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

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5 Mio-Pliocene trans-Drake separation and diversification
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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 50×10^6 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 50×10^6 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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425 426 427 *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.

621 622 4.7 Biogeography in *Aequiyoldia* spp.

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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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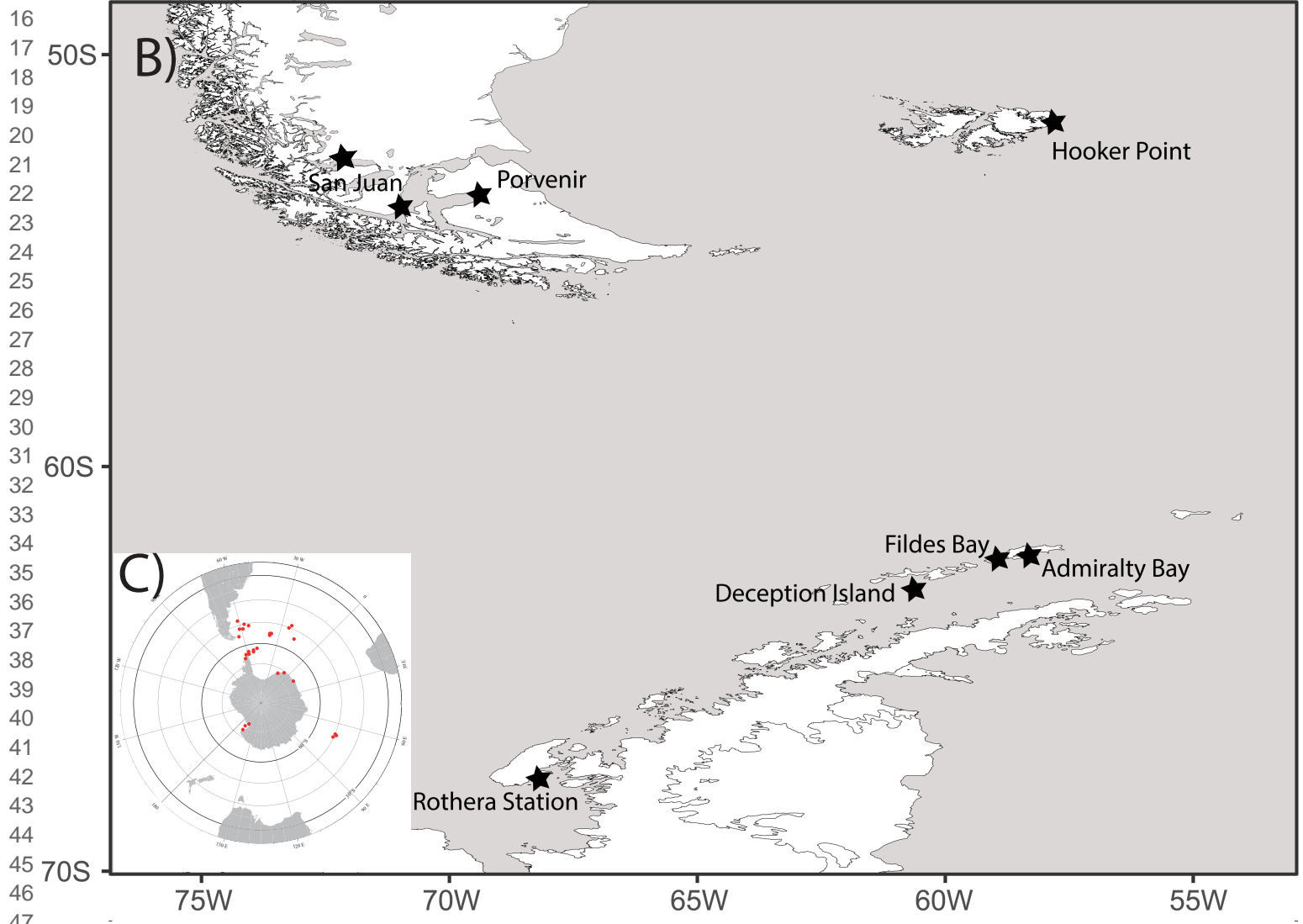
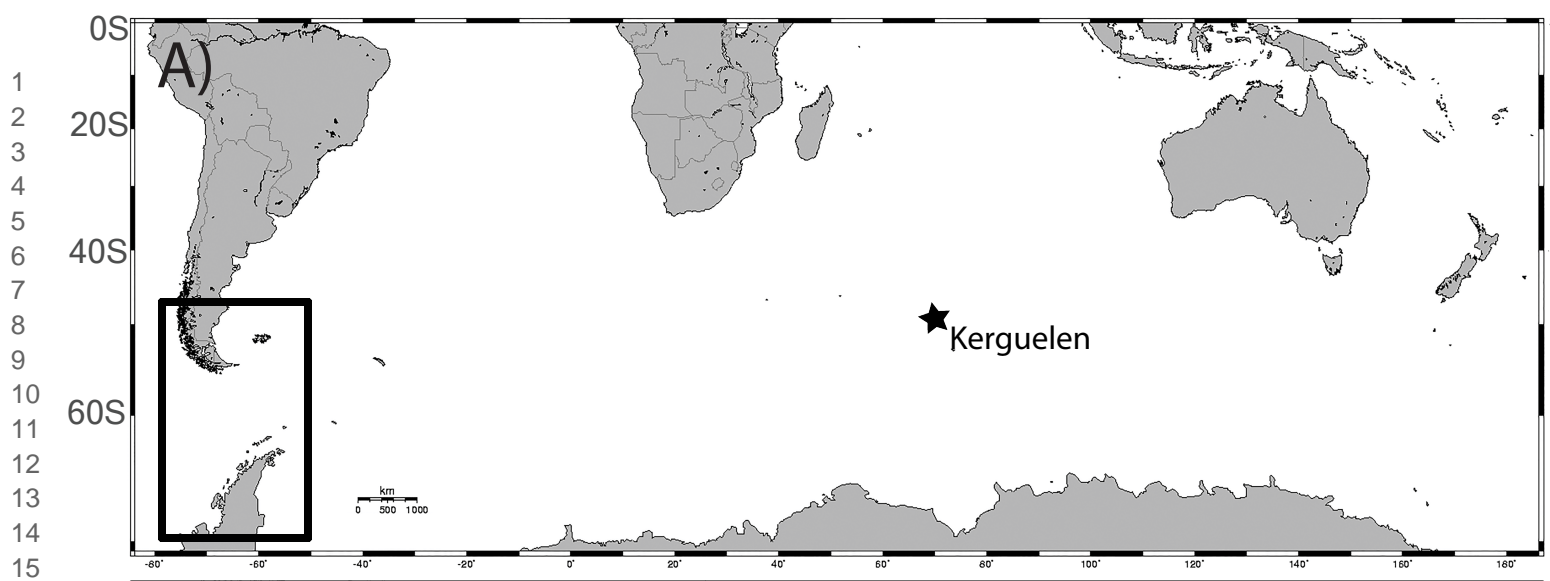
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1190 **Figure 2.** *Aequiyoldia eightsii* individuals from: a-c) Kerguelen Islands (KI = L1), d-f) Rothera Station
1191 (AP = L2), g-i) Admiralty Bay (AP = L3), j-l) South America (SA = L4), m-o), and the
1192 Falkland/Malvinas Islands (FI = L5).
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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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1202 **Figure 4.** Bayesian maximum clade credibility tree based on mtDNA (COI) showing divergence time
1203 estimates within A) protobranch bivalves and B) *Aequiyoldia*. Posterior probabilities are shown above the
1204 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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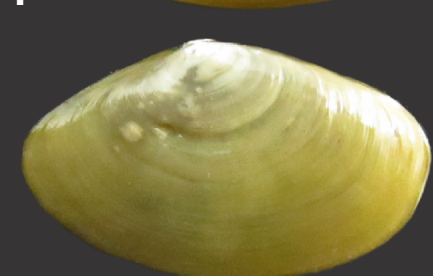
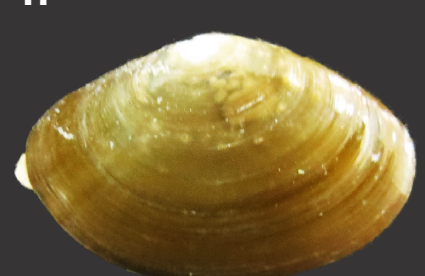
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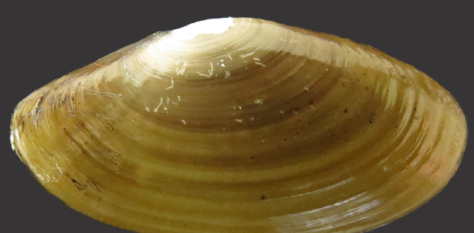
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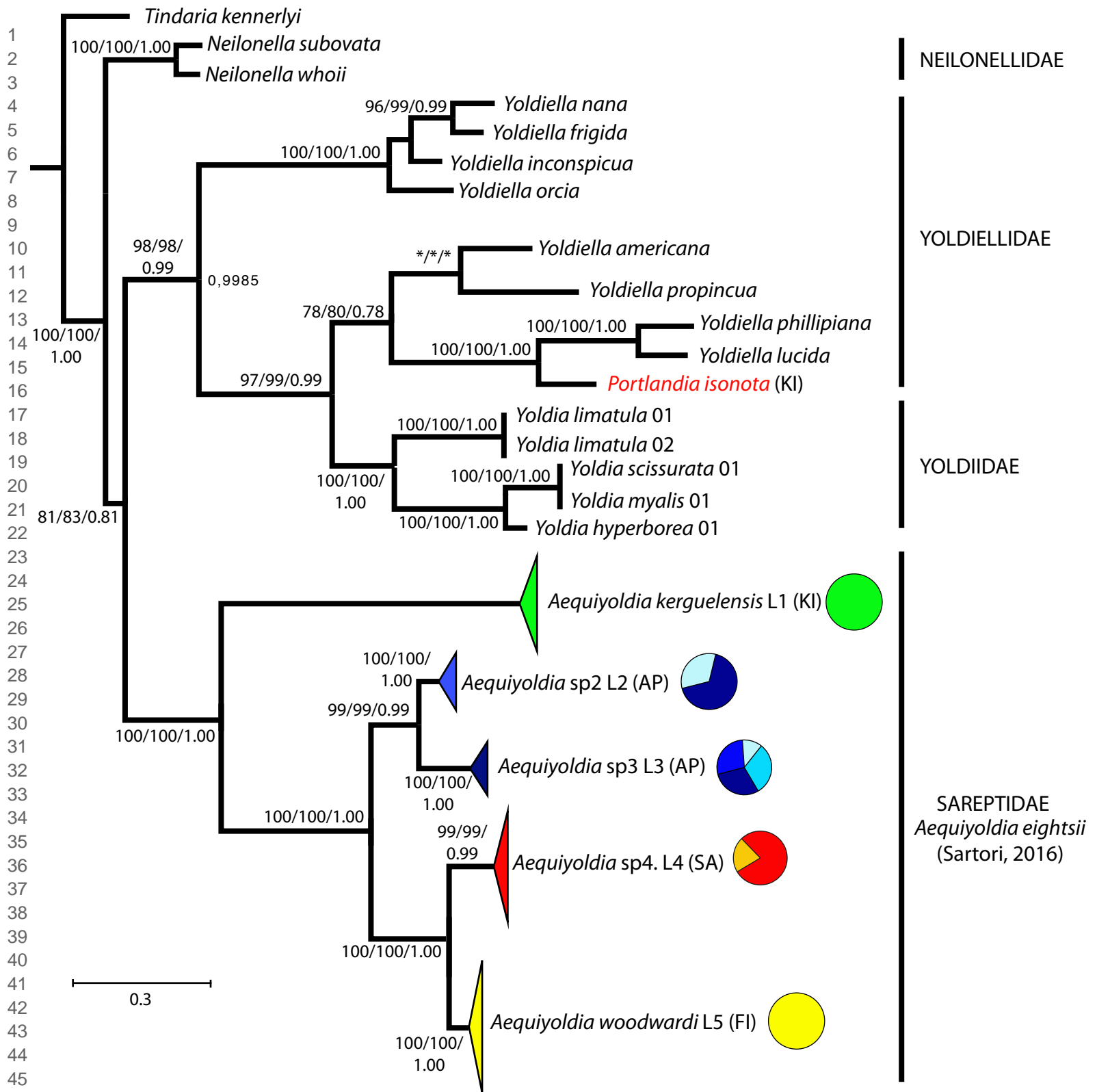


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47 **ANTARCTIC PENINSULA**

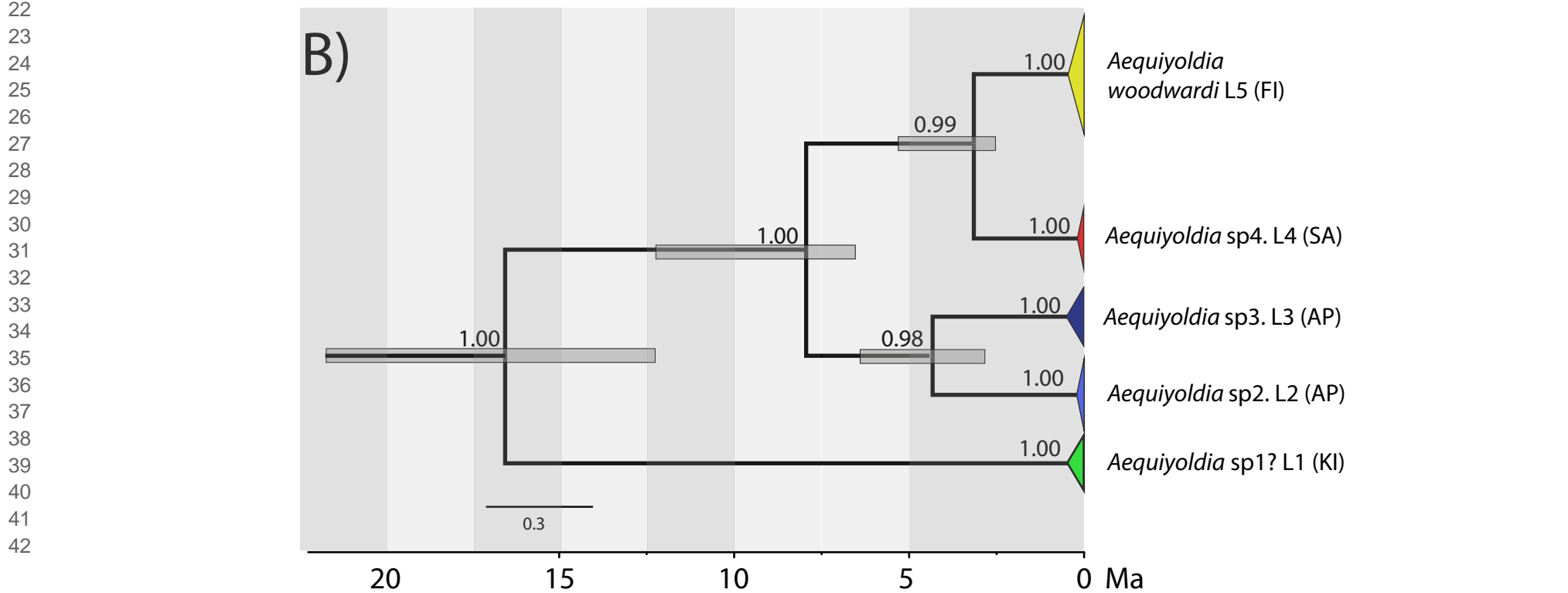
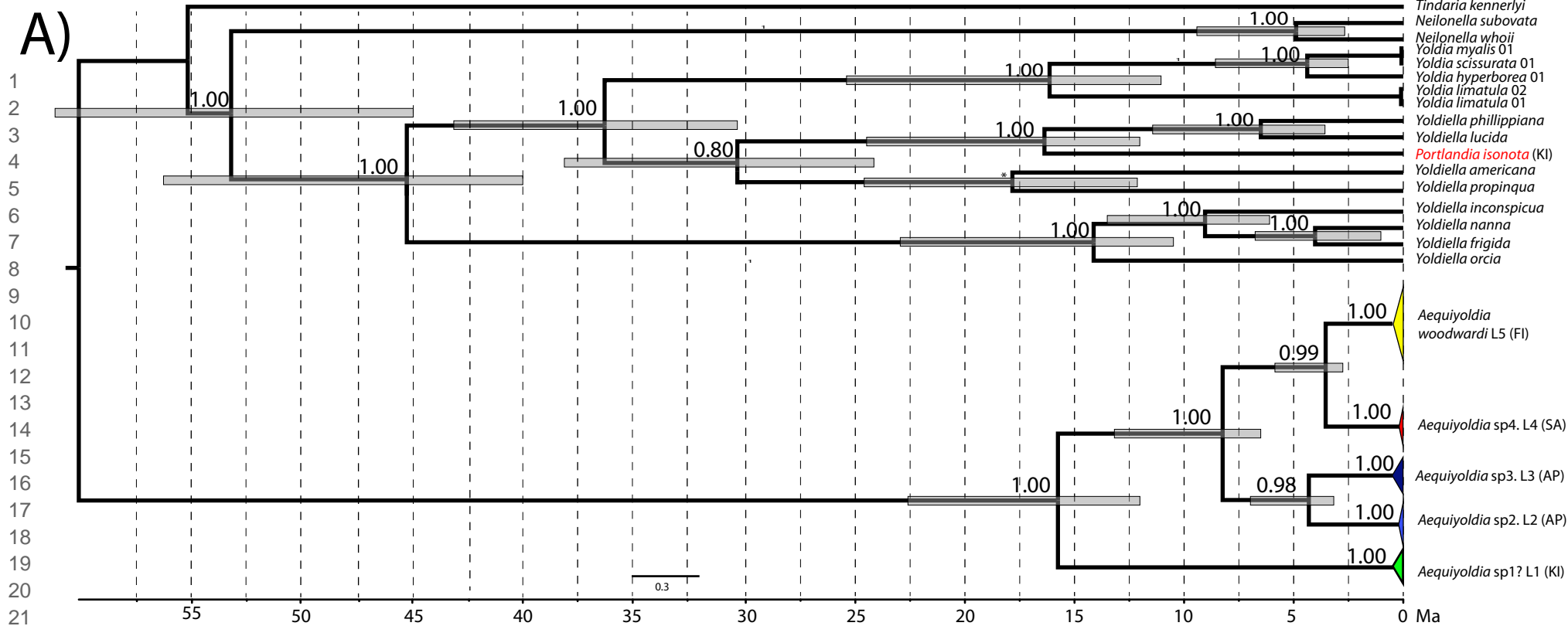
- 48 Admiralty Bay (AP)
- 49 Fildes Bay (AP)
- 50 Deception Island (AP)
- 51 Rothera Station (AP)

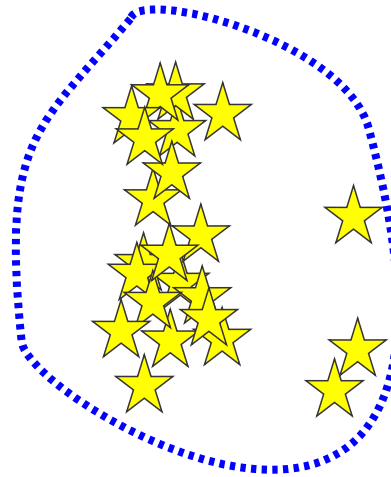
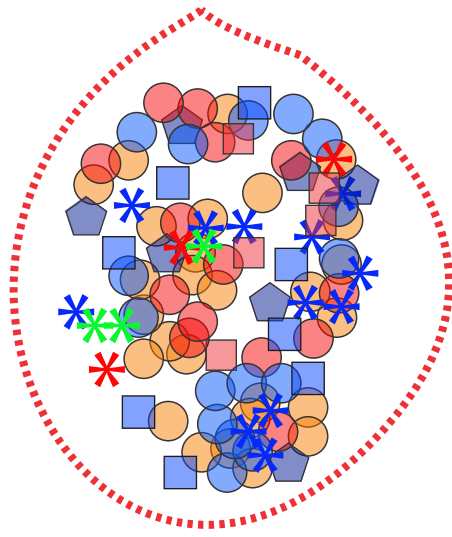
47 **SOUTH AMERICA**

- 48 San Juan (SA)
- 49 Porvenir (SA)
- 50 Hooker Point (FI)

47 **KERGUELEN ISLAND (KI)**

- 48 Port Aux Français





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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>

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SOUTH AMERICA (SA)

- San Juan (Strait of Magellan)
- Porvenir (Tierra del Fuego)

FALKLAND/MALVINAS ISLANDS (FI)

- Hookers Point

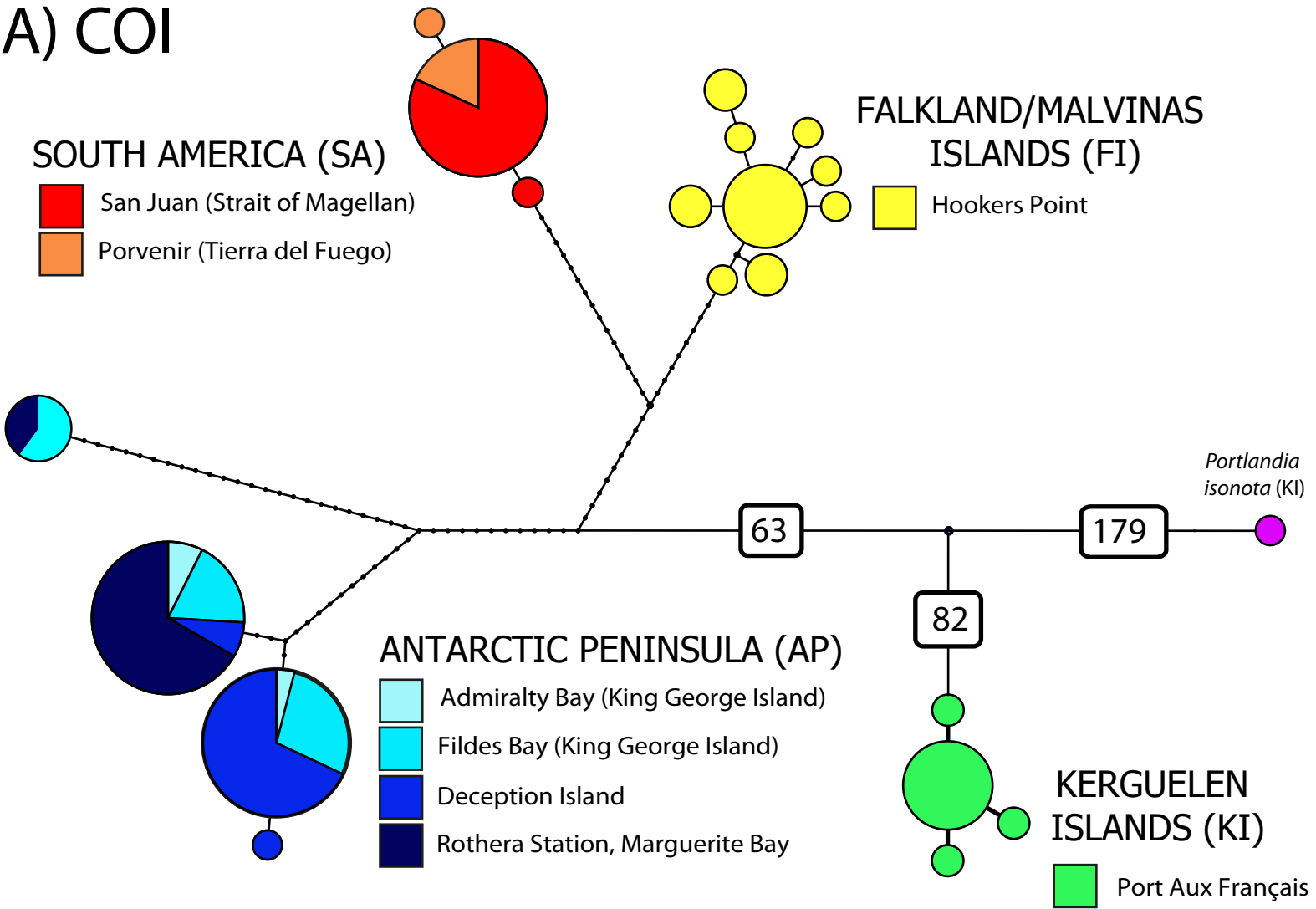
ANTARCTIC PENINSULA (AP)

- Admiralty Bay (King George Island)
- Fildes Bay (King George Island)
- Deception Island
- Rothera Station, Marguerite Bay

KERGUELEN ISLANDS (KI)

- Port Aux Français

*Portlandia
isonota* (KI)



B) ITS1 + ITS2

- Deception Island
- San Juan (Strait of Magellan)
- Porvenir (Tierra del Fuego)
- Hookers Point

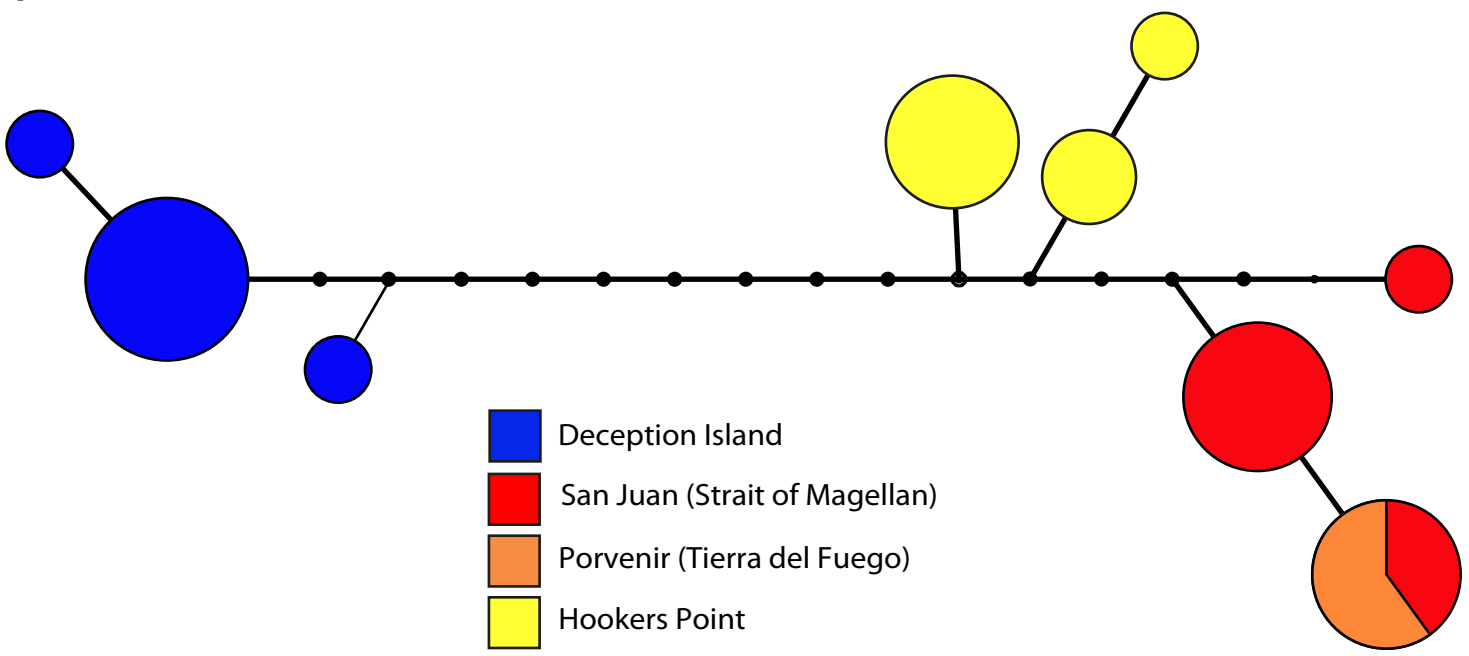


Table 1. Sampling sites of *Aequiyoldia eightsii*: Geographical coordinates, name of collector, n: number of analyzed individuals per locus.

Sites	Lat.	Long.	n (COI)	n (ITS)
Port-aux-Français, Kerguelen Islands	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%	****