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Abstract

The species of the genus *Aequiyoldia* Soot-Ryen, 1951, previously known as *Yoldia*, are common, soft-substratum, sareptid bivalves. In the Southern Ocean, *Aequiyoldia eightsii* (Jay, 1839) was originally described from the Antarctic Peninsula and has also been reported in southern South America, whereas *A. woodwardi* (Hanley, 1960) was recorded for the Falkland/Malvinas Islands and Tierra del Fuego, southern South America. *Aequiyoldia* has received little attention across its distribution in the Southern Ocean, and although its taxonomy and systematics remain uncertain, all species of *Aequiyoldia* have been grouped under the same broadly distributed unit: *A. eightsii*. However, molecular studies have demonstrated a marked mtDNA genetic divergence (> 7%) between *A. eightsii* populations from South America and Antarctic Peninsula. In order to further understand the diversity and biogeography of

Aequiyoldia, we analyzed *A. eightsii* populations from different provinces of the Southern Ocean including South America (SA), the Falkland/Malvinas Islands (FI), Antarctic Peninsula (AP), and Kerguelen Islands (KI). Individuals were characterized according to typical diagnostic morphological measurements, and phylogenetic relationships were reconstructed based on mtDNA (Cytochrome c Oxidase Subunit I). Patterns of genetic divergence of nucDNA intergenic transcribed spacers (ITS1, ITS2) were also estimated. The statistical analysis of external diagnostic characteristics revealed two morphotypes: (1) individuals with the morphology recorded for the nominal FI species, *A. woodwardi*, and (2) individuals from SA, AP, and KI, with the morphology recorded for *A. eightsii*. However, phylogenetic reconstructions based on mtDNA and nucDNA suggest the presence of at least five lineages within *A. eightsii* including: one lineage in Kerguelen Island, two lineages in Antarctic Peninsula, one lineage in South America, and the last one restricted to the Falkland/Malvinas Islands. Despite their very similar morphologies, AP, SA, and KI populations of *Aequiyoldia* exhibited also marked levels of genetic divergence. Such results are evidence that the Antarctic Polar Front represents an historical biogeographic barrier for this group and that after the separation of these lineages, they followed independent evolutionary pathways in different provinces of the Southern Ocean. Estimates of divergence time suggest that KI separated from the other lineages close to the middle Miocene. Following this, the separation between the AP and SA lineages occurred at the end of the Miocene around 7.5 Ma. Finally, *Aequiyoldia* diversified during the Pliocene in Antarctic Peninsula (~4.5 Ma) and South America (~3.0 Ma). Individuals from FI exhibited morphological differences, and 4% of divergence from South American individuals, suggesting that *A. woordwardi* could be revalidated. Similarly, the marked molecular divergence between the KI and the rest of the recorded lineages also support the validity of *A. kerguelensis* (Thiele, 1931).

1. Introduction

Southern-Ocean marine fauna has been traditionally associated with low diversity, mainly because of extreme environmental conditions and several groups including teleost fish, gastropods, bivalves, and some crustacean groups are scarcely represented in this region (Chown et al., 2015). Nevertheless, more than 8000 marine species are currently known and most of them have high levels of endemism (50% to 97%) (De Broyer and Koubbi, 2014). In fact, the expected number of Antarctic species ranges between 11,000 and 17,000 species. Accordingly, this continent seems to have intermediate species richness compared to other tropical, temperate or Arctic environments (Gutt et al., 2004). Therefore, the Antarctic marine biota is much more diverse than previously thought, and its distribution, composition, and abundance is the outcome of major tectonic, oceanographic, and climatic changes operating in the region since the Mesozoic (Crame, 1999; Zachos et al., 2001; Clarke et al., 2005; Linse et al., 2006; Aronson et al., 2007; Griffiths et al., 2009; Poulin et al., 2014).

The opening of major gateways in the region, the isolation of the Antarctic continent and the establishment of the Antarctic Circumpolar Current (ACC) are profoundly coupled to the origin and diversification of several Southern Ocean marine benthic groups (Clarke and Crame, 1989; Clarke et al., 1992, 2005; Aronson et al., 2007; Griffiths and Waller, 2016). The ACC is the major current system on the planet and is delimited by two main fronts: 1) the sub-Antarctic Front (SAF) and 2) the Antarctic Polar Front (APF) that have major influence over the distribution of the marine benthic biota (Dell, 1972; Crame, 1999; Rintoul et al., 2001; Aronson et al., 2007; Knox, 2007; Griffiths et al., 2009; Pierrat et al., 2013; Saucède et al., 2014; Griffiths and Waller 2016; González-Wevar et al., 2017). The APF might limit latitudinal movement of species enough to act as an effective barrier to connectivity between Antarctic and sub-Antarctic provinces. In fact, molecular analyses in several co-distributed Antarctic and South American invertebrates including the nemertean *Parborlasia corrugatus* (Thornhill et al., 2008), the brittle star *Astrotoma agassizzii* (Hunter and Halanych 2008), the pycnogonid *Collossedeis megalonix* (Krabbe et al., 2010), the bivalve *Yoldia* (González-Wevar et al., 2012; Poulin et al., 2014), and the red algae *Gigartina skottsbergii* (Billard et al., 2015) have revealed differences in evolutionary history with marked molecular divergence, showing that they have been isolated for several millions of years (Clarke et al., 2005; Barnes et al., 2006; Aronson et al., 2007; Wilson et al., 2009; González-Wevar et al., 2010, 2017; Moon et al., 2017). Conversely, in the sub-Antarctic, the SAF can transport organisms between geographically distant provinces. This is especially true for species with high dispersive potential (Gérard et al., 2008; Díaz et al., 2011; Mortimer et al., 2011), kelps (Waters, 2007; Fraser et al., 2009; Macaya and Zuccarello, 2010), kelp-associated organisms (Fraser et al., 2011; Nikula et al., 2010; Cumming et al., 2014; González-Wevar et al., 2018; for a review see Moon et al., 2017), and even non-buoyant macroalgae (Fraser et al., 2013).

Recent biogeographic analyses based on taxonomic lists recognized a highly endemic Southern-Ocean biota, the presence of a single Antarctic province, and a clear separation between Antarctica and other

sub-Antarctic provinces (Griffiths et al., 2009; Pierrat et al., 2013; Griffiths and Waller, 2016). During the last decade, the use of molecular tools for the taxonomic identification of organisms with conserved morphology greatly increased the number of taxa in the Southern Ocean through the recognition of cryptic species in several invertebrates groups such as crustaceans (Held and Wägele, 2005; Raupach and Wägele, 2006; Leese et al., 2008; Krabbe et al., 2010; Arango et al., 2011; Baird et al., 2011; Dietz et al., 2015), mollusks (Strugnell et al., 2008, 2012; Wilson et al., 2009; González-Wevar et al., 2010; Allcock et al., 2011), echinoderms (Wilson et al., 2007; Heimeier et al., 2010; Janosik et al., 2011; Hemery et al., 2012; Gubili et al., 2016), nematodes (Lee et al., 2017), and even macroalgae (Billard et al., 2015). In this context, the broadly distributed Southern Ocean bivalve *Yoldia eightsii* shows a similar pattern with the presence of different lineages at both sides of the Drake Passage (González-Wevar et al., 2012; Poulin et al., 2014).

The genus *Yoldia* includes ~ 20 species of saltwater bivalves broadly distributed around the globe. Until 2013, the higher-latitude species, *Yoldia eightsii*, was recognized as broadly distributed in different provinces of the Southern Ocean including South America, Antarctic Peninsula, and the Kerguelen archipelago. Nevertheless, multi-locus molecular reconstructions identified *Yoldia* as paraphyletic with the Antarctic *Y. eightsii* completely separated from the rest of the species (Sharma et al., 2013). Following this, higher latitude *Y. eightsii* has been included in the genus *Aequiyoldia* Soot-Ryen, 1951. This genus of sareptid bivalves is currently distributed in Antarctic and sub-Antarctic soft-substratum ecosystems and includes only two nominal species: *A. defossata*, a Pliocene fossil from East Antarctica (Quilty et al., 2016) and the broadly distributed *A. eightsii* (Jay, 1839). Several synonymous species have been grouped within *A. eightsii*, including *Yoldia kerguelensis* (Thiele, 1931) and *Y. subaequilateralis* (E.A. Smith, 1875) from the Kerguelen archipelago, and *Yoldia woodwardi* (Hanley, 1860) from the Falkland/Malvinas Islands.

The absence of clear diagnostic characters and the highly conserved morphology of protobranch bivalves impose major taxonomic uncertainties within this group (Bouchet and Huber, 2015; Sartori, 2016a, b). In fact, Antarctic Peninsula and South American populations of *A. eightsii* are morphologically identical, although Antarctic individuals are larger than those recorded off South America (Villarroel and Stuardo, 1998). At the same time, morphological characteristics of the Kerguelen Islands species, *A. kerguelensis* and *A. subaequilateralis*, completely overlap those recorded for *A. eightsii* from Antarctic Peninsula and South America (Rabbarts & Whyborn 1979). Following this, *A. eightsii* is currently recognized in different Southern Ocean provinces including the Falkland/Malvinas Islands, Kerguelen Islands, Antarctic Peninsula, and South Shetland Islands (Linse, 2014). However, recent molecular analyses using the mitochondrial locus cytochrome c oxidase subunit I (COI) recognized more than 7% divergence between South American and Antarctic Peninsula populations of *A. eightsii*, which is far beyond the intraspecific level (Gonzalez-Wevar et al., 2012; Poulin et al., 2014).

In this study, we performed phylogenetic reconstructions, divergence-time estimations and morphological comparisons of *Aequiyoldia* populations from different provinces of the Southern Ocean. We extended previous molecular and morphological analyses by including *Aequiyoldia* populations from the Kerguelen Islands and the Falkland/Malvinas Islands. We also included more populations from South America and from Antarctic Peninsula. Through this, we obtained a clear picture about the evolutionary relationships within *Aequiyoldia*, the taxonomic status of different populations across the distribution of the group, the number of evolutionary units, and their respective divergences based on mitochondrial (COI) gene sequences. We also compared *A. eightsii* populations using nucDNA sequences (ITS1, ITS2) to corroborate the biogeographic patterns recorded with the mitochondrial markers. Finally, shell morphometry was conducted by applying the criteria described by Rabarts and Whybrow (1979).

2. Material and Methods

2.1 Sample collection

Aequiyoldia eightsii specimens were collected by SCUBA diving off the Kerguelen (KI) and Falkland Islands (FI), South America (SA) (Porvenir, San Juan River, Skyring Sound), and Antarctic Peninsula (AP) (Admiralty Bay, Fildes Bay, Deception Island, Rothera Station, Marguerite Bay, Adelaide Island) (Fig. 1 and 2, Table 1). All specimens were stored in 96° ethanol for future molecular analyses. For comparative purposes, we also included one individual of *Portlandia isonota* collected off the Kerguelen Islands.

2.2 DNA preparation and sequence edition

Genomic DNA was extracted from mantle and branchial tissues using the E.Z.N.A. Mollusc DNA Kit (Omega bio-tek). A 621-bp fragment of the cytochrome c oxidase subunit I (COI) was amplified using universal primers (Folmer et al., 1994). The PCR program consisted of 5 min at 95°C followed by 40 cycles at 95°C for 40 s, 45°C for 40 s, 72°C for 1 min, and a final extension of 7 min at 72°C. Fragments of up to 1190 bp containing the internal transcribed spacers 1 and 2 (ITS1 and ITS2) were amplified using universal primers (Oliverio and Mariottini, 2001). The PCR program was 5 min at 95°C, then 40 cycles at 95°C for 1 min, 54°C for 1 min, 72°C for 1 min, and a final extension of 7 min at 72°C. Amplified PCR products were purified using QIAquick Gel Extraction Kit (QIAGEN) and sequenced in both direction with an Automatic Sequencer ABI3730 x 1 at Macrogen Inc. (Seoul, South Korea). Chromatograms were inspected visually, and multiple alignments were obtained using MUSCLE (Edgar, 2004) implemented in CodonCode Aligner v7.1 (CodonCode Corporation). Base composition of nucleotide sequences was analysed with MEGA 6 (Tamura et al., 2013) and codon usage was determined using the effective number of codon value (ENC; Wright, 1990) with DnaSP 5.0 (Librado and Rozas, 2009). New *Aequiyoldia* sequences will be submitted to GenBank under accession numbers: XXXXX – XXXXXX.

2.3 Phylogenetic reconstructions

Mitochondrial reconstructions included the different haplotypes of *Aequiyoldia* recorded in SA, AP, FI, and KI. For comparative purposes, we included other protobranch genera (*Yoldiella*, *Yoldia*, *Tindaria*, *Neilonella*) in the reconstructions following Sharma *et al*. (2013). Phylogenetic relationships were estimated using maximum parsimony (MP), maximum likelihood (ML), and Bayesian analyses (BA). MP analyses were performed using MEGA v.6.0 (Tamura et al., 2013) and the following assumptions: characters were treated as equally weighted using a heuristic search and tree bisection reconnection (TBR) with the branch swapping option. Non-parametric bootstrap (BS) values were used to infer nodal support for both the MP and ML with 1000 pseudo-replicates (Felsenstein, 1981). Nucleotide substitution models for ML and BA analyses were selected using the Akaike Information Criterion (AIC) and the Bayesian Information Criterion (BIC), with jmodeltest v.2.0 (Darriba et al., 2012), respectively. The GTR + I + G (COI) substitution model was used for ML and BA analyses using the programs MEGA and MrBayes 3.1.2. (Huelsenbeck and Ronquist, 2001). Bayesian inference posterior probabilities were estimated using the Metropolis coupled Markov chain Monte Carlo algorithm (MCMC), running four chains for 50×10^6 generations with trees sampled every 1000 generations. Stationarity was inferred when the average standard deviation of split frequencies was less than 0.01 (Huelsenbeck and Ronquist, 2001). The initial 10% of the trees were discarded (burn-in), and posterior probabilities were estimated as the fraction of trees showing a particular node. Posterior probability density was summarized as a Maximum Clade Credibility Tree (MCCT) using TreeAnnotator v.1.6.1 (http://beast.bio.ed.ac.uk/TreeAnnotator) and visualized with FigTree v.1.4.3 (http://tree.bio.ed.ac.uk/software/figtree). For comparative purposes, we also reconstructed mtDNA and nucDNA genealogical relationships within sub-Antarctic and Antarctic *Aequiyoldia* populations using maximum-likelihood networks in Hapview (Salzburger et al., 2011). This methodology allowed the reconstructions of phylogenies based on intra- and interspecific molecular data sets.

2.4 Divergence time estimations

Relaxed molecular clock analyses were used for mtDNA sequences with an uncorrelated lognormal (ucln) model of molecular evolutionary rate heterogeneity and the GTR + I + G substitution model implemented in BEAST v.1.6.2 (Drummond and Rambaut, 2007; Drummond et al., 2012). An age prior with a normal distribution was applied (mean, 44; SD 4.4) to the time of the oldest Antarctic *Aequiyoldia* fossil from La Meseta Formation during the Middle Eocene (Beu, 2009). At the same time, we included another prior within *Aequiyoldia* from Antarctica: *A. defossatum* (mean, 5, SD, 0.5), a Pliocene fossil from Vestfold Hills, East Antarctica (Quilty et al., 2016). 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 1000 generations. The convergence of model parameters was estimated by plotting marginal posterior probabilities versus the generation state in TRACER 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 > 1000).

2.5 Morphological analysis

Shell morphology of *Aequiyoldia eightsii* populations from different provinces of the Southern Ocean (Fig. 2) was characterized following the criteria of Rabarts and Whybrow (1979). For the pallial sinus shape, callipers were used to determine three parameters for each individual shell: H = Height, L = Length, and W = Width. Discriminating scores (D) were obtained through the following formula D=6H-(3L+2I). The matrix of D was then analyzed through non-metric, multidimensional scaling (NMDS) using Euclidean distances.

3. Results

3.1 DNA polymorphism

Phylogenetic reconstructions including populations of *Aequiyoldia eightsii* revealed clearly discriminated evolutionary lineages associated to different provinces of the Southern Ocean (Fig. 3-4, Table 2). For instance, *A. eightsii* populations from KI differed markedly (> 19%) from those collected in other the Southern Ocean provinces (AP, SA, FI). In fact, more than 27 amino acid substitutions separated KI from the rest of the analyzed populations (AP, SA, FI). No indels or stop codons were identified within *A. eightsii* populations from AP, SA, or FI. A total of eight amino-acid substitutions were recorded among individuals from these areas (see below). Mitochondrial DNA alignments (621 bp) included 79 variable positions (12.72%) of which 73 (92.4%) were parsimoniously informative. Nuclear ITSs (1190 bp) were more conserved with only 28 variable positions (2.35%), 19 of which (67.85%) were parsimoniously informative. Mitochondrial and nuclear sequences were not saturated at any position and no evidence for codon bias was detected for COI (ENC = 35.89).

3.2 Phylogenetic relationships

Phylogenetic reconstructions from mitochondrial data were consistent with previous analyses in protobranch bivalves (Sharma et al. 2013) that discriminated major taxonomic protobranch groupings with high bootstrap and posterior probabilities. For instance, the single individual of *Portlandia isonota* from KI fell within the diversity of *Yoldiella* from different regions (Fig. 3). At the same time, these analyses highlighted the presence of several evolutionary lineages within the nominal species, *A. eightsii* (Fig. 3). The monophyly of *Aequiyoldia eightsii* was highly supported by the different markers and reconstruction methods. Nevertheless, the high divergence level (> 19%) and phylogenetic position of *A. eightsii* individuals from KI (Fig. 3) allowed us to discriminate them as a different species. However, the exact systematic position of KI individuals remains uncertain. Although KI specimens may represent a basal branch of *Aequiyoldia*, considering the high level of genetic divergence, we tend to think that they

constitute a different *Aequiyoldia* species endemic to KI. We recognized the presence of at least two evolutionary lineages in AP that were sympatric at two sites (i.e., Rothera Station and Admiralty Bay) (Fig. 3). We also recognized the presence of a different lineage in SA and another endemic to the FI (Fig. 3). Genetic divergence between Antarctic and South American *A. eightsii* populations ranged between 6.3% and 7.5% (Table 2), whereas these levels were lower between SA and FI populations (4%) and among the different AP units (~3%).

3.3 Divergence time estimates

Divergence time estimates based on mtDNA sequences suggested that the *A. eightsii* populations from KI separated from the remaining lineages during the middle Miocene c. 16.5 Ma (22.5–12 Ma) (Fig. 4). Following this, the separation between Antarctic and South American lineages occurred at the end of the Miocene, c. 8.5 Ma (13–6.5 Ma) (Fig. 4). Finally, the diversification of *Aequiyoldia* in AP and SA occurred during the Pliocene, beginning with the separation between the AP lineages ~ 4.5 Ma (6.5–3.5 Ma), followed by the separation between SA and FI ~ 3.0 Ma (5.5–2.5 Ma) (Fig. 4).

3.4 Morphological analysis

The nMDS analysis separated individuals of *A. eightsii* into two main groups (Fig. 5). The first included all individuals assigned to the broadly distributed nominal species *A. eightsii* collected at AP, SA, and KI, which exhibited a u-shaped pallial sinus. The second group included individuals from FI that represented a different morphological entity that exhibited a v-shaped pallial sinus.

4. Discussion

4.1 Cryptic speciation in the Southern Ocean

Recent molecular studies in broadly distributed Southern-Ocean organisms have demonstrated the presence of several cryptic species complexes. Examples include crustaceans of the genus *Eusirus* (Baird et al., 2011) and *Serolis* (Leese et al., 2008), the nudibranch *Doris kerguelenensis* (Wilson et al., 2009), octopuses of the genus *Pareledone* (Allcock et al., 2011), pycnogonids of the genus *Nymphon* (Arango et al., 2011), *Pallenopsis* (Weis et al., 2014; Harder et al., 2016), *Colossendeis* (Dietz et al., 2015), the brittle star *Astrotoma* (Heimeier et al., 2010) the ophiurid *Promachochrinus* (Hemery et al., 2012), the macroalgae genus *Gigartina* (Billard et al., 2015), and *Durvillaea* (Fraser et al., 2009). Given the high number of cryptic species complexes discovered in the Southern Ocean, the region's biodiversity has been severely underestimated. As recorded in *Aequiyoldia*, cryptic species complexes consist of closely related species with incomplete morphological differentiation as opposed to distantly related species that have acquired a misleading degree of morphological similarity through other evolutionary processes (i.e., convergent evolution) (Koubbi et al., 2014). Besides increasing diversity patterns and species counts, the discovery of cryptic species often results in a shift from a small number of broadly distributed,

morphologically variable species to a series of less variable species with smaller, often allopatric distributions. Notable exceptions of newly delimited species with larger distributions in the Southern Ocean do exist (Fraser et al., 2009; Nikula et al., 2010; Cumming et al., 2014; González-Wevar et al., 2018). New molecular advances have started to change our understanding of circumpolar and cosmopolitan species, indicating the need for further, more detailed taxonomic and integrative biogeographic work on potential cryptic species (Grant et al., 2010). Biogeographic patterns may change with increased knowledge of diversity both at the genetic and species levels and how much these patterns are likely to change is still unclear. Nevertheless, it seems probable that the overall pattern of rich Antarctic fauna distinct from that of its neighbours will hold true and even strengthen (Koubbi et al., 2014). Species numbers in the Southern Ocean are likely to increase with further sampling, taxonomic work, and molecular analyses, helping provide a better understanding of global diversity, distribution, and evolutionary history (Griffiths et al., 2011).

4.2 Cryptic speciation in Aequiyoldia

The highly conserved morphology and absence of genetic data led researchers to synonymize several nominal species of *Aequiyoldia* (i.e., *A. kerguelensis*, *A. subaequilateralis*, *A. woodwardi*) into a single, nominal, broadly distributed species: *A. eightsii*. Accordingly, *A. eightsii* has exhibited an extended distribution across the sub-Antarctic from South America to the Kerguelen Island as well as a circum-Antarctic distribution (Linse, 2014). Herein, the monophyly of *Aequiyoldia* in the Southern Ocean was supported by mtDNA sequences and different reconstruction methods. Mitochondrial and nuclear diversity within *Aequiyoldia* across its distribution (AP, SA, FI, and KI) as well as its morphological characterization uncovered the presence of at least five cryptic species currently distributed in AP, SA, FI and KI. We found a clear distinction between sub-Antarctic and Antarctic lineages of *A. eightsii*, corroborating previous molecular studies in the group (González-Wevar et al., 2012, 2017; Poulin et al., 2014). In Falkland/Malvinas Islands, we recorded the presence of an endemic lineage clearly separated from its closest continental South American relative. Finally, along the Antarctic Peninsula, we recorded at least two sympatric evolutionary units in Marguerite Bay (West Antarctic Peninsula) and Admiralty Bay (South Shetlands).

4.3 A highly divergent Aequiyoldia lineage in Kerguelen Islands

The *A. eightsii* population from KI was the most divergent and showed a basal position in the phylogenies. All COI haplotypes from KI clustered together in a monophyletic group separated from the remaining lineages from AP, SA, and FI by more than 19% divergence (Table 2). Such level of divergence could not be corroborated by nuclear data, since ITSs sequences from KI did not align properly with those from AP, FI, and SA. However, it may also reflect a long divergence history between KI lineage from the South American and Antarctic ones. Hence, the amount of molecular divergence

accumulated in KI was extreme, despite morphological similarities among the KI, AP, and SA populations. Because of its geographic isolation, Kerguelen Archipelago is indeed characterized by a high level of endemism in a variety of terrestrial and marine species (Briggs, 1966; McDowall, 1968; Poulin and Féral, 1995; Hennion and Walton, 1997; Brandt et al., 1999; Chapuis et al., 2000; Emerson, 2002; Ledoux et al., 2012; González-Wevar et al., 2017). Consequently, this sareptid bivalve endemic to KI should clearly be considered as a different *Aequiyoldia* species. Accordingly, and considering that an *Aequiyoldia* species has been previously described in Kerguelen, our study supports the re-validation of *A. kerguelensis* (Thiele, 1931).

4.4 Trans-Drake speciation: new Aequiyoldia *species in South America*

Phylogenetic reconstructions and molecular divergence within *Aequiyoldia* support previous Antarctic and South American population-based comparisons in the group (González-Wevar et al., 2012; Poulin et al., 2014) by recognizing the presence of different evolutionary lineages on both sides of the Drake Passage. Such conclusions are supported by the mtDNA (6.5% - 7.5%) and nucDNA (1.2%) molecular divergence recorded between Antarctic Peninsula and South American *Aequiyoldia* populations (Suppl. Fig. 1). Consequently, we suggest that South American *A. eightsii* deserves specific status and that integrative taxonomic revisions are required within *Aequiyoldia*.

4.5 Re-validating Aequiyoldia woodwardi (Hanley, 1860)

The morphological revision of *Aequiyoldia* shells confirmed the current presence of a broadly distributed morphotype in different provinces including AP, SA, and KI (Fig. 4). In a revision of the group, Villarroel and Stuardo (1998) concluded that the external anatomy of *A. eightsii* individuals from South America and Antarctic Peninsula is very similar but they differ in their respective sizes, being the Antarctic populations bigger than the South American ones. However, molecular data do not support the presence of a single genetic unit across the Southern Ocean. Rather, the data highlighted the presence of hidden cryptic diversity with at least five evolutionary lineages. Together with this broadly distributed morphotype, we also recorded another one currently restricted to FI. Mitochondrial (COI) and nuclear (ITSs) sequences of *Aequiyoldia* individuals from FI revealed the presence of a monophyletic group in these islands separated from its sister South-American lineage by 4% and 0.5% of divergence, respectively (Suppl. Fig. 1). Such molecular results corroborated previous morphological revision in the group (Rabarts and Whybrow, 1979) that recorded the presence of a different species off these islands: *Yoldia woodwardi* Hanley, 1860. Consequently, based on morphological and molecular evidence, we propose a revalidation of the specific status conferred to the *Aequiyoldia* from FI and propose *Aequiyoldia woodwardi* be used for this species. These results support recent population-based studies of several marine, near-shore, benthic invertebrates including isopods of the genus *Serolis* (Leese et al., 2008), the patellogastropods *Nacella magellanica* (González-Wevar et al., 2012) and *Nacella mytilina* (González-

Wevar et al., 2016), and pulmonates of the genus *Siphonaria* (González-Wevar et al., 2018); all those studies recognized FI and SA populations as different genetic and sometimes as evolutionary units. As stated by Leese et al. (2008), whereas the biota of FI shared most of their faunal inventory with SA, molecular studies have demonstrated that shallow benthic species may, in fact, be strongly differentiated or even reproductively isolated. The genetic differentiation between SA and FI populations of *Aequiyoldia* may be a consequence of their distinct glaciological histories during the coldest glacial periods. The southern tip of South America was almost completely covered by ice during the last Glacial Maximum (LGM), and shallow marine habitats were largely absent. In contrast, little evidence of ice advances during the LGM existed in FI apart from small cirques and short glacially eroded valleys (Clapperton and Sugden, 1976; Clapperton, 1994; Hodgson et al., 2014), and no study shows evidence of LGM glaciers extending offshore (Wilson et al., 2002, 2008).

4.6 Divergent lineages *in* Aequiyoldia *off the West Antarctic Peninsula*

Phylogenetic reconstructions and levels of mtDNA divergence, as well as divergence time estimates indicated the presence of at least two different *Aequiyoldia* lineages off the Antarctic Peninsula. These lineages are separated by 5.78% of mtDNA divergence and are currently found in sympatry in Marguerite Bay and off the South Shetland Islands. These divergent Antarctic *Aequiyoldia* lineages could either be the consequence of sympatric cryptic species or persistent ancestral polymorphism with incomplete lineage sorting. To discriminate between these hypotheses, additional nuclear genetic data are required.

4.7 *Biogeography in* Aequiyoldia *spp.*

Molecular data suggest that the tmrca of *Aequiyoldia* spp., separating KI from the rest of the analyzed provinces of the Southern Ocean (AP, SA and FI), occurred c. 16.5 Ma (22.5–12 Ma), close to the middle Miocene transition (MMCT). This period represents an epoch of major oceanographic and climatic changes in the Southern Ocean that were probably associated with the intensification of the ACC and the re-establishment of a continental Antarctic Ice Sheet (Zachos et al., 2001; DeConto and Pollard, 2003; Mackensen, 2004; Verducci et al., 2009). Major oceanic circulation changes during this period were probably associated with the full development of a deep ACC, a process that was only achieved during the late Miocene around 12 Ma (Dalziel et al., 2013). As recorded for other Southern-Ocean invertebrates, fluctuations in latitudinal positioning and strengthening of the ACC during this period seemed to be important drivers in the separation of lineages over geographically distant Southern Ocean provinces (González-Wevar et al., 2017). Alternatively, the establishment of a strong and deep ACC may have generated an effective oceanographic barrier that can be invoked to explain the diversification of *Aequiyoldia* in different areas of the Southern Ocean. Following this, the separation between Antarctic and South American lineages of *Aequiyoldia* occurred at the end of the Miocene c. 8.5 Ma (13–6.5 Ma).

In this context, late Miocene phases of Southern Ocean diversifications have been identified in different groups of marine invertebrates including some octopus clades (Strugnell et al., 2008), octocorals (Dueñas et al., 2016), patellogastropods (González-Wevar et al., 2017), muricid gastropods (Barco et al., 2012), and fishes (Near et al., 2012). For instance, the limpet genus *Nacella* shows a particular radiation at the end of the Miocene 8.5–7.0 Ma (González-Wevar et al., 2017), and the muricid gastropod of the subfamily Pagodulinae diversified in the Southern Ocean between the late Miocene and Pliocene (Barco et al., 2012). Following this, during the Pliocene, *Aequiyoldia* diversified both off South America and across the Antarctic Peninsula. In both Antarctic and South America, this diversification started around ~ 4.5 Ma (6.5–3.5 Ma) and ~ 3.0 Ma (5.5–2.5 Ma), respectively. Diversification processes during the Pliocene have been reported for other Southern-Ocean marine benthic invertebrates including the Antarctic amphipods of the genus *Epimeria* (Verheye et al., 2017), deep-sea octopuses (Strugnell et al., 2008), and deep-sea octocorals (Dueñas et al., 2016). Molecular population-based studies have provided strong evidence from a wide range of marine and terrestrial taxa that fragmentation of populations into refugia during glacial maxima and subsequent expansions during interglacial periods may represent an important mechanism enhancing cryptic speciation processes (Allcock and Strugnell, 2012; Fraser et al., 2012; Poulin et al., 2014). Hence, the presence of isolated refugia during glacial maxima may have enhanced such process and the re-colonization of newly de-glaciated habitats could have favoured the recent radiation recorded in *Aequiyoldia* off South America and the Antarctic Peninsula.

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Figure Captions

Figure 1. A) Main Southern Ocean provinces included in the study. B) Sampling sites of *Aequiyoldia eightsii* off South America (SA), Falkland/Malvinas Islands (FI), and Antarctic Peninsula (AP). C) Distribution of *Aequiyoldia eightsii* in the Southern Ocean (Linse et al., 2014).

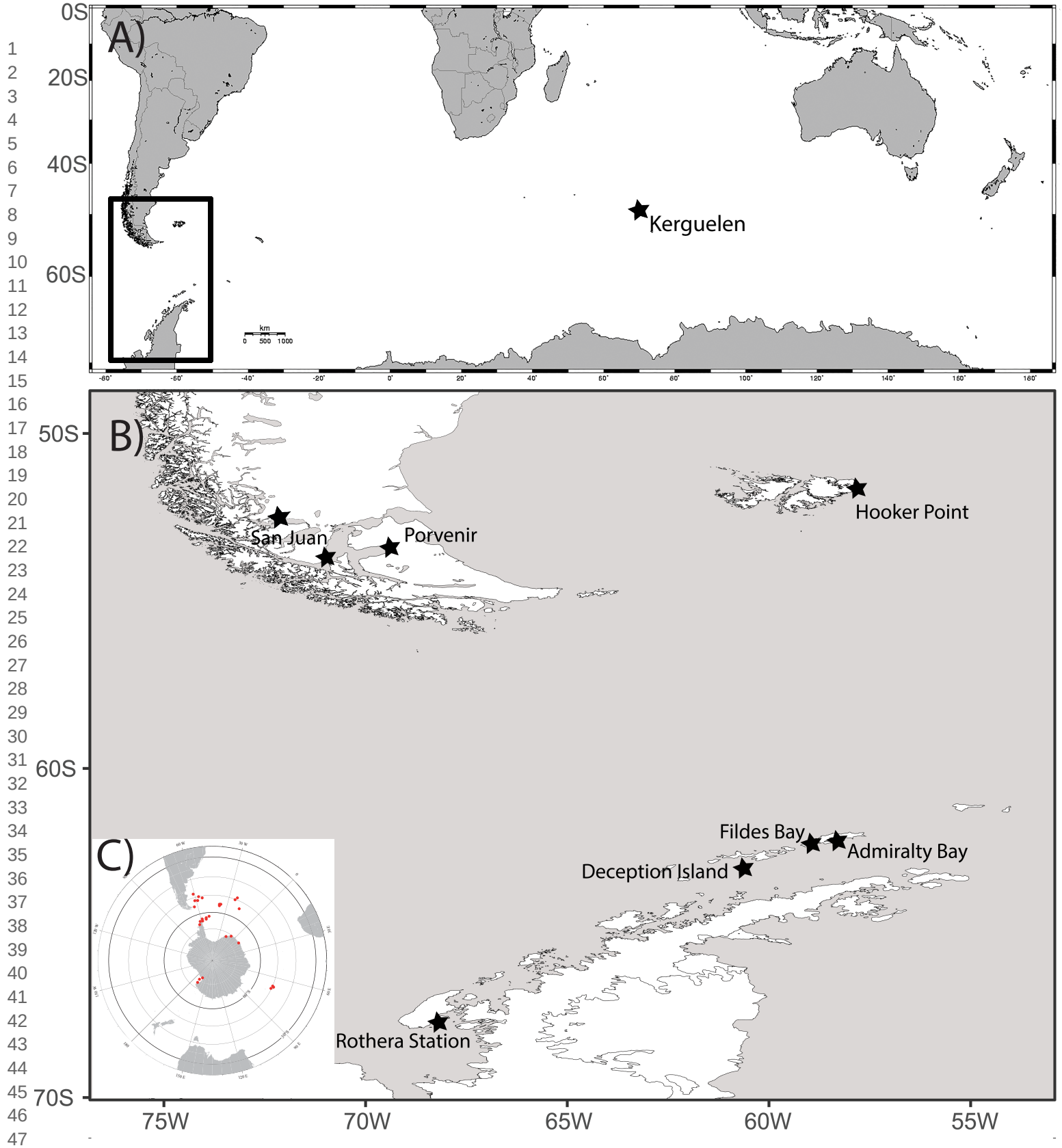
Figure 2. *Aequiyoldia eightsii* individuals from: a-c) Kerguelen Islands (KI = L1), d-f) Rothera Station (AP = L2), g-i) Admiralty Bay (AP = L3), j-l) South America (SA = L4), m-o), and the Falkland/Malvinas Islands (FI = L5).

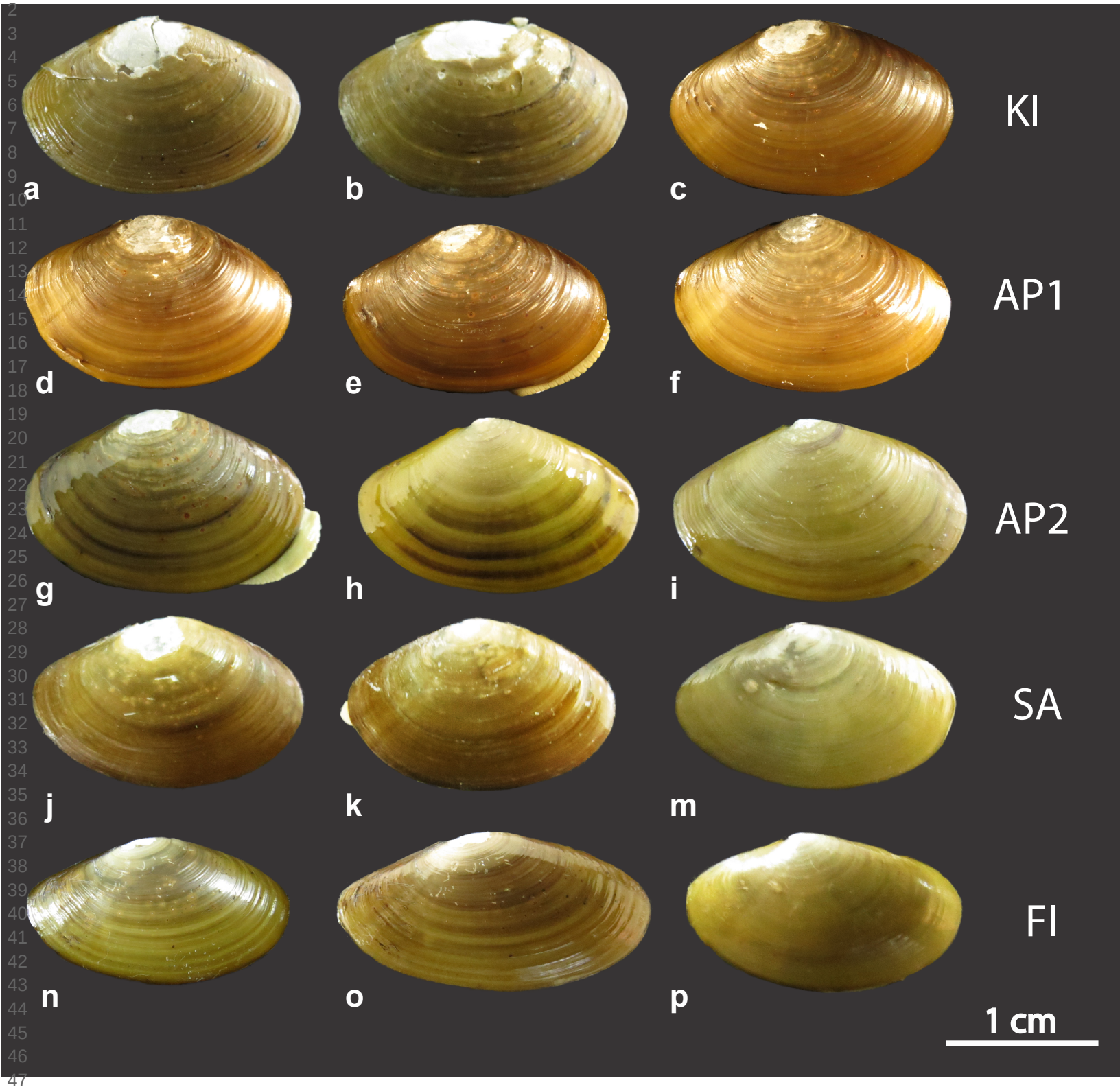
Figure 3. Bayesian maximum clade credibility tree of *Aequiyoldia* relationships based on mtDNA (COI) sequences with special emphasis on Antarctic Peninsula and South American populations. Bootstrap support values (MP and ML) and BPP are shown above the nodes (in that order). Map and colors indicate major sampled areas. Round charts indicate the frequency of the recorded *Aequiyoldia* lineages across the sampling sites.

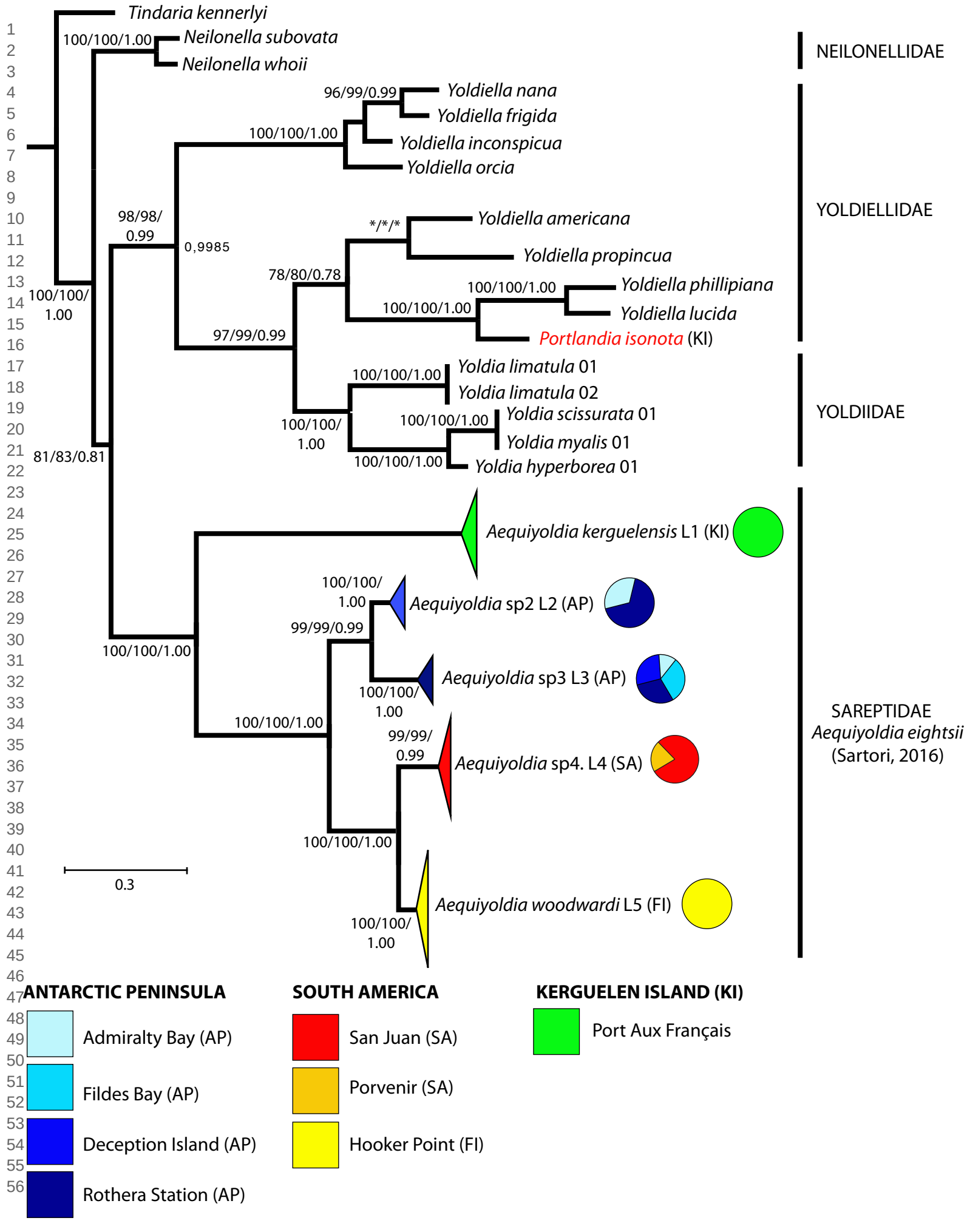
Figure 4. Bayesian maximum clade credibility tree based on mtDNA (COI) showing divergence time estimates within A) protobranch bivalves and B) *Aequiyoldia*. Posterior probabilities are shown above the clades. Grey boxed regions depict 95% Bayesian credible intervals (BCIs) for relative divergence times.

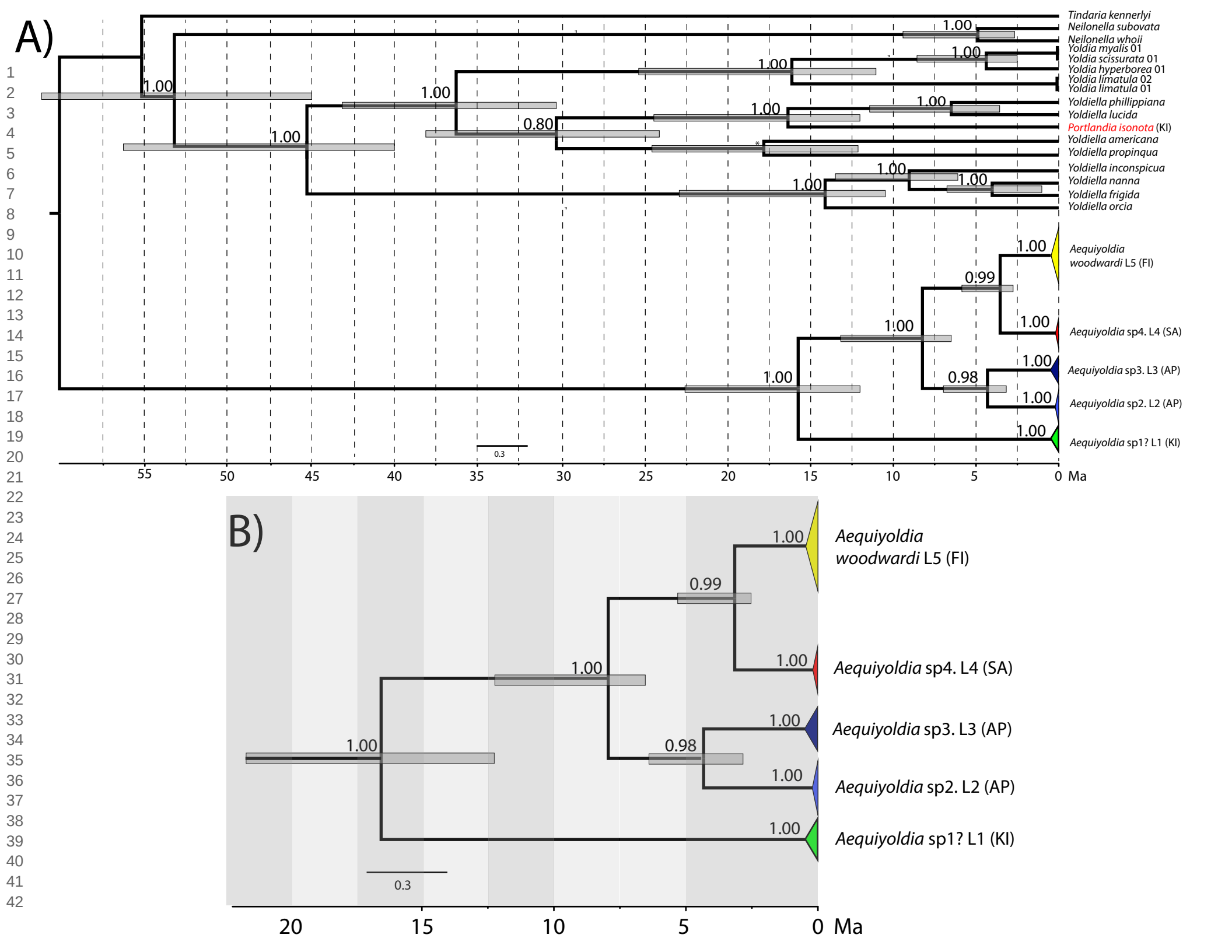
Figure 5. Non-metric, Multidimensional Scaling (NMDS) ordination of *A. eightsii* samples from the Kerguelen Islands (KI), Falkland/Malvinas Islands (FI), South America, and the Antarctic Peninsula using shell morphological data following Rabarts and Whybrow (1979).

Supplementary Figure 1. A) mtDNA (COI) maximum parsimony networks including Aequiyoldia individuals collected at different localities in the Southern Ocean. B) nucDNA (ITS1 and ITS2) maximum parsimony network including Aequiyoldia individuals from different localities in the Southern Ocean. 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.

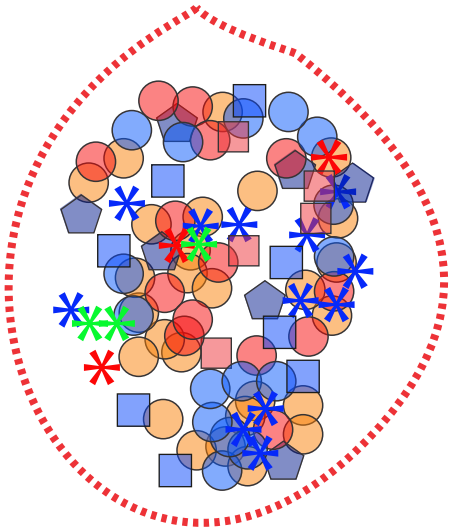

A)
0S
20S
40S
60S
Kerguelen
km
0 500 1000
B)
50S
60S
70S
75W
70W
65W
60W
55W
San Juan
Porvenir
Hooker Point
Fildes Bay
Admiralty Bay
Deception Island
Rothera Station
C)

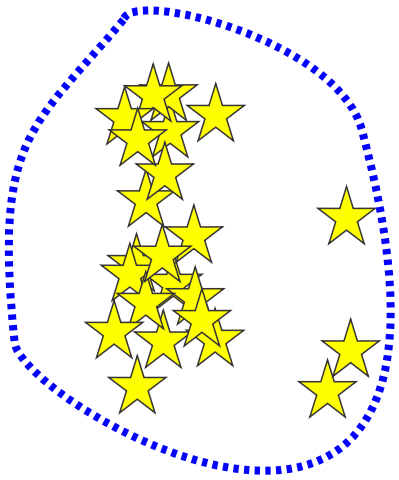

a
b
c
KI
d
e
f
AP1
g
h
i
AP2
j
k
m
SA
n
o
p
FI
1 cm


Tindaria kennerlyi
100/100/1.00
Neilonella subovata
Neilonella whoii
NEILONELLIDAE
96/99/0.99
Yoldiella nana
Yoldiella frigida
100/100/1.00
Yoldiella inconspicua
Yoldiella orcia
98/98/
0.99
//*
Yoldiella americana
0,9985
Yoldiella propincua
78/80/0.78
100/100/1.00
Yoldiella phillipiana
100/100/
1.00
100/100/1.00
Yoldiella lucida
97/99/0.99
Portlandia isonota (KI)
YOLDIELLIDAE
100/100/1.00
Yoldia limatula 01
Yoldia limatula 02
100/100/
1.00
100/100/1.00
Yoldia scissurata 01
Yoldia myalis 01
81/83/0.81
100/100/1.00
Yoldia hyperborea 01
YOLDIIDAE
Aequiyoldia kerguelensis L1 (KI)
100/100/
1.00
Aequiyoldia sp2 L2 (AP)
99/99/0.99
100/100/1.00
Aequiyoldia sp3 L3 (AP)
100/100/
1.00
100/100/1.00
99/99/
0.99
Aequiyoldia sp4. L4 (SA)
100/100/1.00
0.3
Aequiyoldia woodwardi L5 (FI)
100/100/
1.00
SAREPTIDAE
Aequiyoldia eightsii
(Sartori, 2016)
ANTARCTIC PENINSULA
Admiralty Bay (AP)
Fildes Bay (AP)
Deception Island (AP)
Rothera Station (AP)
SOUTH AMERICA
San Juan (SA)
Porvenir (SA)
Hooker Point (FI)
KERGUELEN ISLAND (KI)
Port Aux Français


A)
Tindaria kennerlyi
Neilonella subovata
Neilonella whoii
Yoldia myalis 01
Yoldia scissurata 01
Yoldia hyperborea 01
Yoldia limatula 02
Yoldia limatula 01
Yoldiella phillippiana
Yoldiella lucida
Portlandia isonota (KI)
Yoldiella americana
Yoldiella propinqua
Yoldiella inconspicua
Yoldiella nanna
Yoldiella frigida
Yoldiella orcia
Aequiyoldia woodwardi L5 (FI)
Aequiyoldia sp4. L4 (SA)
Aequiyoldia sp3. L3 (AP)
Aequiyoldia sp2. L2 (AP)
Aequiyoldia sp1? L1 (KI)
1.00
0.99
0.98
0.80
0.3
55
50
45
40
35
30
25
20
15
10
5
0 Ma
B)
Aequiyoldia woodwardi L5 (FI)
Aequiyoldia sp4. L4 (SA)
Aequiyoldia sp3. L3 (AP)
Aequiyoldia sp2. L2 (AP)
Aequiyoldia sp1? L1 (KI)
1.00
0.99
0.98
0.3
20
15
10
5
0 Ma

Stress 0.02





SOUTH AMERICA	FALKLAND/MALVINAS ISLANDS	ANTARCTIC PENINSULA	KERGUELEN ISLANDS
Porvenir San Juan Skyring Sound	Hooker Point	Admiralty Bay Fildes Bay Deception Island Rothera Station	Port Aux Français

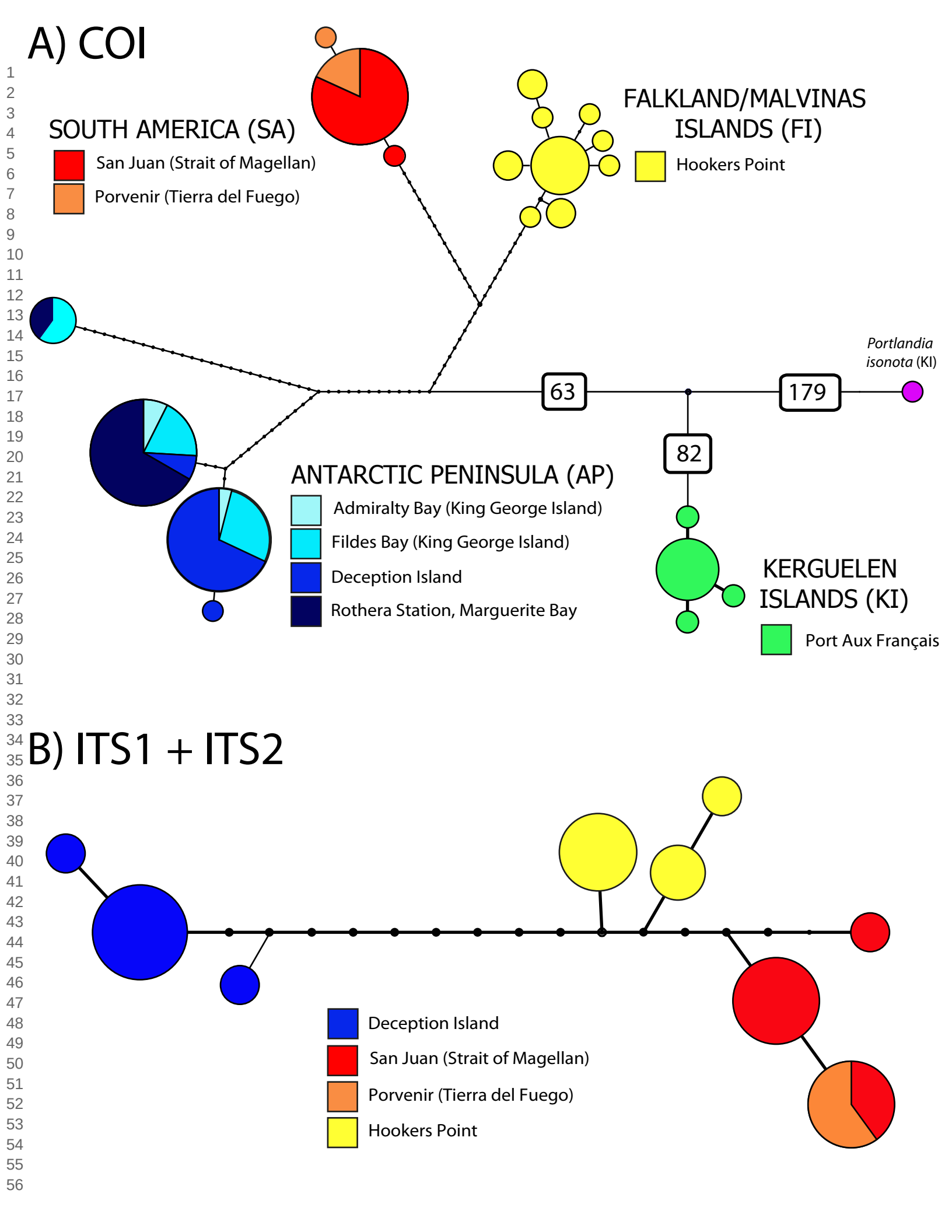

A) COI
SOUTH AMERICA (SA)
San Juan (Strait of Magellan)
Porvenir (Tierra del Fuego)
FALKLAND/MALVINAS ISLANDS (FI)
Hookers Point
Portlandia isonota (KI)
63
179
82
ANTARCTIC PENINSULA (AP)
Admiralty Bay (King George Island)
Fildes Bay (King George Island)
Deception Island
Rothera Station, Marguerite Bay
KERGUELEN ISLANDS (KI)
Port Aux Français
B) ITS1 + ITS2
Deception Island
San Juan (Strait of Magellan)
Porvenir (Tierra del Fuego)
Hookers Point

Table 1. Sampling sites of *Aequiyoldia eightsii*: Geographical coordinates, name of collector, n: number of analyzed individuals per locus.

Sites	Lat.	Long.	n (COI)	n (ITS)
Port-aux-Français, Kerguelen Islands	49º21'S	70º13'W	11	9
Hooker's Point, Falkland/Malvinas Islands	51º42'S	57º46'W	20	7
San Juan River, Strait of Magellan	53º38'S	70º56'W	20	9
Porvenir, Tierra del Fuego	53º18'S	70º22'W	5	-
Skyring Sound, Strait of Magellan	52º34'S	70º56'W	-	3
Admiralty Bay, South Shetland Islands	62º09'S	58º27'W	6	-
Fildes Bay, South Shetland Islands	62º12'S	58º57'W		-
Deception Island, South Shetland Islands	62º56'S	60º36'W	20	8
Rothera Station, Antarctic Peninsula	67º34'S	68º07'W	20	-

Table 2. Uncorrected p-distances based on mtDNA COI sequences between the recorded lineages of *Aequiyoldia* from Kerguelen Island (KI), the Antarctic Peninsula (AP), South America (SA), and the Falkland/Malvinas Islands (FI).

	KI	AP1	AP2	SA	FI
KI	****				
AP1	19.96%	****			
AP2	20.61%	5.79%	****		
SA	20.45%	6.60%	8.53%	****	
FI	19.80%	5.47%	6.92%	3.86%	****