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## **Cryptic speciation in Southern Ocean *Aequiyoldia* *eightisii* (Jay, 1839): Mio-Pliocene trans-Drake Passage separation and diversification.**

Claudio A. González-Wevar, Karin Gérard, Sebastián Rosenfeld, Thomas Saucède, Elie Poulin, Javier Naretto, Angie Díaz, Simon A. Morley, Paul Brickle

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4 Cryptic speciation in Southern Ocean *Aequiyoldia eightsii* Jay, 1839:  
5 Mio-Pliocene trans-Drake separation and diversification  
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9 Claudio González-Wevar<sup>a,b,c</sup>, Karin Gérard<sup>a,b\*</sup>, Sebastian Rosenfeld<sup>a</sup>, Elie Poulin<sup>c</sup>, Thomas  
10 Saucède<sup>d</sup>, Javier Naretto<sup>e</sup>, Angie Díaz<sup>e</sup>, Simon Morley<sup>f</sup>, Paul Brickley<sup>g</sup>  
11  
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- 13  
14 a) Departamento de Recursos Naturales, Universidad de Magallanes, Bulnes 01890, Punta Arenas,  
15 Chile  
16  
17 b) Laboratorio de Ecología Molecular Antártica y Subantártica, Bulnes # 01890, Universidad de  
18 Magallanes, casilla 113-D, Punta Arenas, Chile  
19  
20 c) Instituto de Ecología y Biodiversidad (IEB), Departamento de Ciencias Ecológicas, Facultad de  
21 Ciencias, Universidad de Chile, Las Palmeras # 3425, Ñuñoa, Santiago, Chile  
22  
23 d) Biogéosciences, UMR CNRS 6282, Université de Bourgogne, 6, boulevard Gabriel, 21000,  
24 Dijon, France  
25  
26 e) Departamento de Zoología, Facultad de Ciencias Naturales y Oceanográficas, Universidad de  
27 Concepción, Concepción, Chile  
28  
29 f) British Antarctic Survey (BAS), Natural Environment Research Council, Madingley Road, High  
30 Cross, Cambridge CB3, 0ET, UK  
31  
32 g) South Atlantic Environmental Research Institute (SAERI), PO Box 609, Stanley Cottage,  
33 Stanley, Falkland Islands  
34  
35

36 \* Corresponding author: [karin.gerard@umag.cl](mailto:karin.gerard@umag.cl)  
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42 **Abstract**  
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44 The species of the genus *Aequiyoldia* Soot-Ryen, 1951, previously known as *Yoldia*, are common, soft-  
45 substratum, saredtid bivalves. In the Southern Ocean, *Aequiyoldia eightsii* (Jay, 1839) was originally  
46 described from the Antarctic Peninsula and has also been reported in southern South America, whereas *A.*  
47 *woodwardi* (Hanley, 1960) was recorded for the Falkland/Malvinas Islands and Tierra del Fuego,  
48 southern South America. *Aequiyoldia* has received little attention across its distribution in the Southern  
49 Ocean, and although its taxonomy and systematics remain uncertain, all species of *Aequiyoldia* have been  
50 grouped under the same broadly distributed unit: *A. eightsii*. However, molecular studies have  
51 demonstrated a marked mtDNA genetic divergence (> 7%) between *A. eightsii* populations from South  
52 America and Antarctic Peninsula. In order to further understand the diversity and biogeography of  
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62 *Aequiyoldia*, we analyzed *A. eightsii* populations from different provinces of the Southern Ocean  
63 including South America (SA), the Falkland/Malvinas Islands (FI), Antarctic Peninsula (AP), and  
64 Kerguelen Islands (KI). Individuals were characterized according to typical diagnostic morphological  
65 measurements, and phylogenetic relationships were reconstructed based on mtDNA (Cytochrome c  
66 Oxidase Subunit I). Patterns of genetic divergence of nucDNA intergenic transcribed spacers (ITS1,  
67 ITS2) were also estimated. The statistical analysis of external diagnostic characteristics revealed two  
68 morphotypes: (1) individuals with the morphology recorded for the nominal FI species, *A. woodwardi*,  
69 and (2) individuals from SA, AP, and KI, with the morphology recorded for *A. eightsii*. However,  
70 phylogenetic reconstructions based on mtDNA and nucDNA suggest the presence of at least five lineages  
71 within *A. eightsii* including: one lineage in Kerguelen Island, two lineages in Antarctic Peninsula, one  
72 lineage in South America, and the last one restricted to the Falkland/Malvinas Islands. Despite their very  
73 similar morphologies, AP, SA, and KI populations of *Aequiyoldia* exhibited also marked levels of genetic  
74 divergence. Such results are evidence that the Antarctic Polar Front represents an historical biogeographic  
75 barrier for this group and that after the separation of these lineages, they followed independent  
76 evolutionary pathways in different provinces of the Southern Ocean. Estimates of divergence time  
77 suggest that KI separated from the other lineages close to the middle Miocene. Following this, the  
78 separation between the AP and SA lineages occurred at the end of the Miocene around 7.5 Ma. Finally,  
79 *Aequiyoldia* diversified during the Pliocene in Antarctic Peninsula (~4.5 Ma) and South America (~3.0  
80 Ma). Individuals from FI exhibited morphological differences, and 4% of divergence from South  
81 American individuals, suggesting that *A. woodwardi* could be revalidated. Similarly, the marked  
82 molecular divergence between the KI and the rest of the recorded lineages also support the validity of *A.*  
83 *kerquelenensis* (Thiele, 1931).  
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## 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 agassizii* (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



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180 sub-Antarctic provinces (Griffiths et al., 2009; Pierrat et al., 2013; Griffiths and Waller, 2016). During the  
181 last decade, the use of molecular tools for the taxonomic identification of organisms with conserved  
182 morphology greatly increased the number of taxa in the Southern Ocean through the recognition of  
183 cryptic species in several invertebrates groups such as crustaceans (Held and Wägele, 2005; Raupach and  
184 Wägele, 2006; Leese et al., 2008; Krabbe et al., 2010; Arango et al., 2011; Baird et al., 2011; Dietz et al.,  
185 2015), mollusks (Strugnell et al., 2008, 2012; Wilson et al., 2009; González-Wevar et al., 2010; Allcock  
186 et al., 2011), echinoderms (Wilson et al., 2007; Heimeier et al., 2010; Janosik et al., 2011; Hemery et al.,  
187 2012; Gubili et al., 2016), nematodes (Lee et al., 2017), and even macroalgae (Billard et al., 2015). In this  
188 context, the broadly distributed Southern Ocean bivalve *Yoldia eightsii* shows a similar pattern with the  
189 presence of different lineages at both sides of the Drake Passage (González-Wevar et al., 2012; Poulin et  
190 al., 2014).

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192 The genus *Yoldia* includes ~ 20 species of saltwater bivalves broadly distributed around the globe. Until  
193 2013, the higher-latitude species, *Yoldia eightsii*, was recognized as broadly distributed in different  
194 provinces of the Southern Ocean including South America, Antarctic Peninsula, and the Kerguelen  
195 archipelago. Nevertheless, multi-locus molecular reconstructions identified *Yoldia* as paraphyletic with  
196 the Antarctic *Y. eightsii* completely separated from the rest of the species (Sharma et al., 2013). Following  
197 this, higher latitude *Y. eightsii* has been included in the genus *Aequiyoldia* Soot-Ryen, 1951. This genus  
198 of sareptid bivalves is currently distributed in Antarctic and sub-Antarctic soft-substratum ecosystems and  
199 includes only two nominal species: *A. defossata*, a Pliocene fossil from East Antarctica (Quilty et al.,  
200 2016) and the broadly distributed *A. eightsii* (Jay, 1839). Several synonymous species have been grouped  
201 within *A. eightsii*, including *Yoldia kerguelensis* (Thiele, 1931) and *Y. subaequilateralis* (E.A. Smith,  
202 1875) from the Kerguelen archipelago, and *Yoldia woodwardi* (Hanley, 1860) from the  
203 Falkland/Malvinas Islands.

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205 The absence of clear diagnostic characters and the highly conserved morphology of protobranch bivalves  
206 impose major taxonomic uncertainties within this group (Bouchet and Huber, 2015; Sartori, 2016a, b). In  
207 fact, Antarctic Peninsula and South American populations of *A. eightsii* are morphologically identical,  
208 although Antarctic individuals are larger than those recorded off South America (Villarroel and Stuardo,  
209 1998). At the same time, morphological characteristics of the Kerguelen Islands species, *A. kerguelensis*  
210 and *A. subaequilateralis*, completely overlap those recorded for *A. eightsii* from Antarctic Peninsula and  
211 South America (Rabbarts & Whyborn 1979). Following this, *A. eightsii* is currently recognized in  
212 different Southern Ocean provinces including the Falkland/Malvinas Islands, Kerguelen Islands,  
213 Antarctic Peninsula, and South Shetland Islands (Linse, 2014). However, recent molecular analyses using  
214 the mitochondrial locus cytochrome c oxidase subunit I (COI) recognized more than 7% divergence  
215 between South American and Antarctic Peninsula populations of *A. eightsii*, which is far beyond the  
216 intraspecific level (Gonzalez-Wevar et al., 2012; Poulin et al., 2014).

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

### 257 *2.1 Sample collection*

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259 *Aequiyoldia eightsii* specimens were collected by SCUBA diving off the Kerguelen (KI) and Falkland  
260 Islands (FI), South America (SA) (Porvenir, San Juan River, Skyring Sound), and Antarctic Peninsula  
261 (AP) (Admiralty Bay, Fildes Bay, Deception Island, Rothera Station, Marguerite Bay, Adelaide Island)  
262 (Fig. 1 and 2, Table 1). All specimens were stored in 96° ethanol for future molecular analyses. For  
263 comparative purposes, we also included one individual of *Portlandia isonota* collected off the Kerguelen  
264 Islands.  
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### 269 *2.2 DNA preparation and sequence edition*

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271 Genomic DNA was extracted from mantle and branchial tissues using the E.Z.N.A. Mollusc DNA Kit  
272 (Omega bio-tek). A 621-bp fragment of the cytochrome c oxidase subunit I (COI) was amplified using  
273 universal primers (Folmer et al., 1994). The PCR program consisted of 5 min at 95°C followed by 40  
274 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  
275 of up to 1190 bp containing the internal transcribed spacers 1 and 2 (ITS1 and ITS2) were amplified using  
276 universal primers (Oliverio and Mariottini, 2001). The PCR program was 5 min at 95°C, then 40 cycles at  
277 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  
278 products were purified using QIAquick Gel Extraction Kit (QIAGEN) and sequenced in both direction  
279 with an Automatic Sequencer ABI3730 x 1 at Macrogen Inc. (Seoul, South Korea). Chromatograms were  
280 inspected visually, and multiple alignments were obtained using MUSCLE (Edgar, 2004) implemented in  
281 CodonCode Aligner v7.1 (CodonCode Corporation). Base composition of nucleotide sequences was  
282 analysed with MEGA 6 (Tamura et al., 2013) and codon usage was determined using the effective  
283 number of codon value (ENC; Wright, 1990) with DnaSP 5.0 (Librado and Rozas, 2009). New  
284 *Aequiyoldia* sequences will be submitted to GenBank under accession numbers: XXXXXX – XXXXXX.  
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### 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  $50 \times 10^6$  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  $50 \times 10^6$  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

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357 sample size values were estimated for each parameter to ensure adequate mixing of the MCMC (ESSs >  
358 1000).  
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### 360 361 362 *2.5 Morphological analysis*

363 Shell morphology of *Aequiyoldia eightsii* populations from different provinces of the Southern Ocean  
364 (Fig. 2) was characterized following the criteria of Rabarts and Whybrow (1979). For the pallial sinus  
365 shape, callipers were used to determine three parameters for each individual shell: H = Height, L =  
366 Length, and W = Width. Discriminating scores (D) were obtained through the following formula  $D=6H-$   
367  $(3L+2I)$ . The matrix of D was then analyzed through non-metric, multidimensional scaling (NMDS) using  
368 Euclidean distances.  
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## 373 374 **3. Results**

### 375 376 *3.1 DNA polymorphism*

377 Phylogenetic reconstructions including populations of *Aequiyoldia eightsii* revealed clearly discriminated  
378 evolutionary lineages associated to different provinces of the Southern Ocean (Fig. 3-4, Table 2). For  
379 instance, *A. eightsii* populations from KI differed markedly (> 19%) from those collected in other the  
380 Southern Ocean provinces (AP, SA, FI). In fact, more than 27 amino acid substitutions separated KI from  
381 the rest of the analyzed populations (AP, SA, FI). No indels or stop codons were identified within *A.*  
382 *eightsii* populations from AP, SA, or FI. A total of eight amino-acid substitutions were recorded among  
383 individuals from these areas (see below). Mitochondrial DNA alignments (621 bp) included 79 variable  
384 positions (12.72%) of which 73 (92.4%) were parsimoniously informative. Nuclear ITSs (1190 bp) were  
385 more conserved with only 28 variable positions (2.35%), 19 of which (67.85%) were parsimoniously  
386 informative. Mitochondrial and nuclear sequences were not saturated at any position and no evidence for  
387 codon bias was detected for COI (ENC = 35.89).  
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### 394 395 *3.2 Phylogenetic relationships*

396 Phylogenetic reconstructions from mitochondrial data were consistent with previous analyses in  
397 protobranch bivalves (Sharma et al. 2013) that discriminated major taxonomic protobranch groupings  
398 with high bootstrap and posterior probabilities. For instance, the single individual of *Portlandia isonota*  
399 from KI fell within the diversity of *Yoldiella* from different regions (Fig. 3). At the same time, these  
400 analyses highlighted the presence of several evolutionary lineages within the nominal species, *A. eightsii*  
401 (Fig. 3). The monophyly of *Aequiyoldia eightsii* was highly supported by the different markers and  
402 reconstruction methods. Nevertheless, the high divergence level (> 19%) and phylogenetic position of *A.*  
403 *eightsii* individuals from KI (Fig. 3) allowed us to discriminate them as a different species. However, the  
404 exact systematic position of KI individuals remains uncertain. Although KI specimens may represent a  
405 basal branch of *Aequiyoldia*, considering the high level of genetic divergence, we tend to think that they  
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416 constitute a different *Aequiyoldia* species endemic to KI. We recognized the presence of at least two  
417 evolutionary lineages in AP that were sympatric at two sites (i.e., Rothera Station and Admiralty Bay)  
418 (Fig. 3). We also recognized the presence of a different lineage in SA and another endemic to the FI (Fig.  
419 3). Genetic divergence between Antarctic and South American *A. eightsii* populations ranged between  
420 6.3% and 7.5% (Table 2), whereas these levels were lower between SA and FI populations (4%) and  
421 among the different AP units (~3%).  
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### 426 427 3.3 Divergence time estimates

428 Divergence time estimates based on mtDNA sequences suggested that the *A. eightsii* populations from KI  
429 separated from the remaining lineages during the middle Miocene c. 16.5 Ma (22.5–12 Ma) (Fig. 4).  
430 Following this, the separation between Antarctic and South American lineages occurred at the end of the  
431 Miocene, c. 8.5 Ma (13–6.5 Ma) (Fig. 4). Finally, the diversification of *Aequiyoldia* in AP and SA  
432 occurred during the Pliocene, beginning with the separation between the AP lineages ~ 4.5 Ma (6.5–3.5  
433 Ma), followed by the separation between SA and FI ~ 3.0 Ma (5.5–2.5 Ma) (Fig. 4).  
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### 439 3.4 Morphological analysis

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

### 449 4.1 Cryptic speciation in the Southern Ocean

451 Recent molecular studies in broadly distributed Southern-Ocean organisms have demonstrated the  
452 presence of several cryptic species complexes. Examples include crustaceans of the genus *Eusirus* (Baird  
453 et al., 2011) and *Serolis* (Leese et al., 2008), the nudibranch *Doris kerguelenensis* (Wilson et al., 2009),  
454 octopuses of the genus *Pareledone* (Allcock et al., 2011), pycnogonids of the genus *Nymphon* (Arango et  
455 al., 2011), *Pallenopsis* (Weis et al., 2014; Harder et al., 2016), *Colossendeis* (Dietz et al., 2015), the  
456 brittle star *Astrotoma* (Heimeier et al., 2010) the ophiurid *Promachochrinus* (Hemery et al., 2012), the  
457 macroalgae genus *Gigartina* (Billard et al., 2015), and *Durvillaea* (Fraser et al., 2009). Given the high  
458 number of cryptic species complexes discovered in the Southern Ocean, the region's biodiversity has been  
459 severely underestimated. As recorded in *Aequiyoldia*, cryptic species complexes consist of closely related  
460 species with incomplete morphological differentiation as opposed to distantly related species that have  
461 acquired a misleading degree of morphological similarity through other evolutionary processes (i.e.,  
462 convergent evolution) (Koubbi et al., 2014). Besides increasing diversity patterns and species counts, the  
463 discovery of cryptic species often results in a shift from a small number of broadly distributed,  
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475 morphologically variable species to a series of less variable species with smaller, often allopatric  
476 distributions. Notable exceptions of newly delimited species with larger distributions in the Southern  
477 Ocean do exist (Fraser et al., 2009; Nikula et al., 2010; Cumming et al., 2014; González-Wevar et al.,  
478 2018). New molecular advances have started to change our understanding of circumpolar and  
479 cosmopolitan species, indicating the need for further, more detailed taxonomic and integrative  
480 biogeographic work on potential cryptic species (Grant et al., 2010). Biogeographic patterns may change  
481 with increased knowledge of diversity both at the genetic and species levels and how much these patterns  
482 are likely to change is still unclear. Nevertheless, it seems probable that the overall pattern of rich  
483 Antarctic fauna distinct from that of its neighbours will hold true and even strengthen (Koubbi et al.,  
484 2014). Species numbers in the Southern Ocean are likely to increase with further sampling, taxonomic  
485 work, and molecular analyses, helping provide a better understanding of global diversity, distribution, and  
486 evolutionary history (Griffiths et al., 2011).

#### 494 495 *4.2 Cryptic speciation in Aequiyoldia*

496 The highly conserved morphology and absence of genetic data led researchers to synonymize several  
497 nominal species of *Aequiyoldia* (i.e., *A. kerguelensis*, *A. subaequilateralis*, *A. woodwardi*) into a single,  
498 nominal, broadly distributed species: *A. eightsii*. Accordingly, *A. eightsii* has exhibited an extended  
499 distribution across the sub-Antarctic from South America to the Kerguelen Island as well as a circum-  
500 Antarctic distribution (Linse, 2014). Herein, the monophyly of *Aequiyoldia* in the Southern Ocean was  
501 supported by mtDNA sequences and different reconstruction methods. Mitochondrial and nuclear  
502 diversity within *Aequiyoldia* across its distribution (AP, SA, FI, and KI) as well as its morphological  
503 characterization uncovered the presence of at least five cryptic species currently distributed in AP, SA, FI  
504 and KI. We found a clear distinction between sub-Antarctic and Antarctic lineages of *A. eightsii*,  
505 corroborating previous molecular studies in the group (González-Wevar et al., 2012, 2017; Poulin et al.,  
506 2014). In Falkland/Malvinas Islands, we recorded the presence of an endemic lineage clearly separated  
507 from its closest continental South American relative. Finally, along the Antarctic Peninsula, we recorded  
508 at least two sympatric evolutionary units in Marguerite Bay (West Antarctic Peninsula) and Admiralty  
509 Bay (South Shetlands).

#### 518 519 *4.3 A highly divergent Aequiyoldia lineage in Kerguelen Islands*

520 The *A. eightsii* population from KI was the most divergent and showed a basal position in the  
521 phylogenies. All COI haplotypes from KI clustered together in a monophyletic group separated from the  
522 remaining lineages from AP, SA, and FI by more than 19% divergence (Table 2). Such level of  
523 divergence could not be corroborated by nuclear data, since ITSs sequences from KI did not align  
524 properly with those from AP, FI, and SA. However, it may also reflect a long divergence history between  
525 KI lineage from the South American and Antarctic ones. Hence, the amount of molecular divergence  
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534 accumulated in KI was extreme, despite morphological similarities among the KI, AP, and SA  
535 populations. Because of its geographic isolation, Kerguelen Archipelago is indeed characterized by a high  
536 level of endemism in a variety of terrestrial and marine species (Briggs, 1966; McDowall, 1968; Poulin  
537 and Féral, 1995; Hennion and Walton, 1997; Brandt et al., 1999; Chapuis et al., 2000; Emerson, 2002;  
538 Ledoux et al., 2012; González-Wevar et al., 2017). Consequently, this sereptid bivalve endemic to KI  
539 should clearly be considered as a different *Aequiyoldia* species. Accordingly, and considering that an  
540 *Aequiyoldia* species has been previously described in Kerguelen, our study supports the re-validation of  
541 *A. kerguelensis* (Thiele, 1931).  
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#### 547 4.4 Trans-Drake speciation: new *Aequiyoldia* species in South America

549 Phylogenetic reconstructions and molecular divergence within *Aequiyoldia* support previous Antarctic  
550 and South American population-based comparisons in the group (González-Wevar et al., 2012; Poulin et  
551 al., 2014) by recognizing the presence of different evolutionary lineages on both sides of the Drake  
552 Passage. Such conclusions are supported by the mtDNA (6.5% - 7.5%) and nucDNA (1.2%) molecular  
553 divergence recorded between Antarctic Peninsula and South American *Aequiyoldia* populations (Suppl.  
554 Fig. 1). Consequently, we suggest that South American *A. eightsii* deserves specific status and that  
555 integrative taxonomic revisions are required within *Aequiyoldia*.  
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#### 561 4.5 Re-validating *Aequiyoldia woodwardi* (Hanley, 1860)

563 The morphological revision of *Aequiyoldia* shells confirmed the current presence of a broadly distributed  
564 morphotype in different provinces including AP, SA, and KI (Fig. 4). In a revision of the group,  
565 Villarroel and Stuardo (1998) concluded that the external anatomy of *A. eightsii* individuals from South  
566 America and Antarctic Peninsula is very similar but they differ in their respective sizes, being the  
567 Antarctic populations bigger than the South American ones. However, molecular data do not support the  
568 presence of a single genetic unit across the Southern Ocean. Rather, the data highlighted the presence of  
569 hidden cryptic diversity with at least five evolutionary lineages. Together with this broadly distributed  
570 morphotype, we also recorded another one currently restricted to FI. Mitochondrial (COI) and nuclear  
571 (ITSs) sequences of *Aequiyoldia* individuals from FI revealed the presence of a monophyletic group in  
572 these islands separated from its sister South-American lineage by 4% and 0.5% of divergence,  
573 respectively (Suppl. Fig. 1). Such molecular results corroborated previous morphological revision in the  
574 group (Rabarts and Whybrow, 1979) that recorded the presence of a different species off these islands:  
575 *Yoldia woodwardi* Hanley, 1860. Consequently, based on morphological and molecular evidence, we  
576 propose a revalidation of the specific status conferred to the *Aequiyoldia* from FI and propose *Aequiyoldia*  
577 *woodwardi* be used for this species. These results support recent population-based studies of several  
578 marine, near-shore, benthic invertebrates including isopods of the genus *Serolis* (Leese et al., 2008), the  
579 patellogastropods *Nacella magellanica* (González-Wevar et al., 2012) and *Nacella mytilina* (González-  
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593 Wevar et al., 2016), and pulmonates of the genus *Siphonaria* (González-Wevar et al., 2018); all those  
594 studies recognized FI and SA populations as different genetic and sometimes as evolutionary units. As  
595 stated by Leese et al. (2008), whereas the biota of FI shared most of their faunal inventory with SA,  
596 molecular studies have demonstrated that shallow benthic species may, in fact, be strongly differentiated  
597 or even reproductively isolated. The genetic differentiation between SA and FI populations of  
598 *Aequiyoldia* may be a consequence of their distinct glaciological histories during the coldest glacial  
600 periods. The southern tip of South America was almost completely covered by ice during the last Glacial  
601 Maximum (LGM), and shallow marine habitats were largely absent. In contrast, little evidence of ice  
602 advances during the LGM existed in FI apart from small cirques and short glacially eroded valleys  
603 (Clapperton and Sugden, 1976; Clapperton, 1994; Hodgson et al., 2014), and no study shows evidence of  
604 LGM glaciers extending offshore (Wilson et al., 2002, 2008).

#### 609 610 4.6 Divergent lineages in *Aequiyoldia* off the West Antarctic Peninsula

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612 Phylogenetic reconstructions and levels of mtDNA divergence, as well as divergence time estimates  
613 indicated the presence of at least two different *Aequiyoldia* lineages off the Antarctic Peninsula. These  
614 lineages are separated by 5.78% of mtDNA divergence and are currently found in sympatry in Marguerite  
615 Bay and off the South Shetland Islands. These divergent Antarctic *Aequiyoldia* lineages could either be  
616 the consequence of sympatric cryptic species or persistent ancestral polymorphism with incomplete  
617 lineage sorting. To discriminate between these hypotheses, additional nuclear genetic data are required.  
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#### 622 623 4.7 Biogeography in *Aequiyoldia* spp.

624 Molecular data suggest that the tmrca of *Aequiyoldia* spp., separating KI from the rest of the analyzed  
625 provinces of the Southern Ocean (AP, SA and FI), occurred c. 16.5 Ma (22.5–12 Ma), close to the middle  
626 Miocene transition (MMCT). This period represents an epoch of major oceanographic and climatic  
627 changes in the Southern Ocean that were probably associated with the intensification of the ACC and the  
628 re-establishment of a continental Antarctic Ice Sheet (Zachos et al., 2001; DeConto and Pollard, 2003;  
629 Mackensen, 2004; Verducci et al., 2009). Major oceanic circulation changes during this period were  
630 probably associated with the full development of a deep ACC, a process that was only achieved during  
631 the late Miocene around 12 Ma (Dalziel et al., 2013). As recorded for other Southern-Ocean invertebrates,  
632 fluctuations in latitudinal positioning and strengthening of the ACC during this period seemed to be  
633 important drivers in the separation of lineages over geographically distant Southern Ocean provinces  
634 (González-Wevar et al., 2017). Alternatively, the establishment of a strong and deep ACC may have  
635 generated an effective oceanographic barrier that can be invoked to explain the diversification of  
636 *Aequiyoldia* in different areas of the Southern Ocean. Following this, the separation between Antarctic  
637 and South American lineages of *Aequiyoldia* occurred at the end of the Miocene c. 8.5 Ma (13–6.5 Ma).  
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652 In this context, late Miocene phases of Southern Ocean diversifications have been identified in different  
653 groups of marine invertebrates including some octopus clades (Strugnell et al., 2008), octocorals (Dueñas  
654 et al., 2016), patellogastropods (González-Wevar et al., 2017), muricid gastropods (Barco et al., 2012),  
655 and fishes (Near et al., 2012). For instance, the limpet genus *Nacella* shows a particular radiation at the  
656 end of the Miocene 8.5–7.0 Ma (González-Wevar et al., 2017), and the muricid gastropod of the  
657 subfamily Pagodulinae diversified in the Southern Ocean between the late Miocene and Pliocene (Barco  
658 et al., 2012). Following this, during the Pliocene, *Aequiyoldia* diversified both off South America and  
659 across the Antarctic Peninsula. In both Antarctic and South America, this diversification started around ~  
660 4.5 Ma (6.5–3.5 Ma) and ~ 3.0 Ma (5.5–2.5 Ma), respectively. Diversification processes during the  
661 Pliocene have been reported for other Southern-Ocean marine benthic invertebrates including the  
662 Antarctic amphipods of the genus *Epimeria* (Verheye et al., 2017), deep-sea octopuses (Strugnell et al.,  
663 2008), and deep-sea octocorals (Dueñas et al., 2016). Molecular population-based studies have provided  
664 strong evidence from a wide range of marine and terrestrial taxa that fragmentation of populations into  
665 refugia during glacial maxima and subsequent expansions during interglacial periods may represent an  
666 important mechanism enhancing cryptic speciation processes (Allcock and Strugnell, 2012; Fraser et al.,  
667 2012; Poulin et al., 2014). Hence, the presence of isolated refugia during glacial maxima may have  
668 enhanced such process and the re-colonization of newly de-glaciated habitats could have favoured the  
669 recent radiation recorded in *Aequiyoldia* off South America and the Antarctic Peninsula.  
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1183 **Figure Captions**  
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1185 **Figure 1.** A) Main Southern Ocean provinces included in the study. B) Sampling sites of *Aequiyoldia*  
1186 *eightsii* off South America (SA), Falkland/Malvinas Islands (FI), and Antarctic Peninsula (AP). C)  
1187 Distribution of *Aequiyoldia eightsii* in the Southern Ocean (Linse et al., 2014).  
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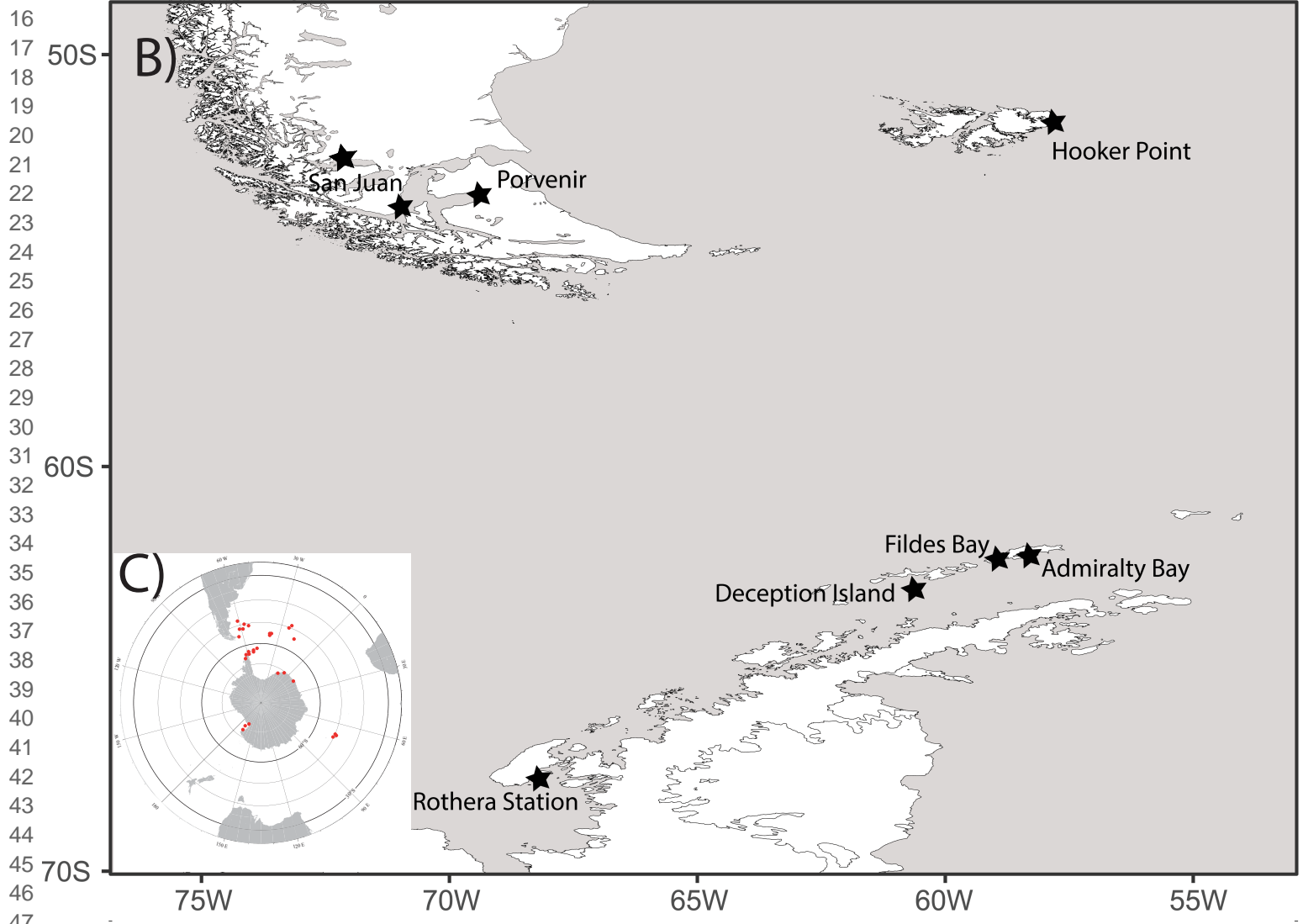
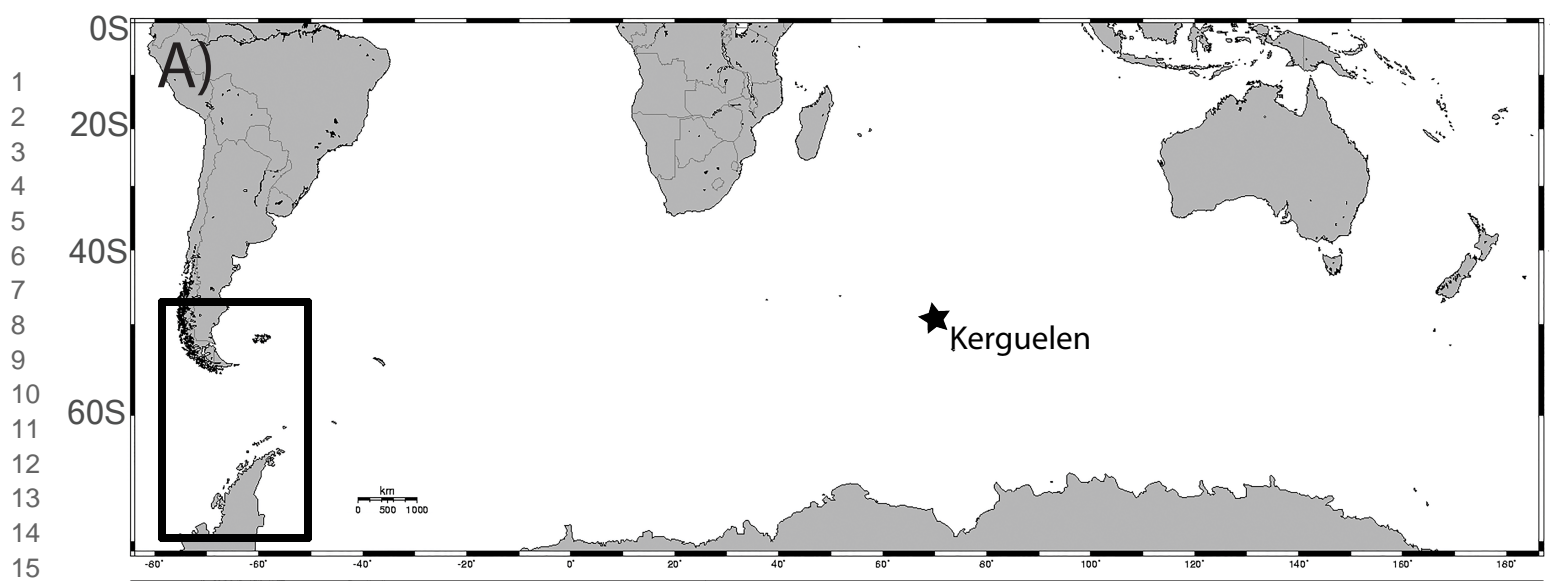
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1190 **Figure 2.** *Aequiyoldia eightsii* individuals from: a-c) Kerguelen Islands (KI = L1), d-f) Rothera Station  
1191 (AP = L2), g-i) Admiralty Bay (AP = L3), j-l) South America (SA = L4), m-o), and the  
1192 Falkland/Malvinas Islands (FI = L5).  
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1195 **Figure 3.** Bayesian maximum clade credibility tree of *Aequiyoldia* relationships based on mtDNA (COI)  
1196 sequences with special emphasis on Antarctic Peninsula and South American populations. Bootstrap  
1197 support values (MP and ML) and BPP are shown above the nodes (in that order). Map and colors indicate  
1198 major sampled areas. Round charts indicate the frequency of the recorded *Aequiyoldia* lineages across the  
1199 sampling sites.  
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1201 **Figure 4.** Bayesian maximum clade credibility tree based on mtDNA (COI) showing divergence time  
1202 estimates within A) protobranch bivalves and B) *Aequiyoldia*. Posterior probabilities are shown above the  
1203 clades. Grey boxed regions depict 95% Bayesian credible intervals (BCIs) for relative divergence times.  
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1206 **Figure 5.** Non-metric, Multidimensional Scaling (NMDS) ordination of *A. eightsii* samples from the  
1207 Kerguelen Islands (KI), Falkland/Malvinas Islands (FI), South America, and the Antarctic Peninsula  
1208 using shell morphological data following Rabarts and Whybrow (1979).  
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1211 **Supplementary Figure 1.** A) mtDNA (COI) maximum parsimony networks including *Aequiyoldia*  
1212 individuals collected at different localities in the Southern Ocean. B) nucDNA (ITS1 and ITS2) maximum  
1213 parsimony network including *Aequiyoldia* individuals from different localities in the Southern Ocean. A  
1214 coloured circle represents each haplotype and indicate the main area where it was collected. The size of  
1215 the circle is proportional to its frequency in the whole sampling effort.  
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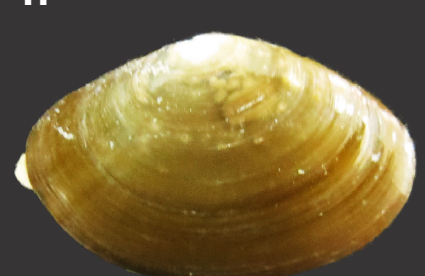
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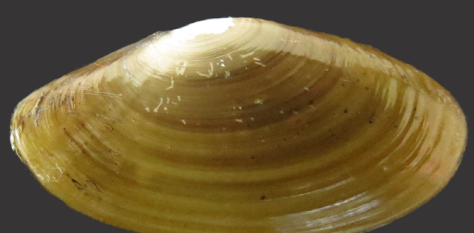
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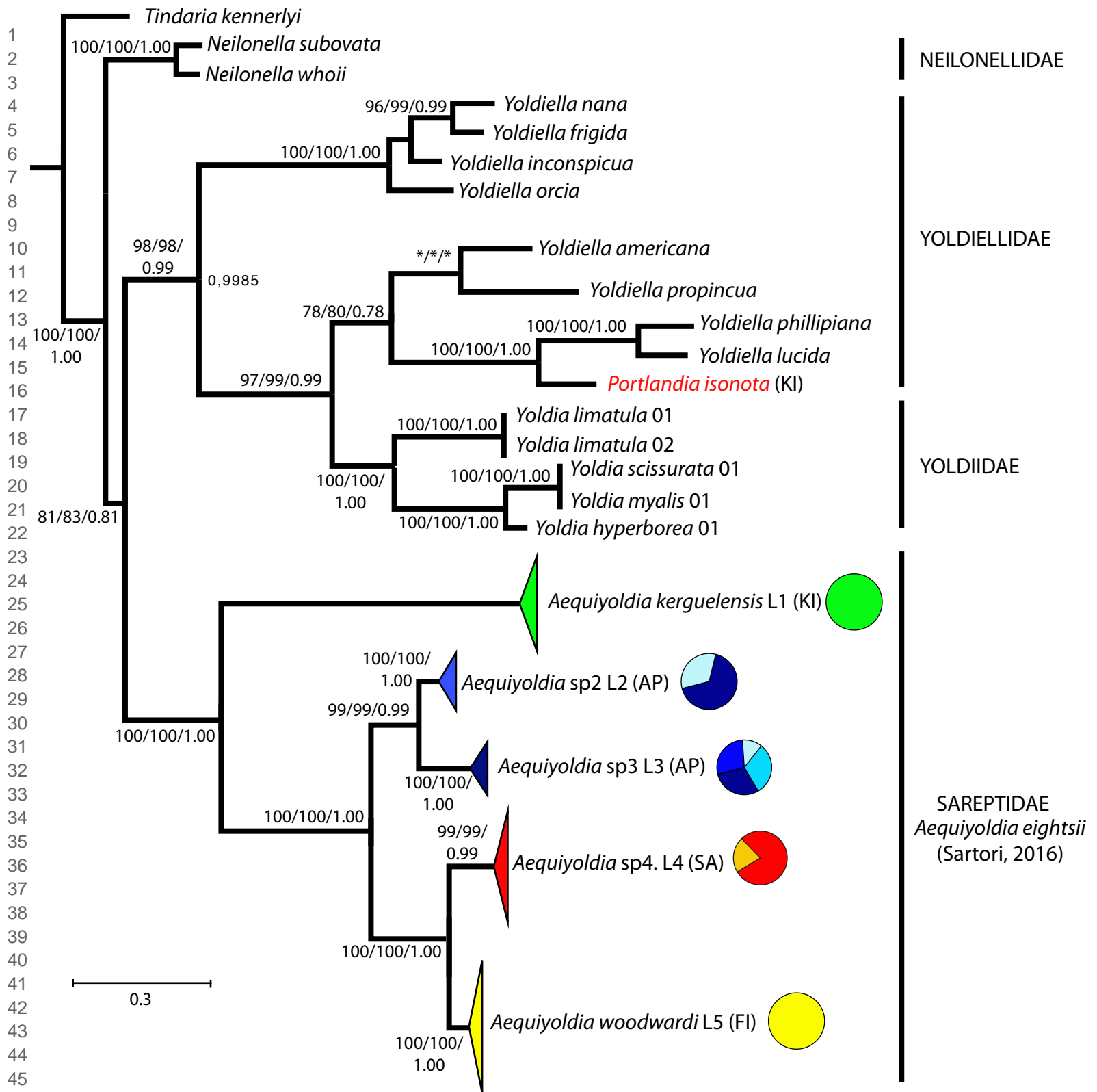


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47 **ANTARCTIC PENINSULA**

47 **SOUTH AMERICA**

47 **KERGUELEN ISLAND (KI)**

48 Admiralty Bay (AP)

48 San Juan (SA)

48 Port Aux Français

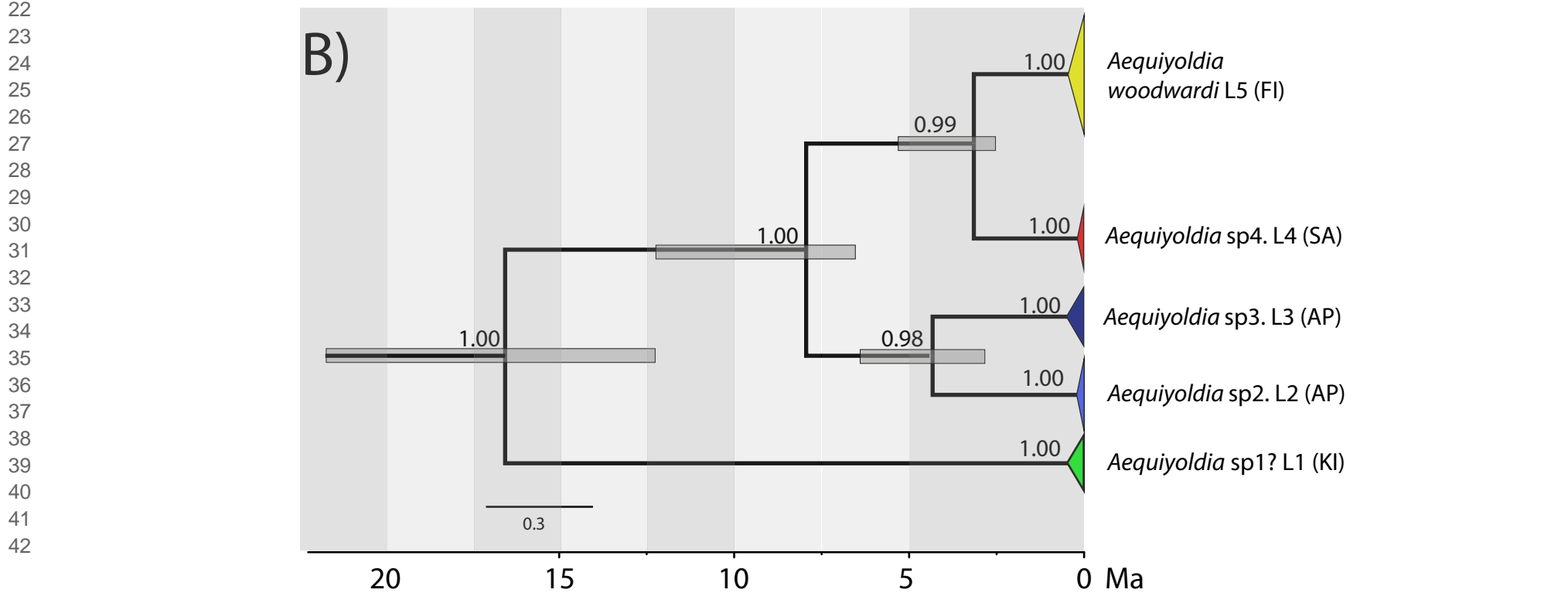
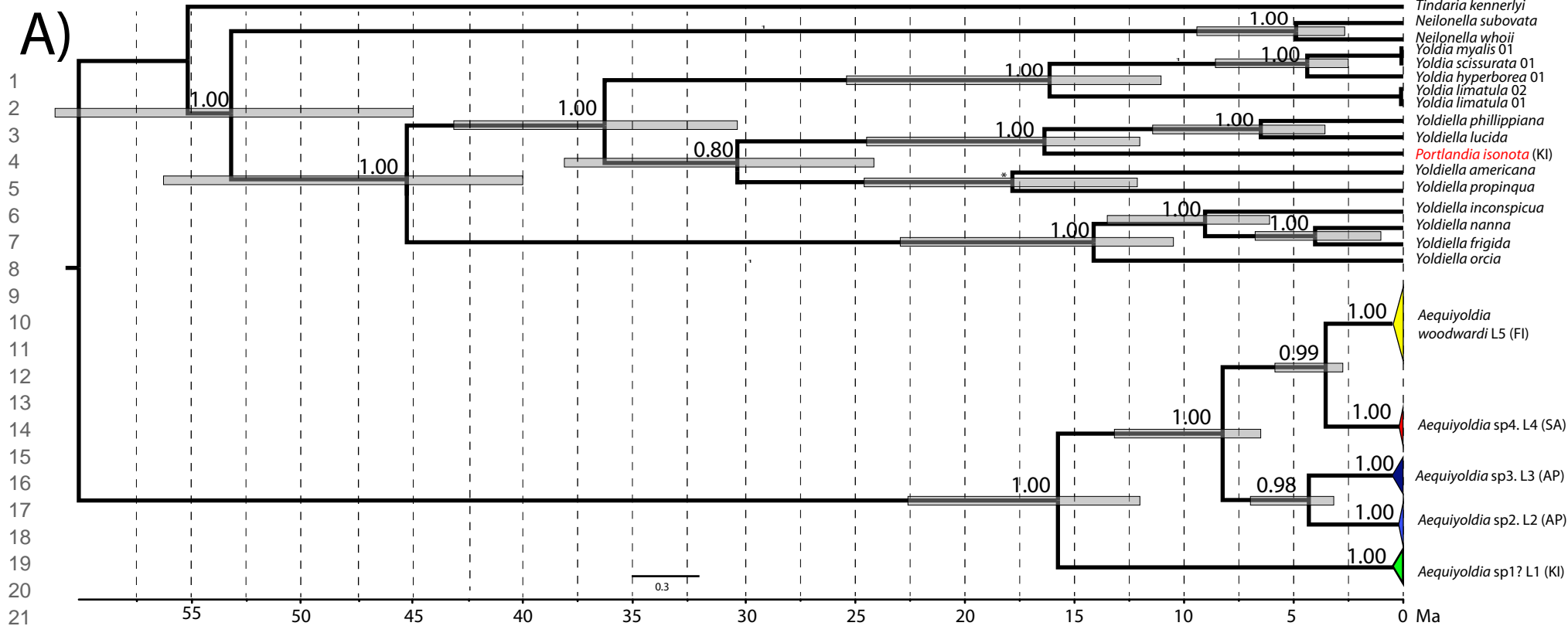
49 Fildes Bay (AP)

49 Porvenir (SA)

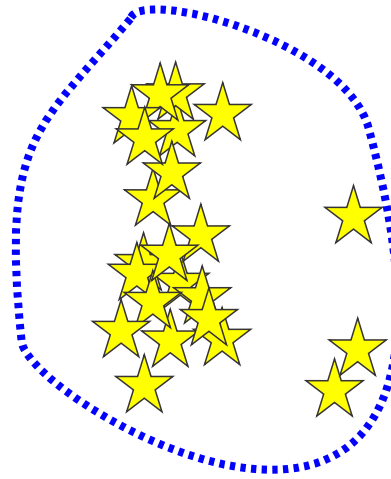
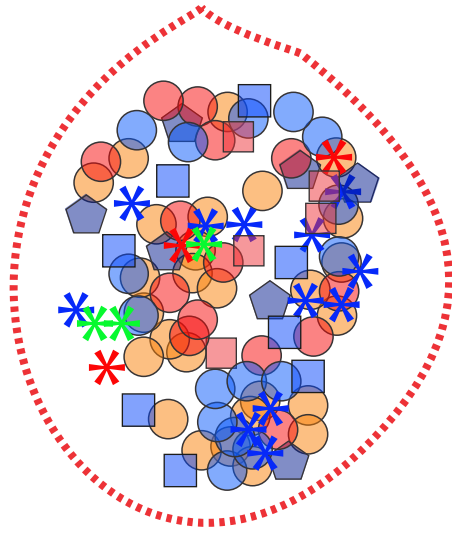
50 Deception Island (AP)

50 Hooker Point (FI)

51 Rothera Station (AP)



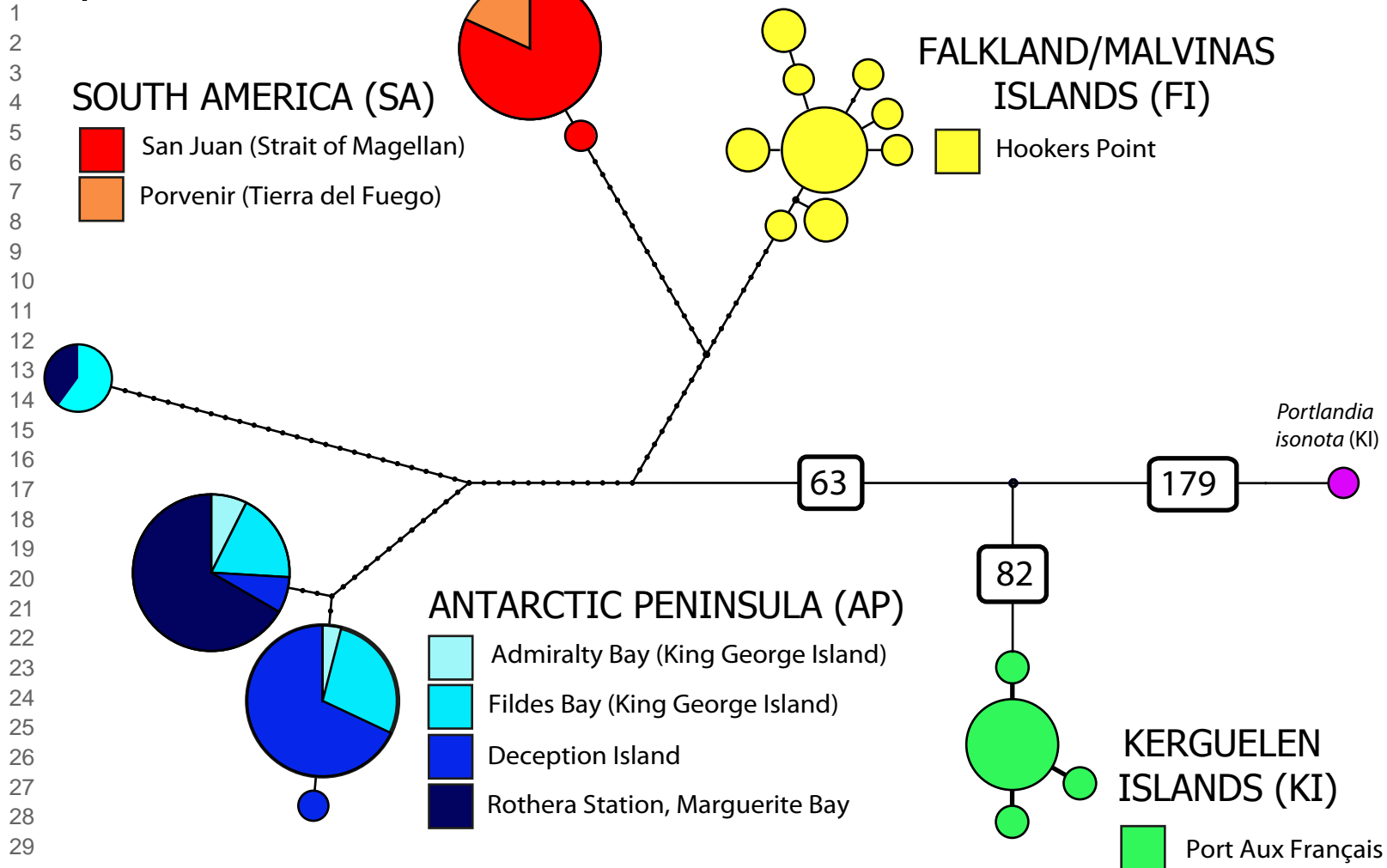




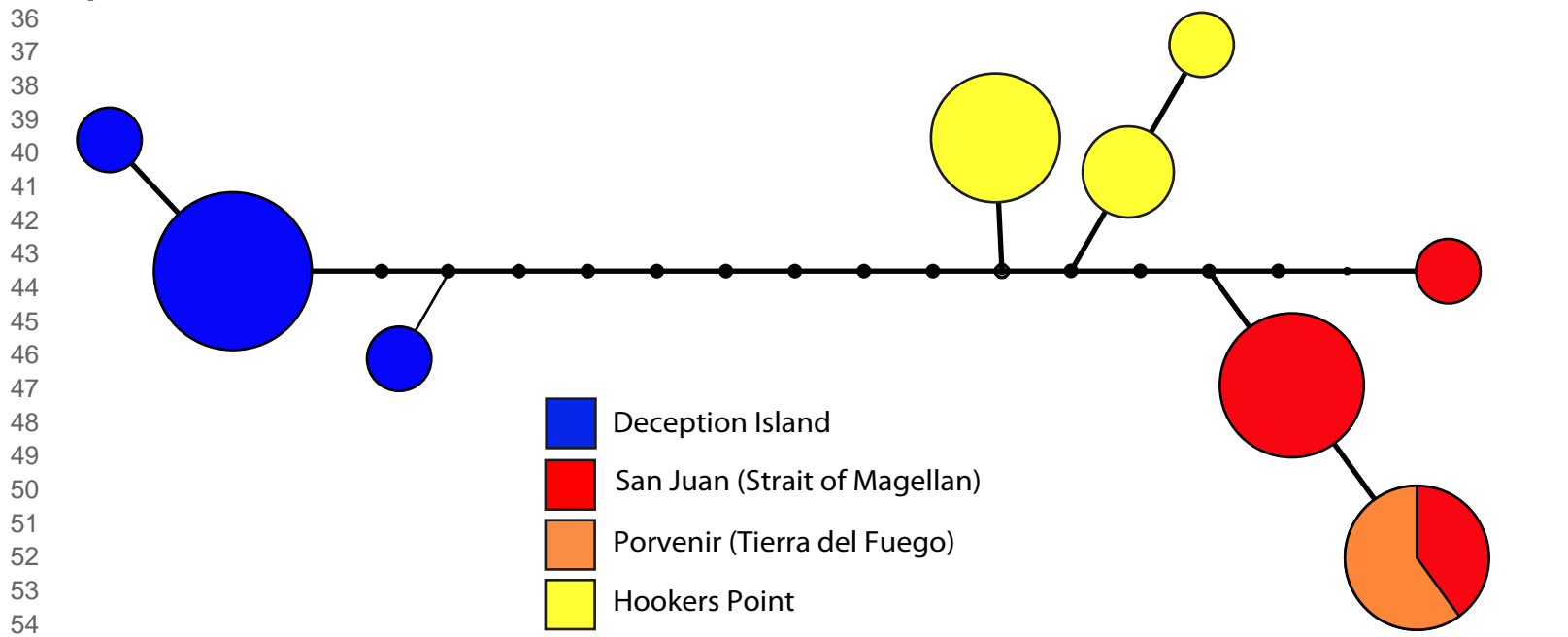
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| SOUTH AMERICA  | FALKLAND/MALVINAS ISLANDS | ANTARCTIC PENINSULA   | KERGUELEN ISLANDS          |
|--|---------------------------|---|----------------------------|
| <p>● Porvenir</p> <p>● San Juan</p> <p>* Skyring Sound</p> | <p>★ Hooker Point</p>     | <p>⬠ Admiralty Bay</p> <p>* Fildes Bay</p> <p>● Deception Island</p> <p>■ Rothera Station</p> | <p>* Port Aux Français</p> |

# A) COI



# B) ITS1 + ITS2





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

| <b>Sites</b>                              | <b>Lat.</b> | <b>Long.</b> | <b>n (COI)</b> | <b>n (ITS)</b> |
|---|-------------|--------------|----------------|----------------|
| Port-aux-Français, Kerguelen Islands      | 49°21'S     | 70°13'W      | 11             | 9              |
| Hooker's Point, Falkland/Malvinas Islands | 51°42'S     | 57°46'W      | 20             | 7              |
| San Juan River, Strait of Magellan        | 53°38'S     | 70°56'W      | 20             | 9              |
| Porvenir, Tierra del Fuego                | 53°18'S     | 70°22'W      | 5              | -              |
| Skyring Sound, Strait of Magellan         | 52°34'S     | 70°56'W      | -              | 3              |
| Admiralty Bay, South Shetland Islands     | 62°09'S     | 58°27'W      | 6              | -              |
| Fildes Bay, South Shetland Islands        | 62°12'S     | 58°57'W      |                | -              |
| Deception Island, South Shetland Islands  | 62°56'S     | 60°36'W      | 20             | 8              |
| Rothera Station, Antarctic Peninsula      | 67°34'S     | 68°07'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% | **** |