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Ocean barriers and glaciation: evidence for explosive radiation of mitochondrial lineages in the Antarctic sea slug *Doris kerguelenensis* (Mollusca, Nudibranchia)

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Abstract

Strong currents and deep passages of water can be barriers for larval dispersal of continental marine animals, but potential effects on direct developers are under-investigated. We examined the genetic structure of *Doris kerguelenensis*, a directly developing sea slug that occurs across the Drake Passage, the body of water separating Antarctica from South America. We found deep mitochondrial divergences within populations on both sides of the Drake Passage, and South American animals formed multiple sister-group relationships with Antarctic animals. A generalised molecular clock suggested these trans-Drake pairs diverged during the Pliocene–Pleistocene, after the formation of the Drake Passage. Statistical parsimony methods recovered 29 separate haplotype networks (many sympatric) that likely correlate with allopatric events caused by repeated glacial cycles. Data from 16S were congruent but more conserved than COI, and the estimated ancestral 16S haplotype was widespread. The marked difference in the substitution rates between these two mitochondrial genes results in different estimates of connectivity. Demographic analyses on networks revealed some evidence for selection and expanding populations. Contrasting with the Northern Hemisphere, glaciation in Antarctica appears to have increased rather than reduced genetic diversity. This suggests orbitally forced range dynamics based on Northern Hemisphere phylogeography do not hold for Antarctica. The diverse lineages found in *D. kerguelenensis* point towards a recent, explosive radiation, likely reflecting multiple refuges during glaciation events, combined with limited subsequent dispersal. Whether recognised as cryptic species or not, genetic diversity in Antarctic marine invertebrates appears higher than expected from morphological analyses, and supports the Antarctic biodiversity pump phenomenon.

Keywords: Antarctica, cryptic speciation, diversity pump, direct development, long-distance dispersal, phylogeography

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Introduction

Growing evidence from mitochondrial DNA (mtDNA) data suggests that the level of gene flow maintained by marine invertebrates may have little to do with predictions inferred from reproductive strategy. Some organisms with no larval dispersal stage appear widespread and with no significant genetic structure (Oosthuizen *et al*. 2004; Waters & Roy 2004; Teske *et al*. 2007). Alternatively, there are many taxa that do not appear to realise their dispersal potential and show significant genetic structure despite having planktonic larvae (Reeb & Avise 1990; Bird *et al*. 2007). This paradox is likely caused by the relative effects of behavioural and mechanical larval retention, selection, the presence of historical barriers, or rafting processes (see Palumbi 1996; Donald *et al*. 2005). Strong currents and deep passages of water can be effective barriers to dispersal to planktonic larvae of marine animals that occur on continental shelves. However, potential effects on adult

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migration of benthic animals lacking a dispersive stage are under-investigated. With a great number of Antarctic invertebrates reproducing without a dispersive larval stage (Poulin *et al*. 2002), the Drake Passage can provide a robust model for understanding patterns of gene flow across other oceanographic barriers.

The Antarctic Circumpolar Current (ACC) flows through the Drake Passage, the body of deep water (approximately 3.5 km on average) that separates South America and Antarctica, resulting in the strongest flowing current in the world's oceans (Barker & Thomas 2004). The Antarctic convergence zone (also known as the Polar Front) occurs in the passage where the cold water of Antarctica meets warmer water from the north. Consequently, the Drake Passage presents a formidable barrier to organisms, with temperature and salinity gradients, shear forces, depth and distance, and is assumed to be a distinct barrier to marine invertebrates with pelagic larvae (Crame 1999; Clarke *et al*. 2005; Thatje *et al*. 2005). However, the Drake Passage's potential as a barrier to animals that lack dispersive larvae, and are capable of living in a wide range of depths (eurybathic), is only beginning to receive attention (e.g. Linse *et al*. 2007; Hunter & Halanych 2008). The opening of the Drake Passage occurred 24–41 million years ago, and if gene flow between continents was permanently interrupted by this event, deep genetic divergence between separated populations should be evident. A connectivity paradigm invoking the Scotia Arc as providing intermediate dispersal points or 'stepping stones' across the Drake Passage (Clarke & Crame 1992) is now being tested empirically (Linse *et al*. 2007).

The Antarctic nudibranch (sea slug) *Doris kerguelenensis* (Bergh, 1884) is a simultaneous hermaphrodite, which lays down cross-fertilised embryos in a gelatinous matrix on the benthos. This species is a direct developer, and young crawl out from the egg mass after a period estimated to be greater than 36 months (Hain 1989; Wägele 1996). This strategy greatly limits the potential dispersal of this species, and if coupled with the probable slow growth and delayed maturity typical of most Antarctic invertebrates (Dayton *et al*. 1974; Arnaud 1977; Brey & Clarke 1993), indicates these slugs might have long generation times. Individual slugs caged by Dayton *et al*. (1974) did not move from their prey sponge ($n = 8$) or reproduce ($n = 15$) after a year of observation. But despite its reduced dispersal capabilities, and 'slow' lifestyle, the geographical range of *D. kerguelenensis* is described as very broad; it shows a circumantarctic and Magellanic (southern South American) shelf distribution (intertidal to 788 m, Burn 1973; Wägele 1990; Schrödl 2003) and is also present in the southern Atlantic and tropical Pacific (off Río de la Plata, Argentina, 740 m, Bouchet 1977; New Caledonia, 500–680 m, Valdés 2001).

Doris kerguelenensis (formerly *Austrodoris kerguelenensis*) was taxonomically reviewed by Wägele (1990) who recognised 12 synonyms within a morphologically variable species. Along with a more recently described species, *Doris georgiensis*, there appears to be a total of two externally indistinguishable species of *Doris* in Antarctica (Taylor *et al*. 2005). The complicated taxonomic history of *D. kerguelenensis*, combined with an increasing recognition of cryptic speciation in Antarctica waters (e.g. Held 2003; Raupach & Wägele 2006; Wilson *et al*. 2007), made us particularly aware that different populations contiguous along the Antarctic continental shelf might also represent different species.

As *D. kerguelenensis* is widespread but predicted to be a poor disperser, we were interested in describing its phylogeographical structure in order to better understand barriers or conduits of genetic connectivity in the marine realm. In particular, we wanted to examine whether the Drake Passage represented a natural barrier to animals without a larval dispersal stage. If lacking a larval dispersal stage restricted gene flow in *D. kerguelenensis*, we predicted that population structure would be congruent with an isolation-by-distance model. We chose the mitochondrial protein-coding gene cytochrome oxidase I (COI) as one of our markers because its widespread use and employment for barcoding allows comparison with many other species-level divergences. We also used partial 16S rRNA, as it is also a commonly employed marker that allows comparison. In summary, we investigated (i) if the deepwater Drake Passage is a barrier to dispersal for *D. kerguelenensis* populations, (ii) if populations were structured, and (iii) if cryptic species were present.

Materials and methods

Sampling

Tissue samples from 144 specimens of *Doris kerguelenensis* (Bergh, 1884) were collected during various Antarctic field expeditions using a Blake trawl, Smith-MacIntyre grab, wire dredge, epibenthic sled or SCUBA diving. Most collections were made from expeditions on the *ARSV Laurence M. Gould* in 2004 and 2006 and the *RV Polarstern* in 2000 and 2002. Other contributing cruises are listed in the Acknowledgements section. Selected vouchers were photographed, and samples were frozen or fixed in ethanol. Collection data and haplotype accession information are available in Table S1, Supporting information. Voucher specimens have been deposited at the Smithsonian Museum of Natural History, and other samples were accessed from collections at the Zoologische Staatssammlung München, and the California Academy of Sciences. Sampling regions are shown in Fig. 1. Three additional *D. kerguelenensis* haplotypes were accessed from GenBank (COI, AF249780; 16S, AF249233-34). Distance measurements between sampling sites were calculated using Google Earth 4.0.2694, measuring a straight line while avoiding coastlines.

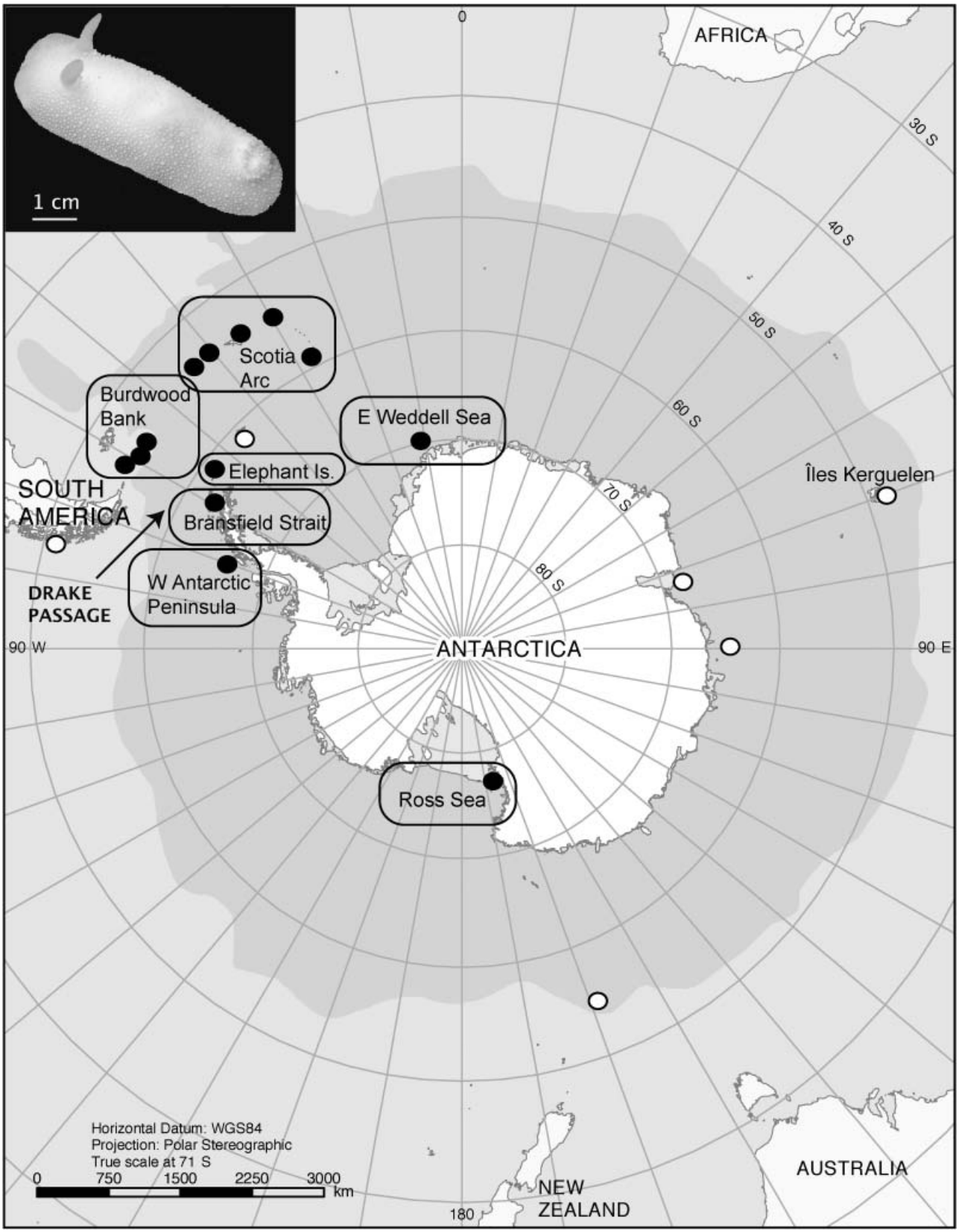

Fig. 1 Map showing sampling regions for *Doris kerguelenensis*. White circles indicate distributional records, black circles indicate sampled sites. Base map supplied by the Census of Antarctic Marine Life, courtesy of the Australian Antarctic Division. Photo by Dirk Schories.

DNA extraction, polymerase chain reaction and sequencing

DNA was extracted from tissue samples using a QIAGEN DNeasy Tissue Kit according to the manufacturer's instructions. A fragment of COI was amplified using the Folmer *et al*. (1994) primers and the following cycling conditions: initial denaturation 95 °C, 2 min; 35 cycles of denaturation 94 °C, 30 s, annealing 44–45 °C, 1 min; extension 68 °C, 1 min; final extension 72 °C, 7 min. A fragment of the 16S rRNA gene was amplified using the universal primers 16Sarl and 16SbrH (Palumbi *et al*. 1991) with the following conditions: initial denaturation 94 °C, 3 min, 35 cycles of denaturation 94 °C, 30 s, annealing 50 °C, 30 s, and extension, 68 °C, 1 min; final extension 68 °C, 5 min. All polymerase chain reaction (PCR) products were bi-directionally sequenced on a Beckman CEQ 8000. Sequences were edited using Seqman II (DNAStar, Lasergene) and aligned manually in Se-Al 2.0a11 (Rambaut 2002). Following alignment, COI data were translated into amino acids to check for premature stop codons indicative of sequencing errors or nuclear pseudogenes. All sequences generated here were deposited to GenBank under accessions EU823127–EU823269 (see Table S1 for more detail). The combined 16S + COI alignment was deposited to TreeBASE.

Genetic diversity, structure and demography

We generated haplotype networks for each gene using TCS 1.21 (Clement *et al*. 2000). Networks were constructed using maximum parsimony, with a 95% probability threshold that infers that character changes defining connections are due to a single mutation. Gaps were treated as a fifth base for 16S, following Ogden & Rosenberg (2007). Geographical data were overlaid on these resulting networks. Compositional heterogeneity was assessed with a chi-squared test in PAUP* (Swofford 2002). Mean uncorrected sequence divergence values (p) and maximum-likelihood corrected values [model TIM + I + Γ selected with Akaike information criterion (AIC)] were estimated using PAUP*, within and between all networks identified by TCS.

Levels of polymorphism in the data were represented by haplotypic and nucleotide diversity indices (Nei 1987) calculated in DnaSP 4.50.2 (Rozas *et al*. 2008). A hierarchical analysis of molecular variance (AMOVA) incorporating the above model parameters on the combined data set assessed variance components based on geographical partitioning, and was carried out in Arlequin 3.11 (Schneider *et al*. 2000). Because molecular data are not normally distributed, the significance was tested by permutating the data. Seven geographical regions were defined a priori and are shown in Fig. 1. We separated the Antarctic Peninsula into three regions: (i) Elephant Island, (ii) the Bransfield Strait, and (iii) west Antarctic Peninsula due to differences in coastal currents and water circulation patterns (Smith *et al*. 1999). For each AMOVA, a sample site was defined as a population.

We used Arlequin 3.11 to calculate COI pairwise fixation indices using ϕ_{ST}, an analogue of F_{ST} that accounts for genetic distance. We used networks estimated by TCS to represent populations, as they were a better estimate of relatedness than geographical partitioning. To consider evidence of selection on COI networks before making demographic estimates, the McDonald–Kreitman test (McDonald & Kreitman 1991), as implemented in DnaSP 4.50.2 was carried out between well-supported sister-group networks containing four or more individuals and a two-tailed Fisher's exact test was employed to test for significance. To infer past population changes and/or deviations from neutrality, the R_2 index (Ramos-Onsins & Rozas 2002) and Fu's *Fs* test (Fu 1997) were calculated in DnaSP 4.50.2 and Arlequin 3.11 respectively, for each well-supported network containing four or more individuals. These two tests are thought to be the most powerful for detecting population growth (Ramos-Onsins & Rozas 2002), with R_2 being the most sensitive for small sample sizes. Fu's *Fs* was calculated using an infinite sites model, with 1000 bootstrap replicates for assessing significance. Positive values indicate a lack of significant recent mutations that may have resulted from balancing selection, population structure, or decline. Negative values reflect excesses of recent mutations that may indicate population expansion or selective sweeps. To identify significant R_2 values, we carried out 1000 coalescent simulations under a population growth-decline model (with no recombination) in DnaSP 4.50.2. Additionally, mismatch analysis in Arlequin 3.11 compared the frequency distribution of pairwise differences among haplotypes against a model of rapid expansion (Rogers & Harpending 1992) on each network and pooled data. Significance was assessed with 1000 parametric bootstrapping replicates (Schneider & Excoffier 1999).

Phylogenetic analyses

Outgroup choice was based on a review and phylogenetic analysis of cryptobranch dorid nudibranchs (Valdés 2002). On the basis of one reproductive character (a direct connection of the seminal receptacle to the bursa copulatrix) Valdés (2002) synonymised several genus names with *Doris* (*Doriopsis*, *Archidoris*, *Austrodoris*, *Neodoris*, *Siraius*, *Doriorbis*). We rooted trees with an outgroup species formerly included in *Archidoris, A. pseudoargus* (Rapp, 1827), which was previously linked with *Austrodoris* (Burn 1973; Wägele 1990) (AF249224, AY345030; AJ225180, AJ223256). We also included two additional outgroup taxa *Doriopsis granulosa* Pease, 1860 (AF249223, AF249798) and *Cadlina laevis* Linnaeus, 1767 (AJ225182, AJ223258). The possibility of substitutional saturation for COI was evaluated statistically in DAMBE 4.5.56 (Xia & Xie 2001).

A combined data set comprised of only unique COI + 16S haplotypes was subjected to phylogenetic analysis using maximum parsimony (MP) in PAUP* (Swofford 2002). MP analyses employed heuristic searches with 10 random additions and tree-bisection–reconnection (TBR) branch swapping, and all characters were unweighted and unordered. Branch support was evaluated by bootstrapping (1000 replicates). For Bayesian analyses in MrBayes 3.1.2 (Huelsenbeck & Ronquist 2001), data were set as two unlinked partitions, using the model GTR + I + Γ selected for both via the AIC (see Posada & Buckley 2004) using MrModelTest 2.2 (Nylander 2002). We ran two replicate analyses of six simultaneous chains (1 cold) run to 10^7 generations. Trees were sampled every 1000 generations, and were used to construct a 50% majority-rule consensus tree with Bayesian posterior probabilities. We used Tracer version 1.3 (Rambaut & Drummond 2005) to observe stationarity of obtained probabilities and discarded the first 3000 trees that represented a pre-stationarity phase.

To roughly date divergences between trans-Drake sister groups, we applied a teguline gastropod COI molecular clock calibrated over the Isthmus of Panama (Hellberg & Vacquier 1999). We used both minimum and mean likelihood corrected pairwise divergences to account for a small part of the variation encompassed by these estimates.

Diversity estimates

An overestimation of the number of species is likely if the number of individuals in a population is undersampled, or the number of populations are undersampled; additional polymorphisms or populations may bridge differences between others, decreasing the number of perceived species (Davis & Nixon 1992). Alternatively, undersampling loci would bias the results into recognising less species that actually exists. How important undersampling is depends on the rate of population/network discovery. To estimate the level of 'species' (= network) diversity that may exist for *D. kerguelenensis*, we applied methodologies more generally used for ecological studies. We assembled species rarefaction curves using statistics generated from EstimateS 8.0.0 (Colwell 2006) to determine if the rate of discovery of new networks slowed down as sampling increased. We assumed each COI network to represent a species, and constructed both individual- and sample-based rarefaction curves. These curves represent the means of repeated resampling (100 replicates without replacement) of all pooled individuals or all pooled samples. Examining the slope of these curves allows us to assess the impact of undersampling on estimating diversity.

In light of the high levels of cryptic diversity, and because rarefaction methods cannot be used for extrapolation (Tipper 1979), we also examined asymptotic and nonparametric richness estimators to assess how much diversity might actually exist. Three broad types of richness estimators exist. Both the Michaelis–Menten (MM) and nonparametric methods are thought to outperform fitting a lognormal abundance (Longino *et al*. 2002), so only the former two were utilised here. The MM equation fits an asymptote to species accumulation curves (here the species observed given the observed samples, S_{obs}, or Mao Tau) and nonparametric estimators use information on the distribution of rare species in the assemblage. The greater the number of rare species in a data set, the more likely it is that still more were not represented (Gotelli & Colwell 2001). Here we used the default setting of 10 as the upper abundance limit for rare or infrequent species. The nonparametric estimators we examined included the incidence-based coverage estimator (ICE), Chao 1 (abundance-based) and Chao 2 (incidence-based) richness estimators. The latter two estimators were constructed utilising the bias-correction setting in EstimateS.

Results

Genetic diversity

The 627-bp alignment of COI from 143 individuals showed 183 parsimony-informative sites (388 conserved), defining 94 haplotypes in TCS (90 by DnaSp), whereas the 484-bp of 16S from 138 individuals showed 45 parsimony-informative sites (382 conserved) defining 51 haplotypes in TCS (48 in DnaSp). COI data showed no indels or stop codons, as expected for a coding region and was not saturated in third codon positions. The 16S data contained three separate indels consisting of a single base pair each. Twenty-eight amino acid position substitutions were detected out of a possible 209 in the COI data (translated with Table 5 NCBI invertebrate mitochondrial), and some had multiple alternative amino acids. Including all singletons, haplotypic diversity for COI = 0.9810 ± 0.000 and for 16S = 0.9270 ± 0.000 (Table 1). By comparison, nucleotide diversity (including all singletons) for COI = 0.0833, and for 16S = 0.0171 (Table 1). When examined by geographical region, haplotypic diversity was very high in all except the Ross Sea (Table 2), which was the only region where samples were collected via SCUBA diving, covering a much smaller area. No evidence for base compositional heterogeneity was detected by a chi-squared test ($P = 1.000$, d.f. = 426).

The average uncorrected pairwise difference between COI-defined networks or subclades ranged from 3–15% and average intraclade differences were 0–5% (Fig. 2). Corrected pairwise differences ranged from 0–1% within subclades, and 2–30% among subclades (Fig. 2). The distribution of distances was multi-modal. The average uncorrected pairwise distance within the single 16S network was 1.9%.

Table 1 COI network and diversity indices for *Doris kerguelenensis* using DnaSP 4.50.2 (excluding gaps and missing data). Demographic tests were calculated for well-supported networks containing four or more haplotypes

Network	*n*	n_h	Fu's *Fs*	R_2	Haplotypic diversity and SD	Nucleotide diversity
1	2	2	—	—	1.0000 ± 0.500	0.0080
2	4	4	–1.012	0.2743	1.0000 ± 0.177	0.0056
3	2	2	—	—	1.0000 ± 0.500	0.0032
4	1	1	—	—	—	—
5	5	4	0.134	0.2634	0.9000 ± 0.161	0.0064
6	2	2	—	—	1.0000 ± 0.500	0.0064
7	6	2	–0.003	0.3727	0.3333 ± 0.215	0.0005
8	3	1	—	—	0.0000 ± 0.000	0.0000
9	2	1	—	—	0.0000 ± 0.000	0.0000
10	4	2	1.099	0.4330	0.5000 ± 0.265	0.0018
11	1	1	—	—	—	—
12	1	1	—	—	—	—
13	2	2	—	—	1.0000 ± 0.500	0.0032
14	3	2	—	—	0.6667 ± 0.314	0.0011
15	8	4	–0.114	0.1948	0.6429 ± 0.184	0.0028
16	2	2	—	—	1.0000 ± 0.500	0.0048
17	2	2	—	—	1.0000 ± 0.500	0.0032
18	5	5	**–1.805**	**0.1375**	1.0000 ± 0.126	0.0061
19	3	3	—	—	1.0000 ± 0.272	0.0043
20	6	4	**–1.454**	0.1805	0.8667 ± 0.129	0.0019
21	2	2	—	—	1.0000 ± 0.500	0.0064
22	1	1	—	—	—	—
23	1	1	—	—	—	—
24	21	12	**–6.382**	**0.0766**	0.9000 ± 0.046	0.0040
25	12	1	—	—	0.0000 ± 0.000	0.0000
26	1	1	—	—	—	—
27	1	1	—	—	—	—
28	6	6	**–2.659**	**0.0899**	1.0000 ± 0.096	0.0063
29	34	20	**–9.534**	**0.0564**	0.8950 ± 0.047	0.0052
Total COI	143	90	–9.146	0.1219	0.9810 ± 0.000	0.0833

n, number of individuals; n_h, number of haplotypes. Bold values were significant, $P \leq 0.05$.

Table 2 COI diversity of *Doris kerguelenensis* individuals and networks by region

Region	No. of sample sites	No. of individuals	No. of haplotypes	Haplotypic diversity	No. of networks
South America (Burdwood Bank)	6	12	12	1.00	3
Scotia Arc	5	6	5	0.933	4
Elephant Island	5	7	7	1.00	6
Bransfield Strait	25	99	61	0.976	17
E Weddell Sea	4	5	5	1.00	5
W Antarctic Peninsula	1	1	1	—	1
Ross Sea	2	13	3	0.154	2
Totals	49	143	94	—	29

Population structure and gene flow

COI data formed 29 distinct and unconnected networks (Fig. S1, Supporting information). Twenty-one of these contained two or more individual haplotypes and eight were represented by single haplotypes (sometimes representing multiple individuals). No COI haplotypes were shared among major geographical regions, but within a region identical haplotypes could be separated by up to 450 km (Fig. 3). Individual networks could cover large distances (up to ~4900 km), crossing regional boundaries. Given that individuals from different networks were found sympatrically (see Fig. 4), we did not find it necessary to explicitly test for isolation by distance. Similarly, no depth correlation

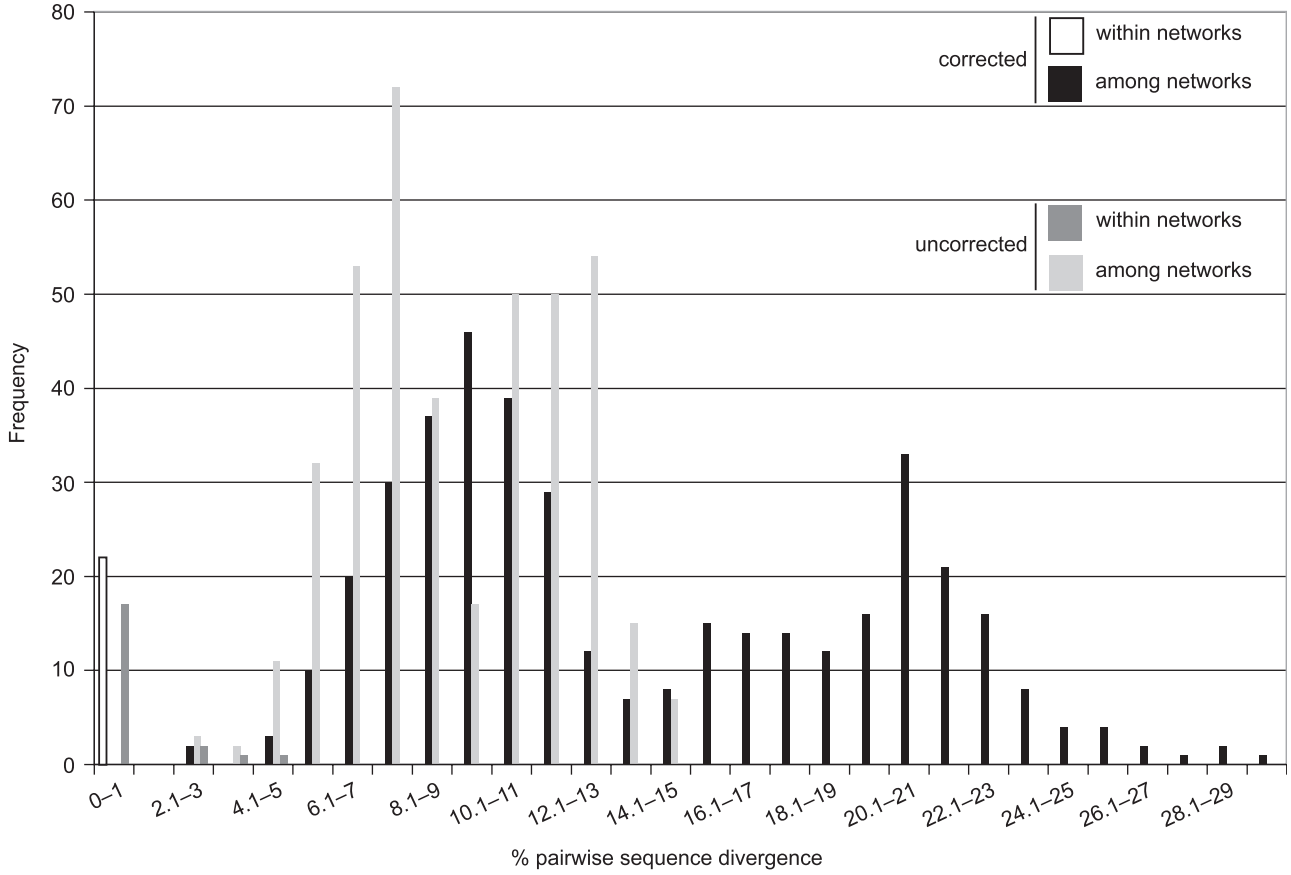

Fig. 2 Frequencies of mean pairwise distances within and among COI networks of *Doris kerguelenensis*.

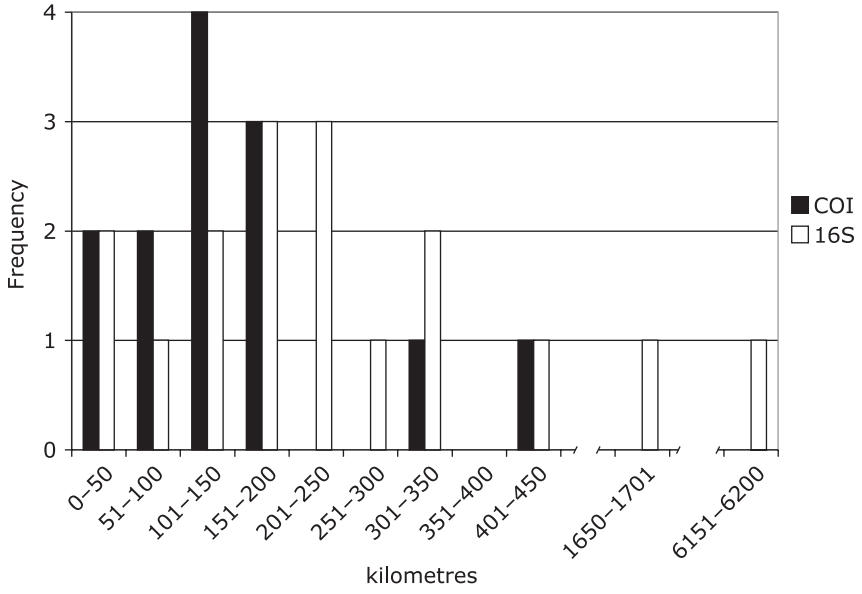

Fig. 3 Histogram showing the frequency of the maximum distances shared by identical haplotypes of *Doris kerguelenensis*. Black bars represent COI and white bars represent 16S. Note *x*-axis is not continuous.

between networks was observed; 64% of individual trawl samples from the same depth contained multiple COI networks (Fig. 4).

In contrast, 16S data formed a single network (Fig. 5). The estimated ancestral haplotype (H5) was widespread, and found in the Bransfield Strait, South America and the Ross Sea (~6176 km, Fig. 3). Haplotypes grouping together in COI networks were congruent with 16S groups. Treating missing data with the recommendations of Joly *et al*. (2007) by placing the few sites that included missing data at the

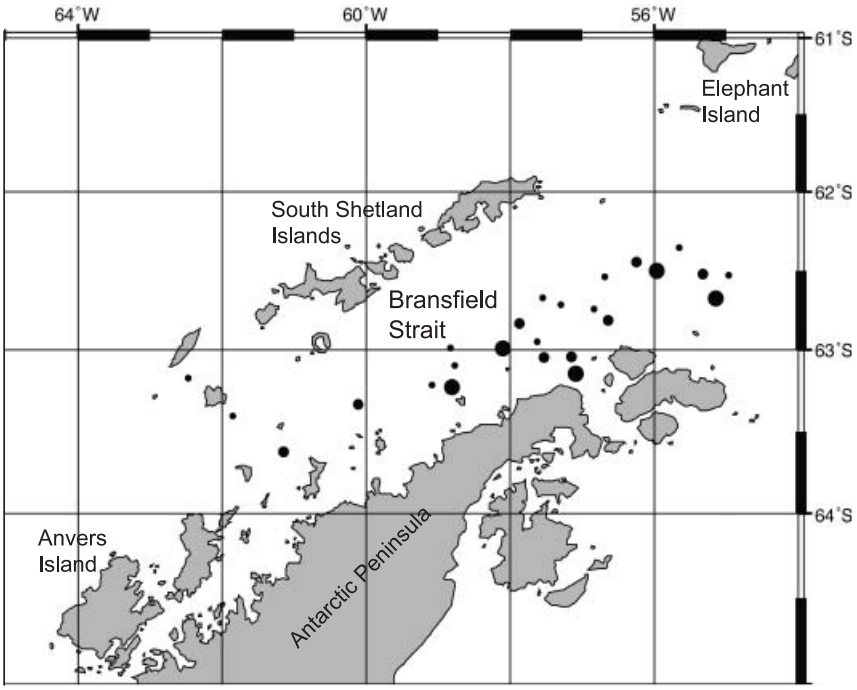

Fig. 4 Detailed map showing haplotype network diversity (combined data) of *Doris kerguelenensis* within the Bransfield Strait. Small (1), medium (2–3) and large (4–6) circles indicate the number of networks recovered per sample site.

Table 3 Analysis of molecular variance (AMOVA) in *Doris kerguelenensis* among regions using combined COI + 16S data. Data for Bransfield Strait, Elephant Island, west Antarctic Peninsula and Scotia Arc are pooled as one region

Source of variation	d.f.	Sum of squares	Percentage of variation	*P* value	Fixation indices
Among regions	3	535.272	**17.93**	0.0000	F_{ct} = 0.17931
Among sites, within regions	42	2130.853	**36.78**	0.0000	F_{sc} = 0.44813
Within sites	88	1409.263	**45.29**	0.0000	F_{ST} = 0.54708

end of the data set did not significantly change the 16S network (results not shown).

Multiple pairwise AMOVA comparisons revealed no basis for regionally partitioning between the Bransfield Strait, Elephant Island, Scotia Arc, and the western part of the Antarctic Peninsula. The final AMOVA compared the Antarctic Peninsula + Scotia Arc group against the Weddell Sea, Ross Sea and South America. All three levels of variance partitioning were highly significant ($P < 0.000$), with most of the variation occurring within a sample site (Table 3). Comparisons between all regional combinations also partly supported combining the Antarctic Peninsula sites with the Scotia Arc, although small but significant subdivision was observed between Bransfield Strait and Elephant Island (results not shown).

Some networks that appeared connected as nearest neighbours by 16S data showed significant pairwise COI ϕ_{ST} values. The most extreme example of this is a group of animals that share the ancestral 16S haplotype (networks 2 and 29) but whose COI data alone infers a significant lack of gene flow between them (ϕ_{ST} 0.89, $P < 0.00$). ϕ_{ST} values for 16S data between the two major groups of 16S (ancestral 2 + 29 vs. remainder) were 0.59, and significant after permutation ($P < 0.00$). Coalescent approaches to determine migration rates were not attempted here because many assumptions were violated (e.g. the presence of additional populations potentially exchanging migrants).

Population demography

Mismatch analyses (raggedness index) significantly rejected an expanding population model ($P < 0.00000$) using parametric bootstrapping for the combined COI + 16S data set. Examining the data separately showed that the distribution of pairwise 16S haplotype differences (Fig. 6a) could not reject the expanding population model. However, the distribution of pairwise COI haplotype differences was clearly multimodal (Fig. 6b) and characteristic of a population at equilibrium. This result could also be

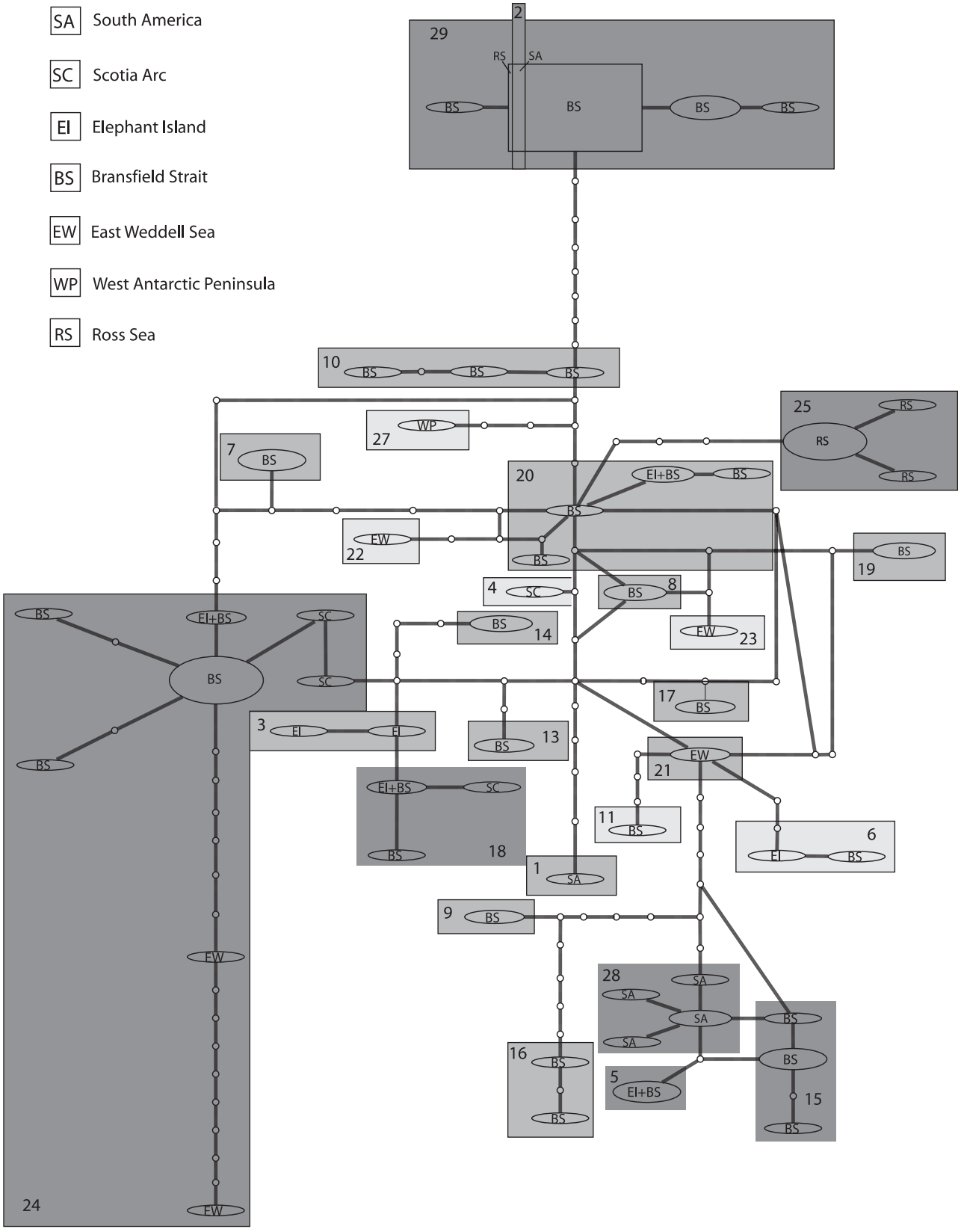

Fig. 5 Statistical parsimony network based on *Doris kerguelenensis* mitochondrial 16S data. Small open circles represent unsampled or extinct haplotypes. Numbered boxes refer to COI mitochondrial networks overlaid on the 16S network. Dark grey boxes indicate COI networks that have significant ϕ_{ST} values to all of their nearest neighbours on the 16S network. Medium grey boxes indicate COI networks that have significant ϕ_{ST} values to at least one of their nearest neighbours on the 16S network. Light grey boxes indicate COI networks that have no significant ϕ_{ST} values to their nearest neighbours on the 16S network. No 16S data were available for taxa included in COI networks 12 and 26, thus they are not represented on this figure.

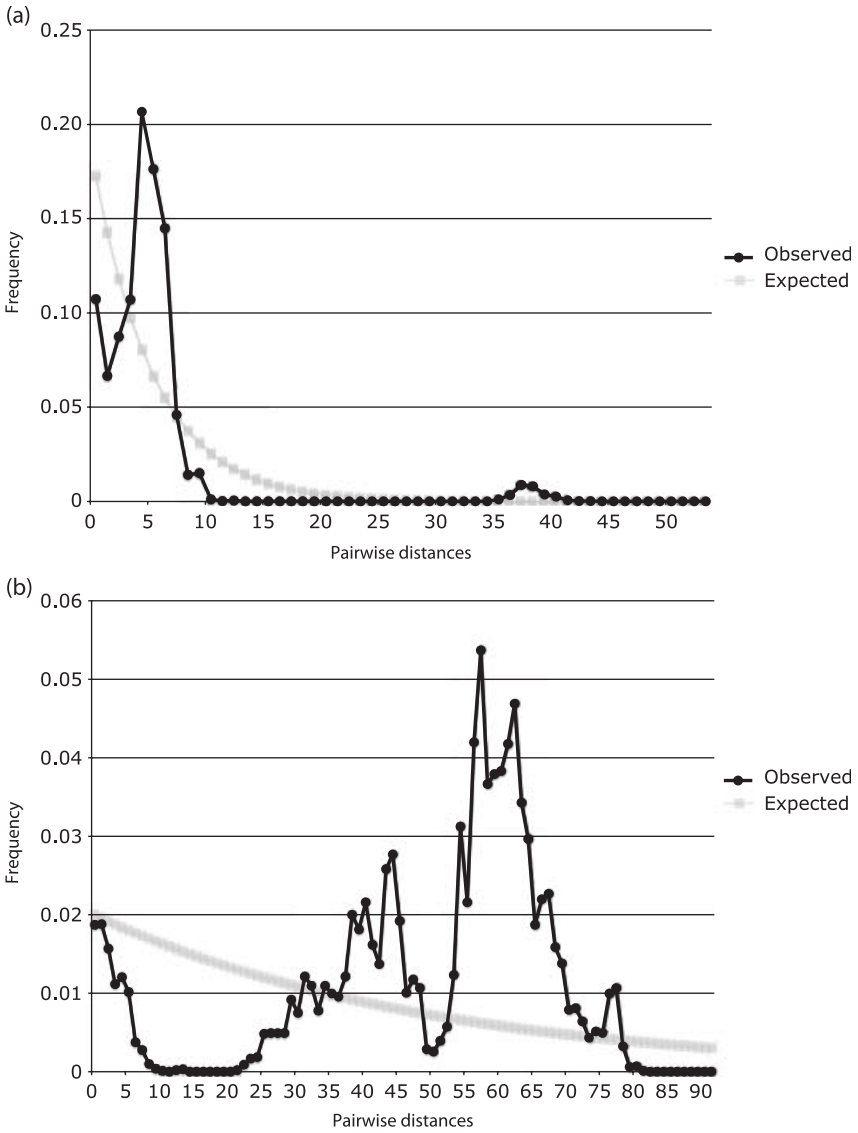

Fig. 6 Mismatch distribution of 16S (a) and COI (b) data for *Doris kerguelenensis*. The population expansion model was significantly rejected for COI ($P < 0.00000$).

explained by forced pooling of different populations that were undergoing expansion. When mismatch analyses were calculated separately on networks with enough variance ($n = 12$), all showed a unimodal distribution, consistent with expanding populations or selection (Rogers 1995).

Negative Fu's *Fs* values indicate excesses of low-frequency haplotypes, generally ascribed to loci under directional selection or population expansion following a severe reduction in population size (bottleneck). Significant R_2 values indicate expanding populations, and were concordant here with Fu's *Fs*. These statistics were calculated for all networks with four or more haplotypes ($n = 11$). Of these, four networks showed significant values for both test statistics (18, 24, 28, 29), and an additional network showed a significant Fu's *Fs* value (20) (see Table 1).

The McDonald–Kreitman test (MK) was used to test evidence of selection, as it is not affected by changes in population size that influence some other demographic tests. The MK test utilises phylogeny to determine evidence of selection and compares the number of synonymous and nonsynonymous changes within clades (polymorphisms) and on the internal branch connecting the clades (fixations) (Skibinski 2000). Because the relationships between different networks groups are not well resolved, we were limited in the comparisons we could make with this test. The only pairwise comparison that met all assumptions could be made between network 2 and 29, and the MK test identified significant departures from a neutral model ($P = 0.021$).

Phylogenetic analyses and estimation of divergence times

Doris kerguelenensis formed a strongly supported monophyletic clade in parsimony and Bayesian analyses (Fig. 7). Many subclades were present and corresponded completely to the

Fig. 7 Bayesian majority-rule consensus tree of *Doris kerguelenensis* showing posterior probabilities and parsimony bootstrap values for nodes of interest. Black circles indicate posterior probability of > 0.95 and parsimony support of > 80. Values below 50 and 0.95 respectively are not shown. – indicates node is not supported, or does not exist in parsimony analysis.

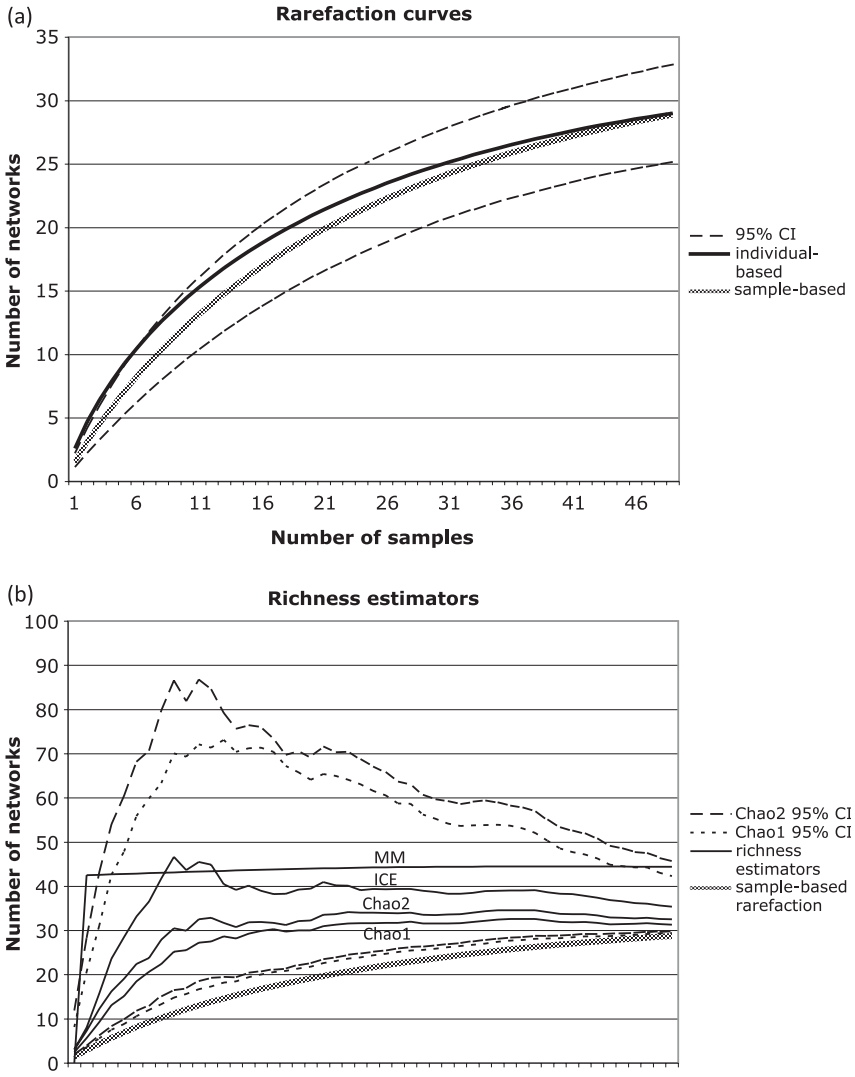

Fig. 8 Estimation of cryptic diversity for *Doris 'kerguelenensis'* based on COI data. (a) Rarefaction curves for observed samples. Confidence intervals are shown for the sample-based rarefaction curve (S_{obs} or Mao Tau) but not for individual-based rarefaction (Coleman method). (b) Richness estimators based on the observed samples. The Michaelis–Menten mean (MM) asymptote was estimated from the sample-based rarefaction curve (illustrated again for comparison). Nonparametric estimators ICE (incidence-based coverage estimator), Chao 1 and Chao 2 are shown, the latter two with 95% confidence intervals.

networks recovered by TCS, thus these terms are synonymous in the rest of the paper. These subclades were common to both methods of phylogenetic analysis and all subclades with multiple terminals were strongly supported (bootstrap > 80; posterior probability > 0.95). Sister-group relationships between these multiple subclades were often unresolved. When resolved, the same relationships were common to both analyses and neither showed strict geographical signal. When COI data were translated to amino acids, the same topology and intraspecific clades were also recovered (not shown).

Mean corrected pairwise distances between well-supported sister group subclades was 5.67 ± 0.77%, and the corresponding within subclade divergence was 0.52 ± 0.38%. The three trans-Drake sister groups identified by the phylogenetic tree (networks 1 + 10, 15 + 28, 2 + 29) show a respective average of 4.35–4.57.8%, 4.31–4.88% and 4.99–5.77% corrected pairwise distance between them, and show corresponding ϕ_{ST} values of 0.914, 0.895, and 0.802. Applying the 2.4%/MY COI molecular clock for gastropods (Hellberg & Vacquier 1999) crudely dated these trans-Drake divergences at 1.81–1.90, 1.80–2.03, and 2.08–2.40 million years ago.

Estimating diversity

The sample- (S_{obs}) and individual-based (Coleman method) rarefaction curves closely resembled each other, but did not approach an asymptotic shape (Fig. 8a). The individual-based curve was slightly higher than the sample-based rarefaction curve, but almost always fell inside the 95% confidence interval limits for S_{obs}. The sharp upward slope of the rarefaction curve indicates that undersampling is likely. However, as sampling increased, no reduction on the number of networks was observed, which would have been expected if individuals that bridged networks together

were increasingly discovered. The MMmeans richness estimator peaked sharply at around 43 on the *y*-axis (number of networks), and then reached stationarity at 45 (Fig. 8b). All of the nonparametric estimators showed some level of fluctuation and gradually dropped and appeared to be converging at values approximately 32–35.

Discussion

Addressing the Drake break

Phylogenetic analysis shows that no single vicariant event separated all populations in the Antarctic Peninsula from all populations in South America. Animals from the Magellan region (South America) appear in three separate parts of the tree, and all three groups of Magellanic *Doris kerguelenensis* show sister group relationships with animals from the Antarctic Peninsula (two with strong support, see Fig. 7). No mitochondrial molecular clock exists for nudibranch molluscs at present, so we applied a teguline gastropod clock (Hellberg & Vacquier 1999) to COI data to roughly date divergence events in this study. The level of sequence divergence among trans-Drake sister groups is similar (5.77–4.99%; 4.88–4.31%; 4.57–4.35%) but the topology of the phylogenetic tree suggests three independent dispersal events from Antarctica into South America. If mutation rates in Antarctica are similar to those of warmer waters (e.g. Held 2001), these trans-Drake divergences date to 1.8 to 2.4 million years ago, corresponding to glaciation events in the Pliocene–Pleistocene rather than the more ancient vicariant separation of Antarctica and South America and opening of the Drake Passage (*c*. 24–41 million years ago). Glaciation events are thought to have rendered most of the Antarctic continental shelf uninhabitable at various times, shifting usually shelf-dwelling benthic organisms northward, to a series of open ocean refuges (Thatje *et al*. 2008), and likely increasing dispersal across the Drake Passage. However, if stronger evidence of selection is uncovered, applying a molecular clock is unreasonable, and the timing of these divergences will have to be re-assessed. Although the phylogenetic data indicate northward expansion events, the ancestral 16S haplotype is present in both South America and Antarctica so the species may have originated on either continent.

The Scotia Arc has been suggested to link populations of animals across the Drake Passage. However, previous work on octopods revealed a distinct genetic break along the Scotia Arc between South Georgia and Shag Rocks (Allcock *et al*. 1997). A short distance (approximately 30 km) of relatively deep water separates these two locations. We also found that samples of *D. kerguelenensis* from these two localities did not appear closely related (networks 18 and 24 respectively). However, our broader geographical sampling revealed that South Georgia and Shag Rock samples independently shared relatives in the Bransfield Strait and Elephant Island. Interestingly, a similar Shag Rocks-Elephant Island connection is demonstrated by a brooding bivalve (Linse *et al*. 2007), and emphasises the importance of broad sampling when inferring specific barriers to gene flow.

Potential episodic long-distance dispersal (discussed below) may occur within the Antarctic continental region for *D. kerguelenensis*, but this study reveals no evidence for contemporary exchange across the Drake Passage. By dating molecular divergences, other studies have indirectly shown historical exchange across the Drake Passage (Page & Linse 2002), and recent, sometimes anthropomorphic, influences may have contributed to organisms crossing to Antarctica (Barnes 2002; Tavares & de Melo 2004; Thatje 2005). Although the molecular clock used here is fairly rudimentary, the present study shows that the Drake Passage may still be a significant contemporary barrier to organismal transport, and this is in accord with other studies of Antarctic marine invertebrates (Hunter & Halanych 2008; Thornhill *et al*. 2008).

Long-distance dispersal in a direct developer?

Identical COI haplotypes in *D. kerguelenensis* were found occurring up to 450 km apart, a scale previously identified in a brooding Antarctic echinoderm and bivalve (approximately 500 and 300 km respectively; Linse *et al*. 2007; Hunter & Halanych 2008). In contrast, one 16S haplotype was shared over a distance of ~6200 km. This particular example involves a 16S haplotype from the Ross Sea falling into a clade of Bransfield Strait haplotypes (Fig. 5, network 29). The corresponding COI haplotype is also more closely related to Bransfield haplotypes (Fig. S1, network 29), and may suggest that infrequent long-distance dispersal occurs in *D. kerguelenensis*. Note that this particular Ross Sea sample was sequenced in a separate laboratory (University of Auckland, New Zealand) that has never handled samples from the Bransfield Strait (S. Lavery, personal communication.). This putative example of long-distance dispersal could be alternatively explained as a consequence of the lower rate of mutation in 16S, and represent the retention of an ancestral haplotype in the Ross Sea. Nonetheless, the corresponding COI haplotype from this sample does not similarly represent the estimated ancestral condition in its network, and is less diverged from the ancestral condition than other Bransfield Strait haplotypes in the same network. Additionally, all other Ross Sea samples formed a more distant cluster of closely related haplotypes, with an analogous COI-defined network (25, see Fig. 5).

Evidence of long-distance dispersal in an organism that does not produce free-swimming larvae is unusual, and has usually been explained by adults living on substrata

that may float and drift with prevailing winds (e.g. Waters & Roy 2004). However, that scenario is not entirely biologically relevant for the sponge-eating *D. kerguelenensis* and so any dispersal mechanism remains somewhat obscure. Possible explanations include (i) adults attached to 'tumbleweed' sponges that have been dislodged from the benthos, (ii) egg masses are being laid on other organisms that are prone to rafting and drifting processes, or (iii) the action of anchor ice removing organisms from the benthos to the sea ice, followed by subsequent break up and dispersal by wind and surface currents (Dayton *et al*. 1970). The latter two explanations seem most likely, given we observed egg masses laid on foliaceous bryozoans that could easily be dislodged, and that Dayton *et al*. observed actual transport of adult organisms via anchor ice, although anchor ice is clearly only important in regions where it is cold enough to physically form (i.e. within the Antarctic-Magellan region).

The occurrence of *D. kerguelenensis* off Río de la Plata in Argentina (Bouchet 1977) is likely to be a northward expansion along the Magellan continental shelf. The record of *D. kerguelenensis* in deep waters off New Caledonia (Valdés 2001) is more unexpected and might have arisen from dispersal pathways arising from the global thermohaline circulation pattern where deep cold water moves northward in the Pacific and Indian Oceans. This would assume animals could survive on abyssal plains between continents, and to date, there is no evidence to support this. Dispersal along the global thermohaline path would also predict *D. kerguelenensis* to occur in deep water along the east African coast, which is unreported to date. Alternatively, the New Caledonian record might simply represent another species that is morphologically similar to *D. kerguelenensis*.

Do these multiple networks represent cryptic species?

The genetic structure of *D. kerguelenensis* reveals multiple groups of related animals whose degree of relatedness cannot be explained by geography. These groups are differentiated best by COI data (nucleotide or amino acids), which show a faster rate of substitution than 16S. Differences between COI and 16S substitution rates were also recovered recently in an Antarctic nemertean (Thornhill *et al*. 2008) and likely indicate a more general pattern. Presumably, the faster rate of evolution in COI has captured historical glaciation events not recorded by 16S, much the same way that mitochondrial data can record phylogenetic signal not observed in the nuclear genome. Although COI resolves many networks, the groups contained in those networks are still congruent with the more conservative 16S data that form a single network. Analyses that utilise differing optimality criteria recover these same groups. Deep mitochondrial divergences between populations of a single species are known for a few invertebrates (e.g. Pinceel *et al*. 2005; Muths *et al*. 2006) although this type of genetic structure is unusual, and typically the consequence of greatly restricted dispersal (Boyer *et al*. 2007) or hybrid breakdown (Ellison & Burton 2006). These types of results are characterised by populations that rarely overlap geographically, which is not true for *D. kerguelenensis*. Here we report sympatry of multiple networks or groups, some single locations housing members from six different networks (Fig. 4).

An increasingly common assumption for marine phylogeographers, summarised by Hart *et al*. (2006), is that haplotype networks (constructed plausibly at 95%) represent a working species concept. The production of multiple networks from a single data set is often the first indication that cryptic species may be present, and can provide a basis for re-examining the differing sets of samples for morphological differences. However, if no definitive morphological characters can be found, the hypothesis rests on the networks themselves. Confidence is gained in the hypothesis if the networks are separated by the types of sequence divergences typically found between other closely related species. Although Hebert *et al*. (2003) suggest a mean molluscan divergence of 11.1 ± 5.1% for COI, examination of the literature for other dorid nudibranch comparisons indicate otherwise (e.g. Pola *et al*. 2007; Turner & Wilson 2008). Well-supported sister species in those studies are separated by divergences on the order of 3.3–5.1%. Pairwise sequence comparisons between the COI networks here range from 2–30%, with all well-supported sister pairs separated by divergences on the order of 5%, much like other dorid nudibranchs.

DNA barcoding methods typically employ a single mitochondrial gene (COI) for species assignment and cryptic species identification (e.g. Hebert *et al*. 2004). Aside from philosophical debate (Moritz & Cicero 2004; Will & Rubinoff 2004), tests of the efficacy of barcoding have focused on the error rate in identification methods (Brower 2005; Meyer & Paulay 2005). Fundamentally, barcoding methods rely on the assumption the amount of variation within and between species is consistent across phyla, or at least within broad groups. Hebert *et al*. (2003) provided a summary of pairwise congeneric divergences in broad animal groups, but these estimates are likely over-inflated as a consequence of pooling of data at higher taxonomic levels, the inadvertent inclusion of cryptic species in comparisons, and not restricting comparisons to sister species. The high rate variation in COI (Mueller 2006) makes estimating acceptable levels of within vs. between group thresholds difficult.

Wägele's (1990) revision of the genus *Austrodoris (*now synonymised with *Doris*) synonymised all of the known species in Antarctic waters with *D. kerguelenensis*, and considered some additional names as *nomen dubia*. Many early species descriptions lacked detail and were often based only on preserved animals. Her work enabled observations

on live animals, and showed that features such as tubercules on the dorsum could change fairly rapidly (on the order of weeks) in each individual. Organs in the reproductive system that are usually variable enough to show species-specific features were conserved across broad geographical ranges (South Georgia, Antarctic Peninsula, Weddell Sea). However, Wägele 1990 did point out considerable morphological variation in the nervous system, the digestive system and the colour and shape of the radula. As these did not show a consistent pattern, she concluded that all animals examined belonged to a single species. Since that time, another species from the Antarctic region has been described (Garcia *et al*. 1993) and was differentiated by the shape of a duct in the reproductive system. This distinction has also been questioned (Valdés 2001; Schrödl 2003) and it is likely that while many synonyms might represent genetically distinguishable units, they were not defined or diagnosed genetically, and it may not be possible to link these units with species names.

Doris kerguelenensis has been recorded feeding on many different sponges. Most data indicate it feeds on hexactinellids *Rossella racovitzae* (Dayton *et al*. 1970), *Rossella nuda* (Dayton *et al*. 1974), *Anoxycalyx joubini* (Dayton *et al*. 1974) and demosponges *Ectyodoryx* cf. *ramilobosa* (Garcia *et al*. 1993), *Tetilla leptoderma* (Dayton *et al*. 1970), *Haliclona dancoi* (Dayton *et al*. 1970), *Polymastia invaginata* (Dayton *et al*. 1970), *Calyx arcuarius* (Dayton *et al*. 1970), and *Dendrilla antarctica* (Barnes & Bullough 1996). Prey choice experiments with hexactinellid and demosponge options failed to show a consistent preference (Iken *et al*. 2002) and this was used as evidence to suggest *D. kerguelenensis* was a generalist predator. These choice experiments may simply have failed to offer the locally preferred sponge. For example, choice experiments with Weddell Sea animals were dominated by hexactinellids, although these sponges have only been demonstrated as preferred prey in the Ross Sea.

Not surprisingly, the defensive chemical compounds found in the body tissue of *D. kerguelenensis* also vary widely in the literature. A series of terpenoid glycerol esters have been characterised and interestingly, different collections resulted in different esters with different carbon skeletons: ent-lambdane (Davies-Coleman & Faulkner 1991; Gavagnin *et al*. 1995), halimane (Gavagnin *et al*. 1995; Gavagnin *et al*. 1999b) and isocopalane diterpenoid glycerols (Gavagnin *et al*. 1999a). In addition, two nor-sesquiterpenes were also discovered (Gavagnin *et al*. 2003). Because *de novo* synthesis of compounds should result in the same metabolites in the same molluscs (Gavagnin *et al*. 1999a), the ability of different populations of *D. kerguelenensis* to display such a wide variety of mantle metabolites was surprising to marine chemists. Similarly intriguing, the metabolite pattern detected in individuals from one location was identical to subsequent collections made from the same location in later studies (Iken *et al*. 2002).

In summary, there appears to be much variation in morphology, prey items, and defensive chemicals in the widespread nudibranch *D. kerguelenensis*. This variation could easily be explained instead by cryptic speciation. Whether the related groups of animals identified by haplotype networks show any consistent patterns in the morphological or ecological traits discussed above remains to be tested. The discovery of 29 distinct mitochondrial lineages, many of which exist in sympatry, indicates an absence of recent gene flow among groups of *D. kerguelenensis*. The most plausible explanation for this data is a recent, explosive radiation within a single morphologically recognised species, *D. kerguelenensis*. This example far surpasses all other known cryptic radiations known for Antarctic invertebrates, and further work will address concordance of these mitochondrial groups with nuclear loci.

Estimating diversity of D. kerguelenensis *in the sampled area*

The closeness of the two types of rarefaction curves indicates that individuals from different networks occur almost randomly and independently among samples in the data set (Colwell *et al*. 2004). The greater the differences between the curves, the more individuals are aggregated nonrandomly among samples. This latter scenario is easily imagined if one assumes a geographically structured species, and trawl samples as independent events. In contrast, the sample-based and individual-based rarefaction curves closely approximate each other (see Fig. 8a), and our study demonstrates multiple networks or subclades living in sympatry.

The various richness estimators behaved relatively differently in this study. As an asymptote, the MMmeans estimate of network richness was highly stable at 45, but the nonparametric methods fluctuated in the earlier part of the observed samples. Clearly, these nonparametric estimators do not approximate reality when examining only a small number of samples. These nonparametric estimates all dropped and appeared to converge around 32–35 as sample size increased, demonstrating the stability afforded by increasing independence of sample size (Longino *et al*. 2002). The difference in the richness estimates of these two methods is clear, however it should be noted that nonparametric methods give minimum estimates, and should be considered a lower bound. After 48 trawl samples, our recorded number of COI networks was 29. This number falls short of the lower 95% confidence interval for Chao 1 and 2, so it appears very likely that further sampling would recover more diversity. Given the mean of all estimator methods approaches 36, it appears that approximately 80% of *D. kerguelenensis* networks expected in the sampled area were recovered by this study. Clearly, with many unsampled regions of Antarctica, and South America, we can expect

a much greater diversity of *D. kerguelenensis* networks to exist in nature.

One of the fundamental differences in calculating diversity estimates using sequence-based haplotype network data is that more sampling may reduce the recorded diversity. This may happen if a haplotype is sampled that connects two or more previously unconnected networks. Although ecological diversity estimates maximally reach an asymptote, the novel network-based approach that we propose has properties that could produce a normal distribution of diversity. Whether this occurs in nature is not known, and the empirical properties of distributions generated by sequence data are yet to be fully explored. A similar rarefaction approach has been tested in assessing microbial communities (Schloss & Handelsman 2004; Curtis *et al*. 2006), and these studies face an additional suite of challenges associated with sequence similarity-based diversity concepts and the decoupling of individuals and their sequences. We suggest that in light of increased recognition of cryptic species diversity in the ocean (Knowlton 1993, 2000), our approach may be valuable for determining the effectiveness of sampling strategies, particularly in challenging sampling environments.

Why does D. kerguelenensis *harbour great diversity?*

Periodic changes in the earth's orbit, known as Milankovitch oscillations, have caused rapid changes in climate, followed by stable periods of approximately a few thousand years (Berger 1988). These changes have been linked to glacial cycles, which have been affecting Antarctica over the last 35 million years. As an ice sheet expands out across the continental shelf, it effectively removes marine benthos, and, if the expansion reaches the continental slope, the debris will be deposited there where mass wasting processes also impact the slope (Thatje *et al*. 2005). These disturbances occur on a huge scale, and ice advances at the last glacial maximum reached the continental slope margin around most of Antarctica (Anderson *et al*. 2002; Thatje *et al*. 2005). This theoretically leaves the deep sea as the only faunal refuge, although it is likely that further shelf refuges must have been available in some areas (Dayton & Oliver 1977; Thatje *et al*. 2008). The process of repeated glacial and interglacial cycles has been termed the Antarctic diversity pump (Clarke & Crame 1989, 1992) for causing major allopatric speciation events that have been invoked to explain some of the marine invertebrate radiations known from the Antarctic, for example, notothenoid fish (Eastman & Clarke 1998), pycnogonids (León 2001), octopods (Allcock 2005), crinoids (Wilson *et al*. 2007) and isopod crustaceans (Held 2000; Raupach *et al*. 2007). In the last 2.4 million years, there may have been as many as 50–60 glacial–interglacial cycles (Imbrie *et al*. 1984; Teidemann *et al*. 1994), allowing massive diversification potential. The recent radiation of *D. kerguelenensis* is one of the most striking examples that can be attributed to the Antarctic diversity pump, and we predict that many others will follow.

Animals such as *D. kerguelenensis*, that move very little as adults, would not be among the first recolonisers after such large-scale disturbances. With lowered motility, it seems likely that a population isolated in a refuge may remain isolated well after new areas of habitat have become available. Small population sizes and long periods of isolation may have increased the role of genetic drift in fixing alleles. If various glacial refugia contained different suites of biotic factors (e.g. different predators in each refuge), selection might also act fairly quickly on a population, particularly as *D. kerguelenensis* is known to host a range of anti-predator chemicals. As each glacial event ended, and secondary contact ensued, diversity in a region would be maintained if a diversity of predators were also moving freely again in that area. Subsequent glaciation cycles would repeat the allopatric separation of animals and selective sweeps, resulting in deep mitochondrial divergences. There is evidence to show that directly developing invertebrates have higher selection rates than those with dispersive larvae (Foltz 2003; Foltz *et al*. 2004), and this may have contributed to within-refuge sweeps. Apparently, potentially advantageous alleles in *D. kerguelenensis* came to fixation rapidly during a glacially mediated allopatric event, because now many monophyletic groups exist in sympatry, with no significant genetic signatures indicating secondary contact between lineages.

The changes in geographical distributions of clades due to Milankovitch oscillations have been termed orbitally forced range dynamics (ORD) (Jansson & Dynesius 2002). This hypothesis suggests that clades that occur in areas that are strongly affected by climate changes are more likely to merge with each other, or go extinct, than to survive intact over one Milankovitch oscillation (~100000 years). Jansson & Dynesius (2002) suggest using species number as a proxy for these long-lived clades that survive a Milankovitch oscillation (termed ß-clades), alluding that areas with high ORD will have a low diversity of species. The inverse of this prediction would be examples of regions with low ORD showing high species diversity, which is arguably the case for salamander radiations in Middle America (Wake 1987) and Lake Baikal species flocks (Zubakov *et al*. 1997; Sherbakov *et al*. 1998). ORD should then also affect micro-evolutionary patterns, and genetic divergence among gene pools should be greater for clades that have experienced low ORD. This is well supported by cases from arctic and boreal regions where recolonisation from glacial refugia has resulted in lowered genetic diversity among gene pools (Hewitt 1996, 2000).

Antarctica provides a stark contrast to expectations under ORD, with a growing number of studies showing that genetic diversity in Antarctica does not seem to be reduced

as in the Northern Hemisphere. Increasing molecular and taxonomic work has revealed many species flocks and levels of intraspecific genetic divergence similar or exceeding to other temperate and even tropical areas (Brandt *et al*. 2007). This suggests that the recolonisation processes affecting genetic diversity in the Antarctic might have been very different to that of the well-characterised Northern Hemisphere, and is likely related to the types of refugia available during glaciation events (Thatje *et al*. 2005, 2008). Alternatively, a simple age-related model of diversification may be invoked, as the Antarctic ice cap has been present since the Oligocene while the Arctic ice cap was established only 2–3 million years ago. Further work examining the genetic structure and consequences of glacial cycling in Antarctic organisms should prove productive in furthering our understanding of these pole-orientated contrasts.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1 COI networks for *Doris kerguelenensis*.

Table S1 Collection data and haplotype accession of *Doris kerguelenensis*

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