Divergence time and species delimitation of microbivalves in the Southern Ocean: the case of Kidderia species

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Received: 16 January 2020 / Revised: 10 May 2021 / Accepted: 19 May 2021 / Published online: 1 June 2021
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Abstract
The systematics of Subantarctic and Antarctic near-shore marine benthic invertebrates requires major revision and highlights the necessity to incorporate additional sources of information in the specimen identification chart in the Southern Ocean (SO). In this study, we aim to improve our understanding of the biodiversity of Kidderia (Dall 1876) through molecular and morphological comparisons of Antarctic and Subantarctic taxa. The microbivalves of the genus Kidderia are small brooding organisms that inhabit intertidal and shallow subtidal rocky ecosystems. This genus represents an interesting model to test the vicariance and dispersal hypothesis in the biogeography of the SO. However, the description of Kidderia species relies on a few morphological characters and biogeographic records that raise questions about the true diversity in the group. Here we will define the specimens collected with genetic tools, delimiting their respective boundaries across provinces of the SO, validating the presence of two species of Kidderia. Through the revision of taxonomic issues and species delimitation, it was possible to report that the Antarctic species is Kidderia subquadrata and the species recorded in the Subantarctic islands Diego Ramirez, South Georgia and the Kerguelen Archipelago is Kidderia minuta. The divergence time estimation suggests the origin and diversification of Kidderia lineages are related to historical vicariant processes probably associated with the separation of the continental landmasses close to the late Eocene.

Keywords Cyamiidae · Microbivalves · Phylogeny · Antarctic · Subantarctic islands

Introduction
In the light of global change, understanding the evolutionary and ecological processes underlying the origin, distribution, and maintenance of Southern Ocean (SO) biodiversity has pivotal importance in mitigating the consequences on biodiversity in this area (Aronson and Blake 2001; Linse et al. 2006; Rogers 2007; Griffiths et al. 2009; Chown et al. 2015; Hawkins et al. 2018). The species of the genus Kidderia (Dall 1876) is one of the few members of the family Cyamiidae in the SO. These small bivalves have an average shell length of 4.5 mm (Simpson 1977) with a maximum size of 7 mm, and belong to a widely distributed group of microbivalves that inhabit across the SO. The current biodiversity value of this group is required to increase knowledge of the diversification and biogeography patterns in the SO, as this is still scarce in various taxa (Geiger and Thacker 2008; Ghiglione et al. 2017). It is especially important as future projections indicate a reduction in the richness of marine species in the SO due to climate change (Griffiths et al. 2017).
Kidderia have elongated, flat shells with an internal ligament located behind the cardinal tooth. They are gregarious with direct development and inhabit intertidal and shallow subtidal rocky ecosystems (Shabica 1974; Simpson 1977). Consequently, a low potential for dispersal and a spatial distribution limited to the ice-free rocky coast of Antarctica is expected. The taxonomy of the species in this group has been developed exclusively using morphological characters and biogeographic records in Subantarctic and Antarctic localities in the SO. The type species of the genus is Kidderia minutata (Dall 1876), originally described in the Kerguelen Islands. Kidderia bicolor (Martens 1885) has been reported in South Georgia, the South Orkney, Heard and Kerguelen Islands as well as in the Weddell Sea and South Shetland Islands (Martens 1885; Hedley 1916; Simpson 1977; Clarke 1996; Zelaya 2005; Barnes et al. 2006; Engl et al. 2016). The species Kidderia subquadrata (Pelseneer 1903) and Kidderia pusilla (Gould 1850) have been reported in Subantarctic ecosystems including Patagonia, Cape Horn, the Falkland/Malvinas and South Georgia Islands (Zelaya 2005, 2015; Barnes et al. 2006). Shabica (1974) reported the presence of K. subquadrata at Doumer Island, Antarctic Peninsula and the most recent record in the literature reports the presence of K. subquadrata in the Antarctic region, on King George Island, South Shetland Islands (Aghmich et al. 2016). These species correspond to the principal species that has been described inhabiting in SO (Online Resource 1). Given this pattern of biogeographical reports, new questions arise: Are the same Kidderia species distributed in Subantarctic and Antarctic areas? What is the effectiveness of the physical barrier, such as the geographic distance that separates the Antarctic continent from the others to gene flow in this genus? What has been the role of the Antarctic circumpolar current (ACC) in promoting speciation and divergence in this genus, an isolation barrier or dispersal vector?

Two main hypotheses have been proposed to explain the current biogeography and species diversification in the SO (Patarnello et al. 1996; Linse et al. 2007; Pearse et al. 2009; Dalziel et al. 2013; Taylor et al. 2018). The vicariance hypothesis is supported by the high degree of genetic differentiation and old divergence-times in some marine taxa such as mollusks, echinoderms, crustaceans and algae (Patarnello et al. 1996; Held 2000; Linse et al. 2007; Medlin and Zingone 2007; Hunter and Halanych 2008; Jackson et al. 2015) distributed across the SO, initiated by the continental separation of the Antarctic from the others in the Jurassic period (Jokat et al. 2003; König and Jokat 2006; Eagles and Vaughan 2009), followed by the opening of Drake passage 42 Ma (Crame 1999; Clarke and Crame 2010; Hayanich and Mahon 2018), and the subsequent deepening of the Tasman Sea (34–33 Ma) (Stickley et al. 2004). These changes modified the oceanographic circulation of the SO (Stickley et al. 2004), the establishment of the ACC and the Antarctic Polar Front (32 Ma) around the Oligocene/Eocene boundary (33.9 Ma) (Clarke and Crame 1989, 1992; Orsi et al. 1995; Clarke and Johnston 2003).

The dispersal hypothesis is related to the function that the ACC fulfills by moving marine organisms around the Antarctic; it is considered the primary vector of dispersal, especially in taxa with high dispersal ability (Clarke et al. 2005; Fraser et al. 2009; Hune et al. 2015; Hayanich and Mahon 2018). The dispersal hypothesis is also supported by molecular studies that reveal the importance of dispersal and recent climatic/oceanographic processes associated with more recent divergences that help to understand the connectivity in the distribution and diversification of species present across the SO (Díaz et al. 2011; González-Wevar et al. 2012, 2019; Near et al. 2012; Poulin et al. 2014; Dueñas et al. 2016). In this study, using molecular analyses we aim to reveal the current biogeographic distribution of species Kidderia to test the hypothesis that best fits the diversity and distribution of this group across in Subantarctic and Antarctic localities in the SO.

Materials and methods

Sample collection and initial specimen identification

Specimens of Kidderia were collected from rocky intertidal ecosystems in different localities of the SO during summer 2016–2019 (Table 1, Fig. 1). In some of these sampled sites, Kidderia species were absent, however, we included records for other microbivalves species found as a confirmation of their distribution, this is the case for Lasaea sp. and Lissarca sp. (Table 1). The initial taxonomic identification was made based on a complete review of the current literature (Güller and Zelaya 2015; Zelaya 2015), as well as using classic taxonomic works (Martens 1885; Martens and Pfeffer 1886; Pelseneer 1903; Odhner 1923), systematic studies on specific taxa (Soot-Ryen 1951; Ponder 1971; Osorio and Arnaud, 1984), and academic databases available on the Internet (Morris and Rosenberg 2005; WORMS editorial board 2018). Finally, for the correct identification of the species we followed the last revision of the group (Zelaya et al. 2020), examining morphological aspects such as: shell outline, sculpture, the position of the umbones and the number and morphology of the hinge teeth.

To assist the morphological identification, we also performed scanning electron microscopy (SEM) analysis to identify key characters in the shell. The SEM analysis was performed using a Hitachi SU3500 scanning microscope at the Universidad Católica del Norte–Chile.
DNA extraction, amplification, sequencing and alignment

Collected samples were stored in 70% alcohol until DNA extraction. Total genomic DNA was isolated using the commercial kit Quick—DNA Plus according to the manufacturer’s protocol (Zymo research). Following this, different fragments of nuclear 28S rRNA (ribosomal large subunit), ITS2 (internal transcribed spacer 2) and mitochondrial (COX1) markers were amplified using polymerase chain reactions (PCR). In the case of 28S rRNA forward primer described by Littlewood et al. (2000) and reverse primer described by Williams et al. (2003) were used. The primers for ITS2 are described by Cheng et al. (2006). Given the absence of genetic information for the genus Kidderia, the strategy to generate molecular markers was to perform MiSeq Illumina sequencing using the genomic DNA and an in-silico enrichment to obtain fragments of the mitochondrial genome. Then, a species-specific primer was designed to amplify a partial fragment of COX1 (approximate 1000 pb): kg8F (TTG GGC TGG GTT AAT AGG TACA-3′) as a forward primer and kg7R (5′-GAA AAC CAG CAA ACA TAG CA-3′) as a reverse primer.

All PCR were performed in a final volume of 25 μl containing 1X PCR buffer, 3.5 mM MgCl₂, 0.2 mM of each dNTP, 0.25 μM of each primer, 0.2X BSA, 1 μl DNA (10–50 ng), 0.6 units GoTaq DNA polymerase (Promega) and H₂O to reach the final volume. For 28S rRNA, the thermocycling program included an initial denaturation step (95 °C for 3 min); 40 cycles of amplification (94 °C for 45 s), annealing at 52 °C for 45 s and extension (72 °C for 2 min), and a final extension step (72 °C for 10 min). For ITS2, the thermocycling program was according to Cheng et al. (2006). The COX1 gene was amplified using

### Table 1 Sampling sites, coordinates and the number of Genbank accession for microbivalves collected to this study

| Nº | Sample sites          | Coordinates         | Species                  | Nº of specimens | 28S rRNA Accession | ITS2 Accession | COX1 Accession |
|----|-----------------------|---------------------|--------------------------|-----------------|--------------------|----------------|----------------|
| 1  | Penguin I. 62°05′S; 57°55′W | Kidderia subquad-rata | 3                        | MT712340-MT712342 | MT712366-MT712368 | MT725757-MT725759 |
| 2  | King George I. (Fildes bay) 62°05′S; 57°56′W | Kidderia subquad-rata | 2                        | MT712344-MT712345 | MT712370-MT712371 | MT725754-MT725755 |
| 3  | Greenwich I. 62°48′S; 59°66′W | Kidderia subquad-rata | 2                        | MT712346-MT712347 | MT712372-MT712373 | MT725752-MT725753 |
| 4  | Livingston I. 62°39′S; 60°36′W | Kidderia subquad-rata | 1                        | MT712343 | MT712369 | MT725756 |
| 5  | Deception I. 62°58′S; 60°33′W | Kidderia subquad-rata | 3                        | MT712351-MT712353 | MT712377-MT712379 | MT725746-MT725748 |
| 6  | Doumer I. 64°52′S; 63°35′W | Kidderia subquad-rata | 3                        | MT712348-MT712350 | MT712374-MT712376 | MT725749-MT725751 |
| 7  | Signy I. 60°43′S; 45°36′W | Kidderia subquad-rata | 3                        | MT712337-MT712339 | MT712363-MT712365 | MT725737-MT725760-MT725761 |
| 8  | Isabel Riquelme I. (Antarctic peninsula) 63°19′S; 57°53′W | Lissarca sp | 1                        | MT712336 | MT712362 | – |
| 9  | Diego Ramírez I. 56°31′S; 68°42′W | Gainardia sp1 | 1                        | MT712361 | MT712387 | MT725738 |
|    |                       | Kidderia sp | 1                        | MT712358 | MT712384 | MT725744 |
|    |                       | Gainardia Baham-ondei | 1                        | MT731649 | – | MT725745 |
| 10 | Hornos I. 55°58′S; 67°13′W | Gainardia sp2 | 1                        | MT712360 | MT712386 | MT725739 |
| 11 | Puerto Williams 54°56′S; 67°37′W | Lasaea sp | –                        | MT732563 | – | – |
| 12 | San Gregorio 52°34′S; 70°4′W | Lasaea sp | –                        | MT732564 | – | – |
| 13 | Falkland/Malvina-nas I. 51°33′S; 50°23′W | Lasaea sp | –                        | MT732562 | – | – |
| 14 | South Georgia I. 54°26′S; 36°33′W | Kidderia minuta | 2                        | MT712356-MT712357 | MT712382-MT712383 | MT725742-MT725743 |
| 15 | Kerguelen I. 49°28′S–69°14′E | Gainardia trapesina | 1                        | MT712354-MT712355 | MT712380-MT712381 | MT725741 |
the following thermocycling profile: an initial denaturation step (95 °C for 5 min); 35 cycles of amplification (94 °C for 30 s, 60 °C for 1 min and 72 °C for 2 min); and a final extension step (72 °C for 8 min). The annealing temperature was modified to 50°C (South Georgia and Kerguelen individuals) and 49 °C (Diego Ramirez individuals) for the PCR performed with Subantarctic individuals of Kidderia. The PCR products were purified using the E.Z.N.A. Cycle Pure PCR Purification Kit (Omega Bio-tek) and sequenced in both directions in the ABI PRISM® 310 Genetic Analyzer of the core-facility AUSTRAL-omics (www.austral-omics.cl).

All sequences were assembled, examined, and aligned in Geneious R10.2.4 software (Kearse et al. 2012) and manually edited to resolve unclear base calls. Sequences of each gene were aligned individually using the MAFFT v7.388 algorithm implemented in Geneious with G-INS-I strategy (Katoh and Standley 2013). Then, the Translate function offered by the Geneious program for coding genes was applied. Finally, the sequences were concatenated using the software Geneious.

**Species delimitation and phylogenetic analysis**

The following approaches were used to delimit the species of this study. First, the General Mixed Yule Coalescence (GMYC) single and multiple threshold model was used, applying the “Species Limits by Threshold Statistics” approach (SPLITS v1.0-19) in the R program (www.r-project.org) (Pons et al. 2006). A second method was used to delimit the species—the Bayesian implementation of the Tree Poisson Process (PTP) model, using a distance-based tree to delimit species implemented in the web server for PTP (www.species.h-its.org/ptp/) (Zhang et al. 2013). The supports of this analysis are presented as posterior probability (PP). For GYMC and PTP, an ultrametric tree was generated in BEAST2. The analysis was run for $30 \times 10^6$ generations with parameters sampled every 1000 generations.

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Fig. 1 a General map shows Subantarctic localities b Map of Antarctic Peninsula localities sampled in this study
and a burn-in value of 10%. A tree file was generated with TreeAnnotator and visualized in FigTree v.1.4.2 (Rambaut 2014). The log file (produced in BEAST) was analyzed in Tracer V1.6 (Rambaut et al. 2016) to assess for convergence. Finally, the automatic barcode gap discovery method (ABGD) was used. This method delivers species boundaries based on patterns of pairwise genetic distances, providing estimates of a maximum limit for intraspecific genetic divergence and using this limit to group sequences belonging to the same species (with less divergence) from sequences belonging to different species (with greater divergence) (Puillandre et al. 2012).

We used maximum likelihood (ML), and Bayesian inference (BI) approaches to explore their phylogenetic relationship. The best evolutionary model was chosen through the JModeltest2 program (Darriba et al. 2012) in XSEDE, available on the CIPRES online platform (www.phylo.org/). The selection of the best model used corrected Akaike criterion (AIC) for ML and Bayesian criterion (BIC) for BI analysis. The selected models for the 28S rRNA gene were GTR+I for ML analysis and TIM3+I for BI analysis. For the ITS2 gene, the selected models were TPM3uf+G for the ML analysis, and TN93 for the BI analysis; and finally, for the Cox1 gene, the TIM1+G models were selected for the ML analysis and HKY+G for the BI analysis. The ML and BI analyses were performed by gene and with concatenated data (ITS2; 28S rRNA; Cox1), using the IQtree web server (http://iqtree.cibiv.univie.ac.at/) (Trifinopoulos et al. 2016) with 1000 bootstrap for ML analysis. BI analysis was implemented in the BEAST2 software package (Bouckaert et al. 2014) with four chains (three heated chains and one cold chain) running for 30 million generations and sampling every 10,000 generations. We discarded the first 10% of trees as “burn-in” and the remaining trees were used to generate a majority-rule consensus tree (Felsenstein 1985). Gaimardia and Lissarca specimens found in this study were sequenced and used as outgroups based on their relationships with Kidderia (sister genus and a more distant outgroup, respectively) (Odhner 1923; Soot-Ryen 1951; Ponder 1971).

Divergence time estimations

Strict clock analysis was used for 28S rRNA/ITS2 concatenated sequences with an uncorrelated lognormal (UCLN) model of molecular evolutionary rate heterogeneity and the GTR+G substitution model implemented in BEAST2. In the Gaimardia clade, an age prior with normal distribution was applied (mean 42.9; S.D. 0.085) to the time of the oldest Antarctic Gaimardia fossil from the La Meseta Formation during the Eocene (Zinsmeister 1984; Stilwell 1992; Beu 2009). We also included another prior in the Kidderia clade from Antarctic specimens: Costokidderia (today synonymized with Kidderia sp.) (mean 3.95; S.D. 0.085), a Pliocene fossil from King George Island (South Shetland Islands) (Gazdzicki and Pugaczewska 1984). We also specified the molecular clock prior in BEAST2 for 28S rRNA and ITS2 of 0.1% and 1% per Ma, respectively, (Anne 2006). A Yule model speciation prior was used for branching rates in the phylogeny and 50×10⁶ generations were run; trees were sampled every 1000 generations. Given the identity of our Gaimardia sequence and the sequence of G. trapesina from Genbank (accession number KX713385), we also included the last one in the analysis.

We repeated the same analysis with mtDNA (Cox1) using relaxed clock analysis with a UCLN model of molecular evolutionary rate heterogeneity, the HKY+G substitution model and the same age priors described above. We performed several initial runs considering a range of Cox1 rates that have been proposed for several other marine bivalves from 0.10 to 2.0% per Ma as lower and upper thresholds (Marko and Moran 2002, 2009; Wilke et al. 2009). We used a Bayes factor analysis to define and select a conservative molecular clock rate of 1% per Ma (Wilke et al. 2009; Li and Drummond 2011). The convergence of model parameters was reviewed in the log file in TRACER v1.6.

Results

In ten of the 15 sample sites, Kidderia species were found (Table 1; Fig. 1). The Subantarctic individuals (Diego Ramírez, South Georgia and Kerguelen Islands) were identified morphologically as K. minuta as well as those from the Antarctic islands King George, Penguin, Greenwich, Livingston, Deception and Doumer identified as K. subquadrata (Table 1). Individuals of K. subquadrata have a small, inequilateral, oblong, rather compressed laterally, white and reddish-brown shell; the sculpture consists of fine dense concentric striae and includes two cardinal teeth in the left valve (Fig. 2a–c). The representative character of the species K. minuta is the presence of rounded, inflated, nearly terminal umbones; the surface of the shell has minute rounded incremental concentric ridges and the cardinal teeth are very minute (Fig. 2d–h).

In total, 22 individuals from Subantarctic (Diego Ramírez, South Georgia and Kerguelen islands) and Antarctic localities (Penguin, King George, Greenwich, Livingston, Deception and Doumer) were sequenced successfully. The alignment of the three concatenated genes was a total of 2639 bp. The 28 s rRNA gene is composed of 1305 bp, with 52 variable sites and 56 parsimonious sites; 473 bp of the alignment correspond to the ITS2 gene, with 88 variable sites and 86 parsimonious sites and finally the Cox1 gene is composed of 861 bp, where 168 sites were variable and 147 parsimoniously informative.
Species delimitation and phylogenetic analysis

We used the concatenated database for species delimitation analysis and constructed unrooted and ultrametric trees (Fig. 2). The summary bars of species delimitation results are shown in Fig. 2i. The GMYC analysis identified three putative species, two in the Antarctic clade (A) and one in the Subantarctic clade (SA). Two groups were delimited with the single-threshold method (confidence limits = 2–4; LR test value = 0.0009, \( p < 0.01 \)) while the multiple thresholds delimited three groups (confidence limits = 3–4; LR test value = 0.0001, \( p < 0.01 \)). The likelihood of the GMYC model for the single and multiple threshold models (LGMYC = 154.40) was compared to the likelihood of the null model (\( L_0 = 147.57 \)). The result of the Bayesian implementation of the Process Tree Poisson model (bPTP) with concatenated rooted trees detected 2 putative species with good bayesian support, Species 1 (\( PP = 0.91 \)) composed of the Subantarctic clade (SA) (Subantarctic Islands Kerguelen, South Georgia and Diego Ramirez), and Species 2 (\( PP = 0.99 \)) composed of an Antarctic clade (A) (Antarctic Islands: Signy, King George, Penguin, Greenwich, Livingston, Deception and Doumer). The ABGD analysis showed a tri-modal pairwise genetic distance (K2P) distribution with a clear and wide barcode gap located between 1 and 14% genetic distance. This method detected two stable putative species with estimated prior maximum divergences of intraspecific diversity as large as 10% (Fig. 2i) (one-tailed 95% confidence interval).

Phylogenetic analysis based on multiple markers and methods recognized the presence of two main clades within Kidderia with high values of bootstrap support and posterior
probability (Fig. 2i). The first includes all the Subantarctic specimens analyzed (Diego Ramirez, South Georgia, Kerguelen), while the second includes the Antarctic specimens (Fig. 2i). The genetic divergence between the Subantarctic and Antarctic lineages ranged from 4.0 to 4.3% (28S rRNA), 18.3 to 19.8% (ITS2) and 16.1 to 18.0% (Cox1). Phylogenetic reconstructions based on each separate gene are shown in Online resource 2.

**Divergence time estimations**

The time estimation analysis for the divergence among Subantarctic and Antarctic Kidderia lineages with 28S rRNA/ITS2 concatenated dataset revealed a time around 36.43 Ma (44.87–28.35 Ma; 95% Height posterior density, HPD) that indicated an ancient divergence during the late Eocene (Fig. 3). A similar result was obtained when the analysis was performed using mtDNA (Cox1), 34.08 Ma in the Eocene/Oligocene boundary (14.94–56.53 Ma; 95% HDP; Online resource 3).

**Discussion**

The present study revealed morphological and genetic evidence that differentiates two Kidderia lineages, with a time of divergence associated with historical events that occurred millions of years ago in the SO. Many of the microbivalve species that inhabit the rocky intertidal and shallow subtidal areas were described between 1850 and 1980, since then there has not been an exhaustive revision in the genus Kidderia (Philippi 1845; Gould 1850; Dall 1876; Martens 1885; Pfeffer 1886; Pelseneer 1903; Odhner 1923; Soot-Ryen 1951; Dell 1964; Ponder 1971). The records of the classical literature are not clear enough to specify the type locality for all described species of Kidderia. In addition, some relatively recent studies based on species checklists may be erroneous, especially if there is no evidence that they were effectively sampled in a specific location. The changes of names and taxonomic assignments generate errors in the identification of species that were described a long time ago and whose presence is currently being re-evaluated. Zelaya (2005) reports that there are 12 species of Cyamiidae.
inhabiting the Subantarctic ecoregions, of which 5 species belong to the genus Kidderia (Online resource 1).

In the revision of Ponder (1971) of the superfamily Cyamiacea, he describes that the species K. minuta, K. pusilla, K. bisulcata (EA Smith 1877), K. campbellitica (Odhner 1924) and K. bicolor belong to the type genus of Kidderia which does not present teeth, or only one in each valve, of which K. minuta is the type species of the genus. However, in the bivalve catalog of Bernard (1983), he reported that K. minuta and K. bicolor are junior synonyms for K. pusilla. Therefore, throughout the history of this genus and by the material reviewed in this study, it has been observed that there are certain morphological similarities between the species of the type genus of Kidderia (Ponder 1971). However, as mentioned by Dall (1876) and observed in this study, the color of the individuals of K. minuta is different (more whitish in the umbones and more reddish in the upper posterior surface), and the shape of the cardinal tooth is also different.

Regarding the present taxonomic status of the Kidderia species, it is important to note that in the Ponder review (1971), he indicated that the species Cyamium subquadratum should be included within the genus Kidderia. The accepted name of K. subquadrata in WORMS is Mysella subquadra-tum (Engl 2012). This genus belongs to the family Lasaeidae (MolluscaBase 2018), although the status is considered as “taxon inquereundum” (MolluscaBase 2018). However, Dell (1964) and Soot-Ryan (1951) considered the species K. subquadra-ta to be a member of the family Cyamiidae. However, in the latest review by Zelaya et al. (2020), these authors concluded that based on morphology, anatomy and DNA, the species of the genus Kidderia and Gaimardia belong to the family Gaimardiidae. However, these authors did not include DNA sequences of Kidderia species. Finally, our results would indicate that the Antarctic lineage would correspond to the species K. subquadrata. In future research of the group, phylogenetic relationship with other taxa such as Heteromactra and Cyamium should be addressed.

Species delimitation

To generate new information and to advance in our understanding of microbivalve evolution, here we introduced the use of a DNA based-identification approach to clarify the taxonomic issues previously mentioned. The multilocus trees provide a valuable source of information for inferring the pattern and processes of diversification (Fujisawa and Barraclough 2013). While PTP analysis is considered faster and more robust (Zhang et al. 2013), ABGD is a simple method to split a sequence alignment dataset based on the distribution of pairwise differences, as such these approaches to species delimitation were concordant in identifying two species of Kidderia. With morphological identification, the Subantarctic lineage is assigned as K. minuta while the Antarctic lineage correspond to K. subquadrata (Fig. 2i). However, the GYMC analysis showed three putative species, specifically in the Antarctic clade. The GYMC analysis may suggest a population genetic structure or a more complex lineage classification (Pons et al. 2006; Reid and Carstens 2012; Talavera et al. 2013; Tang et al. 2014). Furthermore, when a single specimen that differs from the others by one nucleotide substitution is found, GMYC tends to diverge in the presence of a low intraspecific divergence (Pentinsaari et al. 2017; Luo et al. 2018). The occurrence of multiple lineages in the Antarctic could be a consequence of a speciation process explained by repeated glacial–interglacial events that have left characteristic signatures of limited gene flow and the occurrence of multiple independent refuges, fragmented populations, and isolation, especially in brooders (Pearse et al. 2009; Chown et al. 2015; Riesgo et al. 2015; Hayanich and Mahon 2018; Lau et al. 2020).

A serious limitation to the utility of DNA barcoding as a practical resource for regulation and molecular diagnostics is human error and uncertainty in creating and curating reference libraries (Collins and Cruikshank 2013). The microbivalves from Antarctic are not the exception. In fact, to have more confidence in our results we decided to generate sequences from the sister group Gaimardia to deal with unambiguous species-level identification. Moreover, in a recent work about review the taxonomy of the Cyamioidea confirm the placement of Gaimardia and Kidderia in Gaimardiidae (Zelaya et al. 2020). We are confidence that the extensive sampling design, the use of three molecular markers, and the integrative morphology-based and molecular approach are the main strengths of our study. The results presented in our work will contribute to clarifying the systematics, taxonomy and biogeography of the genus along with its diversity across the SO.

Divergence time estimation

The divergence time estimation between Subantarctic and Antarctic Kidderia lineages is related to past geographic and oceanographic events. Relevant tectonic events initiated in Jurassic times, where the South American and Antarctic continents started moving since at least 150 Ma (Jokat et al. 2003; König and Jokat 2006; Eagles and Vaughan 2009). The period between Paleocene and Eocene has been correlated with vicariance hypothesis caused by continental drift (Pearse et al. 2009). Later, a significant cooling event occurred at 37 Ma, well known as the Antarctic cooling, a climatic process started at the Paleocene–Eocene boundary (55 Ma) (Crame 1999; Thatje 2012; Hayanich and Mahon 2018). In the Eocene, the breakup of continents was complete with the opening of the Drake Passage (42 Ma) (Crame 1999; Eagles et al. 2006; Scher and Martin 2006; Clarke and Crame 2010; Hayanich and Mahon 2018), which led to the
deepening of the sTasman Sea (34–33 Ma) (Stickley et al. 2004) and the establishment of the Antarctic Circumpolar Current and the Antarctic Polar Front around Oligocene/Eocene boundary (34 Ma) (Clarke and Crame 1989, 1992; Orsi et al. 1995; Pudsey and Howe 1998; Rintoul et al. 2001; Clarke and Johnston 2003; Stickley et al. 2004; Pfuhl and McCave 2005).

The role of the ACC has been very important in understanding the richness of species in the SO. Its influence on the existence and formation of several species with a varied time of divergence is widely accepted (Pearse et al. 2009). The ACC has been flowing through and around the Antarctic for more than 30 million years with occasional dispersal events, greatly impacting incubator species and establishing new isolated populations that could drive a speciation process before secondary contact of isolated populations, however, it is also known that the ACC currently maintains the link between spatially isolated populations in different taxa inhabiting the SO (Alcock and Strugnell 2012). While the ACC acted as a barrier between Subantarctic and Antarctic populations in species such as Parborlasia corrugatus (Thornhill et al. 2008) and species of the genus Ophiomastus (Hunter and Halanych 2010; Janosik et al. 2011) other species remain genetically connected across the SO, such as Astronima agassizii (Hunter and Halanych 2008) and Siphonaria species (González-Wevar et al. 2018). In other phylogeographic studies in non-buoyant macroalgae species, evidence of recent colonization explained by exceptional events of rafting and current reproduction and recruitment occurring on a very small scale (Guillemin et al. 2020) has been reported. In addition, recent studies have reported a relaxation of the ACC which has been observed through kelp rafting (Durvillaea antarctica and Macrocystis pyrifera), suggesting that the ACC could act as a dispersion agent for marine organisms, specifically, for species that at present are non-native in the Antarctic (Avila et al. 2020; Macaya et al. 2020).

Many successful colonizations appear to have preceded Pliocene intensification of the ACC (Chown et al. 2015). In bivalves, there are some cases reported in the literature. For example, Linse et al. (2007) studied different populations of the brooding bivalve Lissarca notorcadensis and found a divergence between Subantarctic and Antarctic groups. This study presented one of the first pieces of evidence of the action of physical barriers that limit the gene flow between these provinces, particularly in these types of marine organisms. In another study, Jackson et al. (2015) estimated the origins of Philobryidae species around 66 Ma for the. A recent report about the divergence time estimation in the bivalve species Aequioldia eightsii by González-Wevar et al. (2019) suggests that the populations from Kerguelen Island separated from the South American and Antarctic lineages during the middle Miocene (16.5 Ma), while the separation between Antarctic and South American A. eightsii populations occurred at the end of the Miocene (8.5 Ma). In other examples, Page and Linse (2002) concluded a more recent speciation in Limatula species (divergence time of 8.03–5.79 Ma) and questioned the vicariance hypothesis. These examples show the relevance to incorporate and evaluate more species with contrasting development modes. In recent years, molecular tools have revealed that each species from the SO appears to have a particular life history. In this way, obtaining a general biogeographic pattern of marine organisms that inhabit the SO is more complex than previously thought (Eastman and McCune 2000; Clarke et al. 2007; Hunter et al. 2008; Leese et al. 2010; Macaya and Zuccarello 2010; Hoffman et al. 2012; Haynich and Mahon 2018). Our evidence revealed that the divergence of Kidderia species is related to the complete separation of the Antarctic from the other continents and the establishment of the ACC, in the timeframe that supports the vicariance hypothesis, around late Eocene.

**Conclusion**

Here, we used classical and molecular approaches to identify two Kidderia species. This study allowed us to contribute new genetic records to advance the understanding of micro-bivalve evolution. With the verification of their presence in these environments, it was possible to investigate their evolutionary history, and therefore, provide useful information to clarify the evolutionary patterns of diversity on a broad scale (Avise 2009). We found the species K. subquadrata in the Antarctic Peninsula region and K. minuta in Subantarctic islands. Greater sampling effort is necessary to verify the presence of other species described in Subantarctic environments such as K. bicolor, K. pusilla and K. bisulcata and other diversity of the genus described in New Zealand.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s00300-021-02885-6.

**Acknowledgements** This work was supported and funded by the National Commission of Scientific and Technological Investigation of Chile through the Fondo de Financiamiento de Centros de Investigación en Areas Prioritarias (FONDIAP) program Research Center: Dynamics of High Latitude Marine Ecosystems (Grant No. 15150003) and by the Instituto Antartico Chileno through the INACH RT 02-15 and INACH MG 06-17 grants. LC acknowledge to Fondecyt 1170591. SR acknowledge to PIA CONICYT ACT172065 and PIA CONICYT APOYO CCTE AFB170008 from the Institute of Ecology and Biodiversity (IEB). We also acknowledge the support of Dr. Élie Poulin and his group in sampling and the project Píve program PROTEKER (No. 1044) of which they are a part and we would like to thank the Subantarctic Conservation Program of the Universidad de Magallanes for all their logistical support in the expedition to Diego Ramirez. Finally, we would like to thank Dr. Claudio Gonzalez-Wevar for his first review and comments on the manuscript. We are thankful to Tomas Saucede.
and two anonymous reviewers for their meticulous revision that helped to improve this manuscript. We thank the Editor in Chief, Dieter Pippenburg for their work searching reviewers during these COVID-19 times, as well as their final comments to complete the editorial process. Finally, we thank the English language revision service offered by https://traduccionescientificas.cl/.

Author contributions LC and DL designed the project and LC, DL and SR collected the samples. DL performed the experiments and the data analysis. SR identified the species and revised the manuscript. LC and DL wrote the manuscript.

Declarations

Conflict of interest No potential conflict of interest was reported by the authors.

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