the Strait of Magellan, southern South America (L1–L3), and one from the Antarctic Peninsula and geographically distant sub-Antarctic islands of South Georgia, Marion, Crozet and Kerguelen (L4). The second group (Figure 3 blue clade) comprised three lineages (L5–L7) found across the southern tip of South America, the Falkland/

Malvinas Islands and South Georgia. Lineage 5 (L5) was collected from the Beagle Channel, Cape Horn, the Falkland/Malvinas Islands and in South Georgia. Lineage 6 (L6) was limited to Hornos Island, Cape Horn and lineage 7 (L7) was restricted to the Falkland/Malvinas Islands.

3.3 | Species-delimitation analyses and divergence-time estimations

Mitochondrial (COI) and nuclear (28S rRNA) species delimitation analyses using ABGD, mPTP and GMYC corroborated phylogenetic reconstructions, and all recovered a total of eight *Laevilitorina* species. Seven of them were found within a nominal *L. caliginosa*; the eighth included the three Antarctic nominal species *L. antarctica*, *L. umbilicata*, and *L. claviformis* (Figure 3).

Mitochondrial divergence-time estimates are in basic agreement with previous studies of littorinids (Figure S3) (Reid et al., 2012; Williams et al., 2003). Estimates within *Laevilitorina* suggest that the TMRCA of the subfamily Laevilitorininae, (*Laevilitorina* + *Laevilacunaria*) occurred ~56 Ma, whereas the origin of the analysed lineages of *Laevilitorina* was ~38 Ma (HPD 45–34 Ma) (Figure 4). Subsequently, the separation between the ancestor of *L. antarctica/L.claviformis/L. umbilicata* and lineages L1–L4 took place during the Miocene ~20 Ma (27–16 Ma) (Figure 4). The separation between Strait of Magellan (L1–L3) and Antarctic Peninsula + sub-Antarctic islands (L4) lineages occurred ~12 Ma (16–8 Ma). *Laevilitorina* diversified in Cape Horn (L5/L7 from L6) and in the Strait of Magellan (L1 from L2/L3) during the Miocene between 16 Ma (21–12 Ma) and 7 Ma (10–5 Ma), respectively (Figure 4). Finally, *Laevilitorina* diversified in the Strait of Magellan (separation of L2 and L3) and Cape Horn (separation of L5 and L7) during the Pliocene ~3 Ma (5–2 Ma) (Figure 4).

3.4 | Geometric morphometrics

Principal components 1–3 (PC1–PC3) combined explained 70.4% of the total morphological variation of shells. The PCA showed some degree of separation amongst the seven analysed groups. HL3, HL6 and HL7 were the most distinct landmarks but there were significant overlaps amongst others (HL1, HL2, HL4 and HL5). PC1 was associated with variability in landmarks located on the apex and the sutures between major whorls on right and left profiles (HL1–HL4, HL14, HL15). PC2 was associated with variability in landmarks located at the junction between the end of suture and the aperture lip and the most external point on right profile of the last whorl (HL5, HL6, HL8, HL12 and HL13). PC3 was associated with landmark variability on the right profile of the last whorl and the aperture (HL6–HL9). Negative and positive values in PC1–PC2 (x-axis) were associated with rounded and elongated specimens, while negative and positive values in PC3 (x-axis) described narrower and broader individuals, respectively.
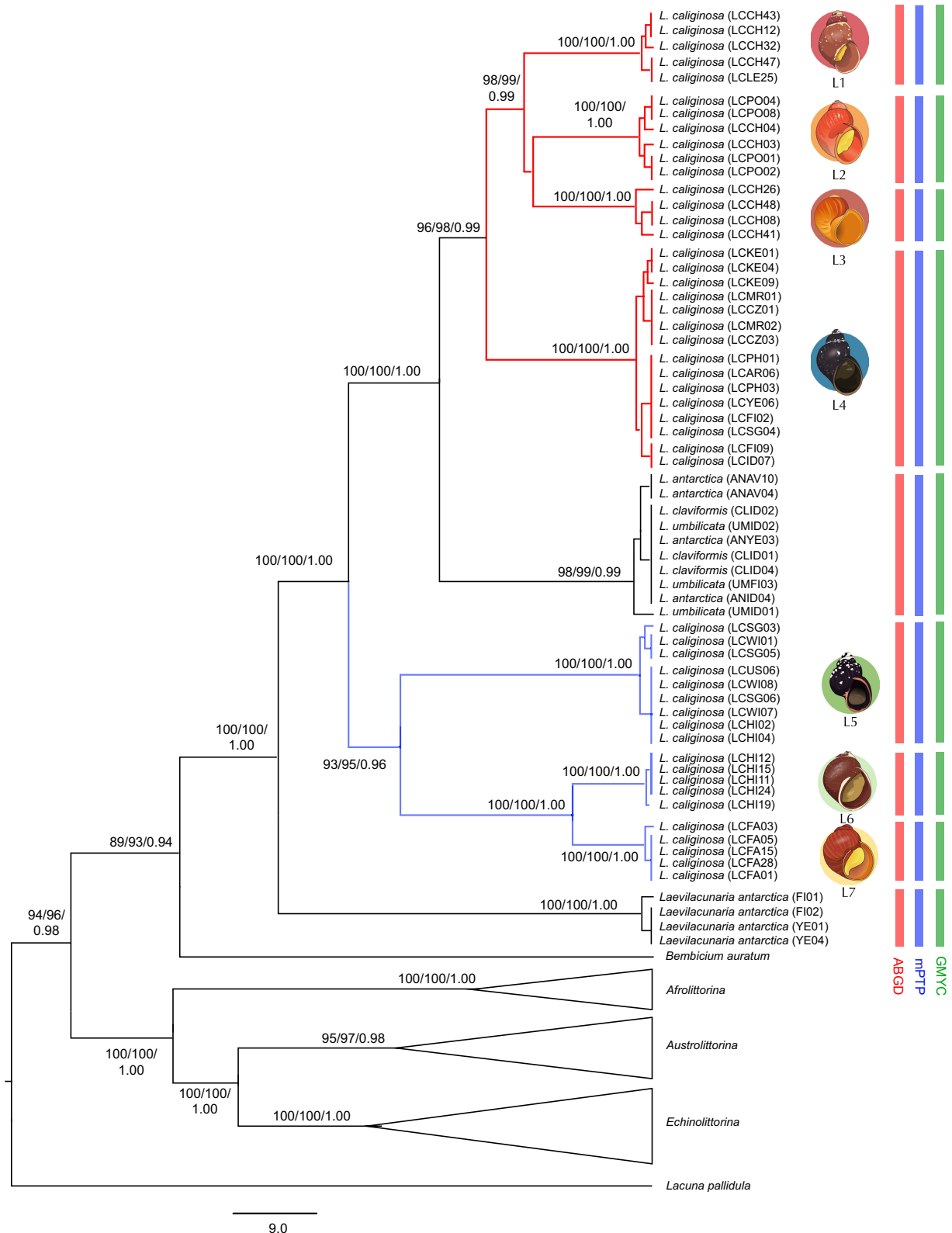
FIGURE 3 Bayesian maximum clade credibility tree of Littorinidae relationships based on multi-locus data with special emphasis on Laevilitorininae (*Laevilitorina* & *Laevilacunaria*) populations collected from around the Southern Ocean. The lineages (L1–L7) recorded within the nominal species *L. caliginosa* are showed. Red and blue rectangles indicate the paraphyletic groups of *Laevilitorina* lineages found within *L. caliginosa*. Bootstrap support (BS) values (MP and ML) and Bayesian posterior probabilities (BPP) are shown above the nodes (in that order).

The CVA based on the first two PCs accounted for 80.9% of the total variance and separated the groups in a similar way to the molecular results, showing marked morphological differences in four of the analysed lineages: L2, L3, L6 and L7 (Figure 5). In contrast, L1, L4 and L5 exhibited some degree of morphological similarity (Figure 5). Nevertheless, permutation tests based on Procrustes distances revealed that all the analysed lineages exhibited significant differences based on their morphological characteristics (Table S4). The DFA based on Procrustes distances established that most of the groups showed high percentage of correct allocation (>89%). Finally, Kendall coefficients of concordance (W) between genetic and morphological distance matrices showed a low and non-significant value ($W = 0.48$; $\chi^2 = 19.11$; $p = 0.52$) suggesting incongruence between genetic and morphological variation among the seven "*caliginosa*" lineages.

4 | DISCUSSION

In this study, we provide robust, yet unexpected, evidence about the biogeography of the marine snail genus *Laevilitorina* around the Southern Ocean. Through genetic and morphological data, we show that the species-level diversity in the genus has been both underestimated in southern South America and overestimated in the Antarctic Peninsula. As previously demonstrated in different groups of near-shore marine organisms including invertebrates, vertebrates and macroalgae, *Laevilitorina* includes different lineages that have been separated by the APF, probably since the separation of the continental landmasses where they are currently distributed. However, one "*caliginosa*" lineage showed a broad trans-APF distribution with populations across the Antarctic Peninsula and geographically distant sub-Antarctic islands. Biogeographical patterns in *Laevilitorina* require new evolutionary explanations, involving the combined role of historical vicariance and recent dispersal, illustrating some wider principles concerning Southern-Ocean biogeography.

The nominal taxon *L. caliginosa* has previously been considered one of the few poorly dispersing species exhibiting a wide circumpolar distribution across the APF. We show that, in fact, it comprises a suite of at least seven lineages, whose levels of genetic divergence clearly indicate species-level status, which is congruent with morphological differentiation in the group. However, CADM analysis showed no relationship between molecular and morphological distances suggesting that morphologic differentiation was uncoupled from species diversification in *Laevilitorina*.

Six of these heretofore unrecognized species have much narrower distributions in and around southern South America, three (L1–L3) in the Strait of Magellan, the remainder (L5–L7) in southern areas of Cape Horn, the Falkland/Malvinas Islands and South Georgia. The broadest distribution is exhibited by the seventh species (L4), which includes populations across the Antarctic Peninsula, South Georgia and geographically distant sub-Antarctic islands of the Indian Ocean (Marion, Crozet and Kerguelen) (Figure 6). Significantly, this range crosses the APF; thus, this *Laevilitorina* lineage (L4) represents the first confirmed example of a near-shore marine benthic invertebrate species found in both Antarctic and geographically distant sub-Antarctic ecosystems.

In contrast to the diversity recorded in southern South America, we show that the nominal species occurring on the Antarctic Peninsula, *L. antarctica*, *L. claviformis* and *L. umbilicata* are genetically indistinguishable, and should probably be considered as a single species. Moreover, phylogenetic reconstructions indicated that this species is nested within the "*caliginosa*" complex.

Geometric morphometric analyses showed that the genetically delineated *Laevilitorina* species exhibit significant morphological differences. Thus, unlike many radiations in the Southern Ocean including nudibranchs (Wilson et al., 2009), bivalves (González-Wevar et al., 2019), octopuses (Allcock et al., 2011), nemerteans (Thornhill et al., 2008), amphipods (Baird et al., 2011), pycnogonids (Arango et al., 2011), and echinoderms (Janosik & Halanych, 2010), *Laevilitorina* is not strictly a cryptic-species complex as speciation
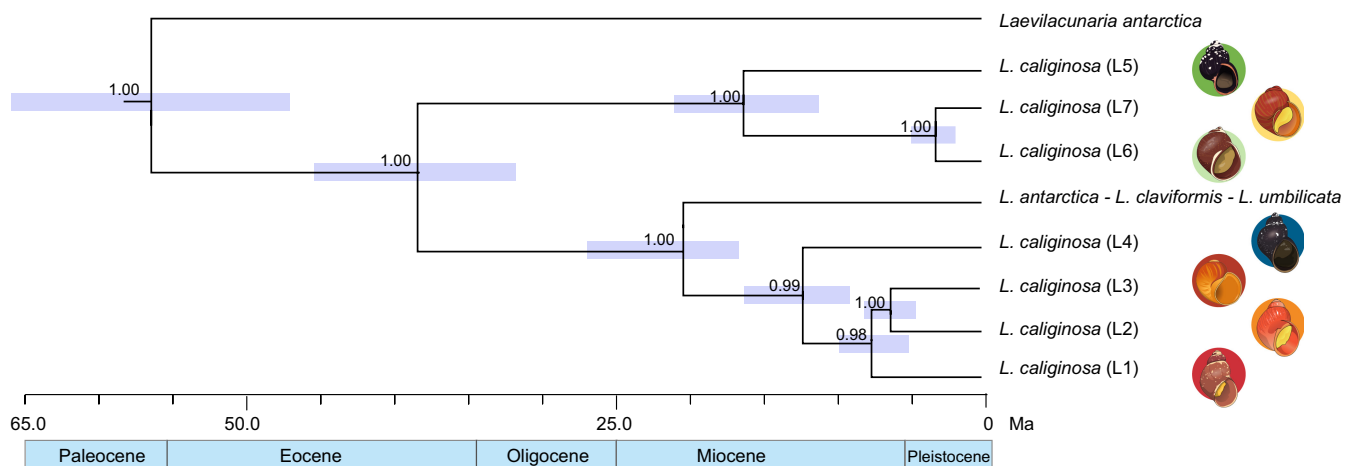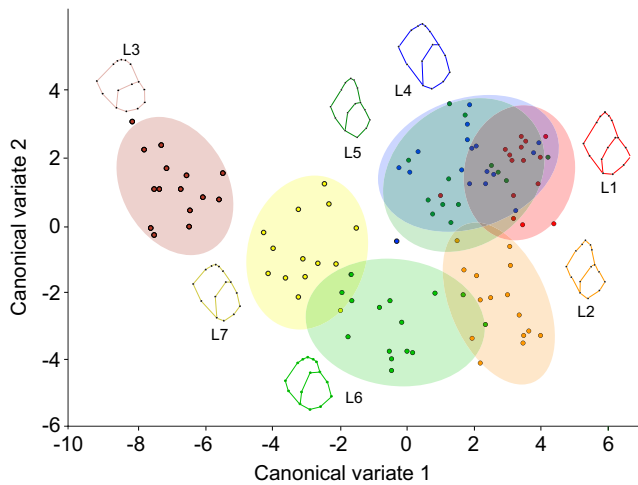

FIGURE 4 Divergence time estimations of Laevilitorininae (*Laevilitorina* + *Laevilacunaria*) based on mtDNA sequences using estimated calibrations by Reid et al. (2012). BPP values are indicated at nodes and shaded bars indicate 95% highest posterior density (HPD) intervals.

FIGURE 5 Canonical variate analyses based on the principal components (PC1 and PC2) of the morphological variation recorded among the lineages (L1–L7) within *L. caliginosa*. 90% mean confidence ellipses are illustrated for each group in matching coloration with the wireframe representations of the average ventral shape variation of each lineage.

has proceeded through molecular differentiation occurring in conjunction with morphological changes.

4.1 | Biogeographic implications

The findings of this study adumbrated above are noteworthy because it gives new and valuable information about actual diversity patterns, evolutionary relationships and the biogeography of nearshore marine benthic invertebrates across its distribution in the Southern Ocean. For instance, before this study, *Laevilitorina* appeared to be a poorly represented genus in South America, with the presence of a single mainland species, *L. caliginosa*, and a second, *L. latior*, restricted to the Falkland/Malvinas Islands. However, through this integrative study we demonstrate that South America represents a species-rich province where this genus diversified over the last 20 Ma (Figure 6). Most of the new diversity in *Laevilitorina* was found in the Strait of Magellan and in Cape Horn, as well as the Antarctic Peninsula, areas that have experienced geological events critical to the understanding of Southern-Ocean biogeography, such as the opening of the Drake Passage and the establishment of the ACC. In addition, during the last 50 years, southern South America and the Antarctic Peninsula have undergone one of the fastest responses anywhere to climate change (Meredith & King, 2005). Several authors (Cavanagh et al., 2021; Morley et al., 2020) have noted the relevance to understand and predict the consequences of global warming in this region, most notably local extinctions, and biological invasions.

During the last decade, several sub-Antarctic marine species have been reported in the Antarctic nearshore, demonstrating the existence of dispersal across the APF (Aronson et al., 2014; Avila et al., 2020; Cárdenas et al., 2020; Fraser et al., 2018). In short ecological timescales, several natural dispersal processes including airborne, oceanic eddy, rafting and hitchhiking on floating objects

may allow the passage to and from Antarctica (Barnes et al., 2006). However, to date, exotic species recorded in Antarctica have failed to establish as permanent populations, probably due to physiological constraints (Fraser et al., 2018; López-Farrán et al., 2021).

The distribution of lineage 4 (L4) is then remarkable, both for its wide geographical extent and because it crosses the APF. These two aspects imply that this lineage is a good long-distance disperser, although why it should be so when its congeners are apparently poor at dispersing is unclear. It is possible that this species is more closely associated with LDD rafting vehicles such as *Durvillaea antarctica*. Future studies of the ecology of the various *Laevilitorina* lineages will be necessary to shed light on this puzzle. Despite these interesting results, the level of resolution of the analysed markers do not allow an accurate estimate of the occurrence, date, and directionality of dispersal events. Future genomic-based analyses including Antarctic lineage 4 (L4) and sub-Antarctic populations will help us understand these major biogeographical issues.

South Georgia is biogeographically interesting for *Laevilitorina* because of the presence of both Antarctic (L4) and sub-Antarctic (L5) lineages. This overlap is surprising, since the coastal gastropods of South Georgia generally have a greater affinity with those from the Weddell Sea sector rather than with South American ones (Zelaya, 2005). In fact, South Georgia represents the northernmost limit of many Antarctic molluscs (e.g., *Nacella concinna*) and, in some exceptional cases, the southernmost limit for several sub-Antarctic ones (e.g., *Siphonaria lateralis*). Nevertheless, we are not aware of any examples, confirmed through molecular analysis, of congeneric near-shore sub-Antarctic and Antarctic molluscan species that co-occur in South Georgia. Thus, *Laevilitorina* may represent the first case of an intertidal gastropod genus in South Georgia, with sympatric—indeed syntopic—South American and Antarctic lineages.

4.2 | Evolutionary diversification of *Laevilitorina* in the Southern Ocean

Our results suggest that the TMRCA of *Laevilitorina* was ~38 Ma (HPD interval 45–34 Ma), close to the Eocene/Oligocene boundary, a period of major change in the Southern Ocean involving the commencement of Antarctic isolation through the opening of major gateways (e.g., the Drake Passage) and the onset of the ACC (Scher et al., 2015; Zachos et al., 2001). Accordingly, the origin of the analysed lineages seems to be deeply connected to ancient vicariant processes driven by continental drift and the initiation of the ACC, as recently suggested for the microbivalve genus *Kidderia* (Levicoy et al., 2021). Subsequently, during the early Miocene, between 20 and 12 Ma, *Laevilitorina* diverged independently in Antarctica and South America, resulting in the ancestors of the eight lineages here found. These radiations were probably associated with the middle Miocene Climatic Transition, a period of drastic climatic shifts marked by the intensification of the ACC and the reestablishment of permanent continental Ice Sheets in East (10 Ma) and West
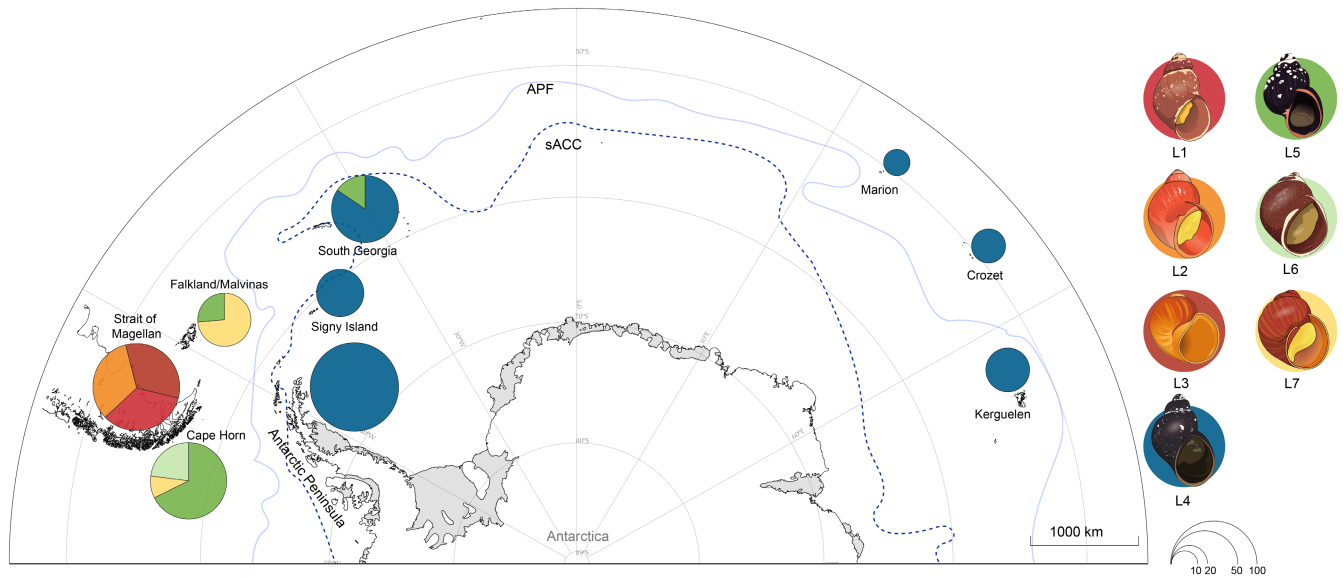
FIGURE 6 Distribution of the recorded lineages (L1–L7) within *L. caliginosa* around the Southern Ocean. Coloured circles show the frequencies and sample sizes. The approximate position of the APF and the sACC are marked with a solid blue and a dashed light blue line, respectively.

(5 Ma) Antarctica (Lewis et al., 2009; Verducci et al., 2009; Zachos et al., 2001). Major oceanographic changes during this period were likely related to the full achievement of a deep ACC ~12 Ma (Dalziel et al., 2013). Fluctuations in the latitudinal position and the strengthening of the ACC seem to be important drivers in the evolution of the Southern Ocean biota (Chenuil et al., 2018; Crame, 2018; González-Wevar et al., 2017, 2019; Halanych & Mahon, 2018; Poulin et al., 2014). The establishment of a strong and deep ACC may have generated an effective oceanographic barrier maintaining the separation of *Laevilitorina* lineages from Antarctica and South America.

More recently, *Laevilitorina* diversified in South America between the middle Miocene and the Pliocene, 16–3 Ma. Similar radiations have been identified in several groups of marine invertebrates during this period across the Southern Ocean including octopods (Strugnell et al., 2008), octocorals (Dueñas et al., 2016), bivalves (González-Wevar et al., 2019), patellogastropods (González-Wevar et al., 2017), trochoid gastropods (González-Wevar et al., 2021) and fishes (Near et al., 2012). Finally, the latest diversification of *Laevilitorina* in South America was probably driven by glacial processes of the Quaternary. Recurrent ice advances and retreats may have enhanced geographical isolation and speciation as inferred for several other South American marine organisms (Fraser et al., 2012; González-Wevar et al., 2011, 2017, 2019; Schächinger et al., 2022). A plausible scenario of this Quaternary diversification in *Laevilitorina* involves small refugial populations of these poorly dispersing snails undergoing substantial genetic drift. Such drift-dominated process is less likely to lead to adaptive morphological differentiation, which would explain our belated recognition of the separate species of *Laevilitorina*. Such pseudocryptic speciation has recently been inferred for the nudibranch genus *Tritoniella*, another poor disperser from the Antarctic and sub-Antarctic, which also separates species either side of the APF (Schächinger et al., 2022).

4.3 | Systematics of *Laevilitorina*

The detailed systematic consequences of our work will be dealt with elsewhere. Nevertheless, it is useful to give here some brief pointers and sound a note of caution. The original description of *L. caliginosa* used specimens collected from Orange Bay, Tierra del Fuego, and included only external morphological characteristics: a dark/brown/green shell coloration, an eroded first whorl and an aperture diameter occupying less than 50% of the total shell length (Gould, 1849). Morphological and coloration patterns found in L5 correspond to this description and specimens were collected from Navarino Island, Puerto Williams and Ushuaia Bay close to the type locality. Accordingly, L5 seems to represent the true *L. caliginosa* and, consequently, it seems that this species is restricted to the southern tip of South America, the Falkland/Malvinas Islands and South Georgia. We note that much of the biological and ecological information on what is purported to be *L. caliginosa* is based on Macquarie Island records (e.g., Reid, 1989; Simpson & Harrington, 1985), but the presence of L5 at Macquarie Island would require confirmation.

The morphological characteristics of *L. latior* (Preston, 1912) correspond well to those of found in L7. Moreover, L7 specimens were collected from very near the type locality of *L. latior*, Port Stanley, in the Falkland/Malvinas Islands (Preston, 1912). This evidence suggest that L7 is in fact *L. latior* and endemic to the Falkland/Malvinas Islands where it coexists with L5 (*L. caliginosa*).

Although the nominal Antarctic species *L. antarctica*, *L. claviformis* and *L. umbilicata* are similar in size and shape (Engl, 2012; personal observations), shells of *L. umbilicata* uniquely exhibit a profound suture and there is significant variation in colour. Thus, before formally synonymizing these taxa it will be necessary to collect and analyse more populations of *L. umbilicata* and *L. antarctica*, especially those from their respectively type localities of South Georgia and East Antarctica.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

New COI and 28S rRNA sequences in *Laevilitorina* have been deposited in GenBank under the following accession numbers: MZ321820–MZ321864 (COI), MZ322329–MZ322390 and MZ381414–MZ381445 (28S rRNA).

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BIOSKETCH

Author contributions: CAG-W, EP, HGS, NIS and SR conceived the idea of the study and designed the analyses. CAG-W, EP, SR, CSM, SM, TS, SAM, PB and NGW collected specimens from different localities across *L. caliginosa*'s distribution. CAG-W, VJ and NIS performed molecular experiments. CAG-W, VJ and NIS performed analyses of the recorded data. VJ, CAG-W and RP performed geometric morphometric analyses. C.A.G-W, EP, HGS, NIS and SR contributed intellectually to the interpretation and discussion of results. CAG-W, HGS, EP and SR wrote the paper. All authors read and approved the final version of the manuscript.

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