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One Antarctic slug to confuse them all: the underestimated diversity of *Doris kerguelenensis*

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ABSTRACT

The Antarctic marine environment, although rich in life, is predicted to experience rapid and significant effects from climate change. Despite a revolution in the approaches used to document biodiversity, less than one percent of Antarctic marine invertebrates are represented by DNA barcodes and we are at risk of losing biodiversity before discovery. The ease of sequencing mitochondrial DNA barcodes has promoted this relatively 'universal' species identification system across most metazoan phyla and barcode datasets are currently readily used for exploring questions of species-level taxonomy. Here we present the most well-sampled phylogeny of the direct-developing, Southern Ocean nudibranch mollusc, *Doris kerguelenensis* to date. This study sampled over 1000 new *Doris kerguelenensis* specimens spanning the Southern Ocean and sequenced the mitochondrial *COI* gene. Results of a maximum likelihood phylogeny and multiple subsequent species delimitation analyses identified 27 new species in this complex (now 59 in total). Using rarefaction techniques, we infer more species are yet to be discovered. Some species were only collected from southern South America or the sub-Antarctic islands, while at least four species were found spanning the Polar Front. This is contrary to dispersal predictions for species without a larval stage such as *Doris kerguelenensis*. Our work demonstrates the value of increasing geographic scope in sampling and highlights what could be lost given the current global biodiversity crisis.

Keywords: allopatry, Antarctica, Antarctic marine biodiversity, cryptic species, cytochrome oxidase I, direct development, mtDNA, nudibranch mollusc, phylogeny, refugia, species delimitation.

Introduction

Biodiversity and natural ecosystems provide humankind with significant economic benefits (e.g. Wallace 1997; McClintock and Baker 2001; Pertierra *et al.* 2021) by way of indirect essential services (e.g. maintaining water cycles – McNeely *et al.* 1990; CO_2 emission mitigation – Domke *et al.* 2020). Despite all known benefits, the rapid destruction of the world's most diverse ecosystems has led most experts to conclude that the earth's biological diversity is in danger (Singh 2002). Although the total number of extant species is currently estimated to be between 5.3 million and 1 trillion, only 1.85 million species have been formally described (Mora *et al.* 2011; Locey and Lennon 2016). We also know that our oceans house a large array of marine species (146 969 accepted species; Ocean Biodiversity Information System, see https://obis.org/), with another 1.4–1.6 million species hypothesised to exist, and are awaiting discovery and description (Bouchet 2006). Concurrent with the evolution of human societies (*c.* 11 000 years ago) in the form of advanced infrastructure, farming and transport, there has been continual biodiversity loss, both of species, and wider ecosystem integrity and functionality (e.g. Rogers-Bennett and Catton 2019; Prates and Perez 2021). This loss is underestimated due to taxonomic uncertainty and data gaps. Throughout geological time, at least five

putative mass extinctions have occurred (Barnosky *et al.* 2011). Unlike past natural events, however, that spanned millions of years, the present anthropogenically driven mass extinction is likely to occur over only hundreds of years (as few as 200 years) (see: Ceballos *et al.* 2015). This loss of biodiversity has significant effects on ecosystem function that has implications for human populations and these may still be vastly underestimated.

Despite being the most isolated continent on Earth, Antarctica has not escaped the negative effects of human activity (Vaughan *et al.* 2003; Aronson *et al.* 2011; Chown *et al.* 2015; Stephens 2018). Such impacts include pollution, overfishing, increased melting of ice and the introduction of invasive or alien species (Tin *et al.* 2009; Aronson *et al.* 2011; Stark *et al.* 2019; Avila *et al.* 2020; De Castro-Fernández *et al.* 2021). Globally, the Antarctic region is known to act as a significant carbon sink and surrounding ecosystems are experiencing temperature increases due to rising global atmospheric carbon dioxide concentrations (Ito *et al.* 2010). Parts of the Antarctic Peninsula, including the west Antarctic Peninsula for example, are particularly vulnerable to these human-induced ecological disasters, as these are already experiencing the greatest increases in mean annual atmospheric temperatures on Earth (Chapman and Walsh 2007; Clarke *et al.* 2007; Ingels *et al.* 2012; Torre *et al.* 2017). Failure to address these global threats will not only result in the degradation of Antarctic marine ecosystems but will potentially lead to enormous losses of global biodiversity.

Technical advances in molecular phylogenetics over the past several decades have resulted in the development of many more sensitive rapid tools for detecting new species (Féral 2002; Goetze 2003; Baird *et al.* 2011). The ease of sequencing mitochondrial DNA barcodes has promoted a relatively 'universal' species identification system across most metazoan phyla and now barcode datasets are more readily used for exploring questions of species-level taxonomy (Hebert *et al.* 2003; Puillandre *et al.* 2012; Taberlet *et al.* 2012; Eberle *et al.* 2020). In current phylogenetic studies the types of molecular information extracted for rapid assessments of animal diversity have evolved from interpreting single-locus (e.g. Hebert *et al.* 2003) to whole-genome datasets (e.g. Jensen *et al.* 2021). This expansion of available sequences, facilitated by the automation of sequencing and decrease in sequencing costs, has accelerated phylogenetic studies and whole-genome-based research (e.g. Johnson *et al.* 2008; Layton *et al.* 2018; Cai *et al.* 2019). This is particularly significant when barcoding cryptic and pseudocryptic species (organisms that are first discerned by non-morphological methods, e.g. Brasier *et al.* 2016; Matsuda and Gosliner 2018; Tyagi *et al.* 2019). When divergent interspecific traits are not morphologically obvious, traditional taxonomic methods fail to detect speciation events and biodiversity will remain under-reported (Knowlton 1993; Baird *et al.* 2011).

The Antarctic continental shelf is one marine realm that has recently revealed apparent high levels of cryptic species (e.g. Linse *et al.* 2007; Wilson *et al.* 2009; Baird *et al.* 2011; Brasier *et al.* 2016), specifically in organisms with poor dispersal abilities (Griffiths 2010; De Broyer and Danis 2011; Grant *et al.* 2011; Neusser *et al.* 2011; Brandt *et al.* 2012). The growth and decay of ice sheets during Antarctica's history has been one of the most significant disturbances acting at an ecosystem level across the shelf and underpins one of the fundamental frameworks for understanding Antarctic diversity, the 'Antarctic Biodiversity Pump' hypothesis (Clarke and Crame 1989, 2010; Gutt and Starmans 2002; Thatje *et al.* 2005). This hypothesis proposes that overall, increased speciation rates are the result of Milankovitch-cycle driven glacial oscillations that drive cryptic and allopatric speciation by gene flow inhibition and speciation events (Clarke and Crame 1989; Crame 1997; Griffiths 2010). A substantial amount of Antarctic species diversity is hypothesised to be the result of species flocks that have been identified as monophyletic taxa displaying high levels of endemic species that are ecologically diverse and abundant in relation to the surrounding habitat (Ribbink 1984; Eastman and McCune 2000; Lecointre *et al.* 2013; Chenuil *et al.* 2018). During these glacial oscillations and ensuing periodic habitat disruptions, ice-free refugia are thought to have sustained reduced populations of once widely distributed species (e.g. Smith *et al.* 2010; Lau *et al.* 2020), allowing many to diverge during this time.

One Southern Ocean marine invertebrate that is emerging as a case study in cryptic speciation is *Doris kerguelenensis* (Bergh, 1884). Recent studies on this nominal species have highlighted either new genetic lineages (Wilson *et al.* 2009, 2013) or new chemical compounds (Iken *et al.* 2002; Maschek *et al.* 2012; Avila 2020). *Doris kerguelenensis* is a direct-developing sea slug with limited dispersal potential and long generation times (Hain and Arnaud 1992; Moles *et al.* 2017*a*). Belonging to the Doridoidea, this nudibranch is a simultaneous hermaphrodite that feeds exclusively on sponges and synthesises secondary metabolites *de novo* (Maschek *et al.* 2012). Wägele (1990) reviewed and ultimately synonymised ten Southern Ocean dorid nudibranch species within the single, morphologically variable species *Austrodoris kerguelenensis* (Bergh, 1884). That work also designated another two species as *nomina dubia* due to misplaced holotypes and inadequate descriptions. The genus *Austrodoris* Odhner, 1926 was subsequently revised and synonymised along with five other cryptobranch dorid nudibranch genera into *Doris* Linnaeus, 1758 (Valdes 2001). Wilson *et al.* (2009) examined the mitochondrial protein-coding gene Cytochrome Oxidase I (*COI*) and revealed 29 putative lineages within this nominal species. These lineages were subsequently corroborated with nuclear and metabolomic trait data to infer biological species (Wilson *et al.* 2013); therefore, this sets a sound basis for using *COI* clades as a proxy for species in this study. Interestingly, three new species were recovered by resampling in the same geographic regions (Wilson *et al.* 2013), hinting at further undetected diversity.

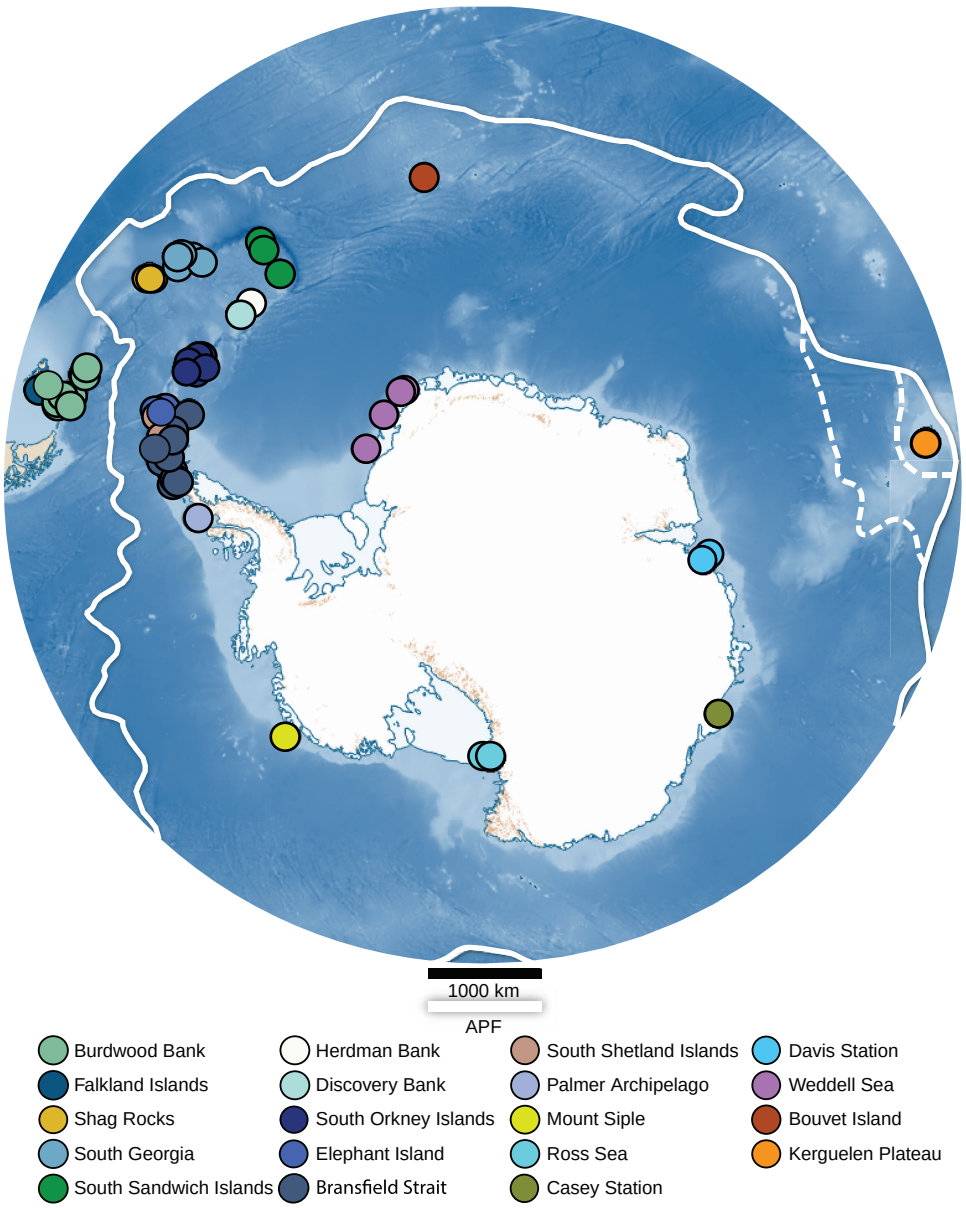

Fig. 1. Map of Antarctica showing the sites of all *Doris kerguelenensis* samples sequenced for the mitochondrial DNA gene *Cytochrome Oxidase I* (*COI*). The Antarctic Polar Front is denoted by the solid white line with two adjustments proposed by Park *et al.* (2014) and Sokolov and Rintoul (2009) (hashed white lines depicting the APF moving south of Kerguelen Island). Colours indicate geographic sampling regions. Base map generated through Quantarctica (ver. 3.2, see https://www.npolar.no/quantarctica/; Matsuoka *et al.* 2021).

In this study, we explored the identity of over 1000 new *Doris kerguelenensis* specimens using mitochondrial DNA sequence data, with the aim of (i) creating an expanded phylogenetic hypothesis for the group, (ii) testing for additional cryptic species within this species complex and (iii) exploring the distributional patterns of these species.

Methods

Specimen collection and preservation

In this study, 1275 individuals of *Doris kerguelenensis* were included from 146 sites from depths between the intertidal and 798 m (Fig. 1, Supplementary Table S1). Our specimens were collected during various Antarctic field expeditions using a Blake trawl, Smith-McIntyre grab, Agassiz trawl, wire dredge, epibenthic sled or hand collected by SCUBA diving. Samples were collected from various locations in the Southern Ocean between 2006 and 2018 (Fig. 1). Nineteen geographical regions were defined *a priori* (Table 1). Owing to differing depths, distances between regions, coastal currents and ocean circulation patterns (Smith *et al.* 1999) we separated the Antarctic Peninsula region into four regions: (i) Palmer Archipelago, (ii) Bransfield Strait, (iii) South Shetland Islands and (iv) Elephant Island. Tissue subsamples were taken from specimens preserved in 96–100% ethanol or frozen. Sequenced specimens were housed in the Western Australian Museum (WAM), Scripps Institution of Oceanography, Benthic Invertebrates Collection (SIO-BIC), Yale Peabody Museum (YPM), California Academy of

Table 1. Table of *COI* diversity of *Doris kerguelenensis* individuals and species by region.

Region	Region code	Number of sites	Number of species	Number of individuals
Bransfield Strait	*BS*	31	21	169
South Orkney Islands	*SOI*	27	19	117
Burdwood Bank	*BB*	17	13	100
Palmer Archipelago	*PAL*	15	13	290
South Shetland Islands	*SSI*	5	13	95
Elephant Island	*EI*	11	11	281
South Georgia	*SG*	11	11	43
Discovery Bank	*DB*	3	9	11
Weddell Sea	*WS*	4	6	7
Shag Rocks	*SR*	6	5	47
Davis Station	*DS*	4	5	18
South Sandwich Islands	*SS*	2	4	40
Herdman Bank	*HB*	2	3	5
Ross Sea	*RS*	3	2	29
Falkland Islands	*FI*	1	1	1
Bouvet Island	*BI*	1	1	1
Mount Siple	*SIP*	1	1	1
Kerguelen Plateau	*KP*	2	1	18
Casey Station	*CS*	1	1	2

Ordered by total number of species per region. $N = 1275$.

Sciences (CAS), National Museum of Natural History, Smithsonian Institution (USNM), University of Barcelona and the Baker Laboratory, University of South Florida. Data for all specimens sequenced in this study are available in Supplementary Table S1 including GenBank accession numbers for outgroup taxa.

In addition to the ingroup specimens, a range of outgroup sequences were selected from GenBank (Wollscheid-Lengeling *et al.* 2001; Shields 2009; Pola and Gosliner 2010; Jung *et al.* 2014; Palomar *et al.* 2014; Hulett *et al.* 2015; Mahguib and Valdés 2015; Goodheart *et al.* 2018) (see Supplementary Table S1) along with other available sequences for *Doris kerguelenensis* (Wilson *et al.* 2009, 2013). This was done as a robust sister group for Dorididae, across the Nudibranchia phylogeny is not yet certain (e.g. Wollscheid-Lengeling *et al.* 2001; Shields 2009; Mahguib and Valdés 2015; Korshunova *et al.* 2020); however, the tree was ultimately rooted with *Prodoris clavigera*.

DNA extraction, amplification and DNA sequencing

Total genomic DNA was extracted from 1077 ethanol-fixed or frozen samples using a DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's instructions (excluding the optional repeat of a final elution step). For analysis, an additional 198 previously published sequences were also incorporated (see Wilson *et al.* 2009, 2013). The same primers were used for PCR and sequencing (LCO1490/HCO2198, Folmer *et al.* 1994; or modified degenerate versions of the former, jgLCO1490/jgHCO2198, Geller *et al.* 2013). These primers amplified a fragment of the cytochrome oxidase I gene (*COI*). Each PCR reaction included: 16.8 µL of molecular grade (deionised) water, 5.0 µL 5 × MyTaq PCR buffer (1 × = 1 mM of dNTPs, 3 mM of $MgC1_2$) (Bioline), 0.8 µL of forward primer, 0.8 µL of reverse primer, 0.2 µL of Platinum Taq polymerase (Invitrogen), with 1.5 µL of DNA template added. The thermocycling protocol for *COI* using primers LCO1490/HCO2198 followed the procedure: 5 min. at 95°C, 7 cycles 30 s at 95°C, 30 s at 45°C, 1 min. at 72°C followed by 35 cycles of 30 s at 95°C, 30 s at 50°C, 1 min. at 72°C with a final extension time of 10 min. with a temperature of 72°C. The thermocycling protocol using primers jgLCO1490/jgHCO2198 was as follows: 3 min. at 95°C, 8 cycles of 30 s at 95°C, 30 s at 50°C, 45 s at 72°C followed by 32 cycles of 30 s at 95°C, 30 s at 48°C and 45 s at 72°C with a final extension time of 5 min at 72°C. Amplicons were screened on E-gels (Invitrogen) and the positive reactions, determined through Bio-Rad Image Laboratory software, were outsourced to the Australian Genome Research

Facility (Perth) for enzyme purification and Sanger sequencing using an Applied Biosystems 3730 capillary sequencer. All sequences were assembled and edited in Geneious Prime 2020.1 (Kearse *et al.* 2012). In cases where amplicon bands were deemed weak or if amplicons failed to sequence, experiments were run to improve sequencing success either by increasing DNA or DNA dilutions (1:10, 1:20, 1:50 and 1:100).

Phylogenetic reconstruction and primary species hypotheses (PSH)

COI sequences from *Doris kerguelenensis* and outgroups were aligned with the MAFFT plugin for Geneious (ver. 1.4.0, see https://www.geneious.com/plugins/mafft-plugin/; Katoh and Standley 2013) using default settings. The sequences were used to create a single gene, maximum likelihood (ML) phylogeny using IQ-TREE (ver. 1.6.12, see http://www.iqtree.org/; Nguyen *et al.* 2015; Trifinopoulos *et al.* 2016), herein referred to as the Primary Species Hypotheses (PSH) (Fig. 2, Supplementary Fig. S1) that will be further tested with delimitation methods. The –m TEST (Kalyaanamoorthy *et al.* 2017) option in IQ-TREE identified TN + F + I + G4 as the best-fit model chosen according to Bayesian Information Criterion (BIC). Nodal support was assessed with 1000 ultrafast bootstrap replicates (Hoang *et al.* 2018). This phylogenetic tree was used to make semi-arbitrary decisions about inferred species clades, much in the same way that taxonomists intuitively assign names based on morphological variation (Dayrat 2005). Some of the 'arbitrary' species-level clades had already been corroborated with nuclear and trait data (see Wilson *et al.* 2013).

In previous publications assessing diversity in *D. kerguelenensis*, species-level clades were numbered (Wilson *et al.* 2009, 2013) from 1 to 32. These original numbers have been incorporated into this new work and we have continued numbering new clades from 33 onwards, with one exception. The original clade 12, originally consisting of a single specimen (USNM1120712) has now been synonymised within clade 11. The newly named clade 12 in this study comprises a previously unknown clade.

Pairwise distances (uncorrected *p*-distances) or Tamura and Nei (1993) (TN93) corrected genetic distances were both calculated from the mtDNA sequences using the 'dist.dna' function from the R.cran (ver. 4.2.0, R Foundation for Statistical Computing, Vienna, Austria, see https://cran.r-project.org/) package Ape (ver. 5.6, see https://cran.r-project.org/web/packages/ape/index.html; Paradis and Schliep 2019) with either 'raw' or 'TN93' selected as the evolutionary model (Supplementary Fig. S2). The gamma shape α and base frequencies were also parameters specified for the distance calculations. Intra- and interspecific (PSH clades) *p*-distances and TN93 corrected genetic distances were also calculated in MEGA X (ver. 10.2.6, see https://www.megasoftware.net/; Kumar *et al.* 2018) (Supplementary Table S2).

Species delimitation

The process of delimitation was carried out in a series of steps. We tested our PSH through a range of species delimitation methods that included the Multi-rate Poisson Tree Process analysis ((m)PTP, Kapli *et al.* 2017), statistical parsimony networks (TCS Java program, ver. 1.21, see https://bio.tools/tcs; Clement *et al.* 2000; Hart and Sunday 2007), the Automatic Barcode Gap Definition (ABGD, Puillandre *et al.* 2012) and Assemble Species by Automatic Partitioning (ASAP, Puillandre *et al.* 2021). All delimitation analyses were run without outgroups included in the input files.

Multi-rate PTP is designed to better accommodate sampling and population specific characteristics of a broad range of datasets as this incorporates different levels of intraspecific genetic diversity (based on specific branching events or species-specific sampling) (Kapli *et al.* 2017). This analysis has been shown to outperform PTP (Zhang *et al.* 2013) in yielding more accurate delimitations with respect to taxonomy (i.e. identifies more taxonomically accepted species).

TCS (Clement *et al.* 2000) bins sequences into haplotypes and calculates the frequencies of the haplotypes in the sample (Clement *et al.* 2000). Relationships among haplotype networks were explored using this method using 'parsimnet', a function in the R.cran package Haplotypes (ver. 1.1.2, C. Aktas, see https://cran.r-project.org/web/packages/haplotypes/index.html) that finds the most parsimonious networks and is an implementation of the TCS methods proposed by Templeton *et al.* (1992). TCS (Clement *et al.* 2000) has been shown to be useful for species delimitation as the parsimony connection limit (95%) successfully delimits the same number of subnetworks as taxa (see Hart and Sunday 2007).

ABGD and ASAP are both ascending hierarchal clustering programs that merge sequences into groups defined as partitions. The partitions are defined by probability and barcode gap width however, unlike ABGD that is solely based on pairwise distances (Puillandre *et al.* 2012), there is no need for an *a priori* defined *P* (when *P* is the prior maximum divergence of intraspecific diversity) within ASAP's input functions. ASAP produces a scoring system that ranks the partitions based on a combination of both probability and gap width ('*asap-score*') (Puillandre *et al.* 2021). In ABGD and ASAP analyses, there are two available substitution models, Jukes and Cantor (1969) (JC69) and Kimura (1980) (K80). However, the evolutionary model selected by IQ-TREE (a variant of Tamura and Nei 1993) is not an available model option when partitioning sequences in these programs, so Tamura and Nei (1993) corrected genetic distances were imported into these delimitation analyses for comparison. We used ABGD alongside ASAP because although ASAP has been developed to replace ABGD, comparisons between the two programs will allow for direct comparision to results from previously published literature. The default parameters employed within ABGD were $P_{min} = 0.001$, $P_{max} = 0.10$, 10 steps, $X = 1.5$, Nb bins of 20. Only the initial partitions were examined

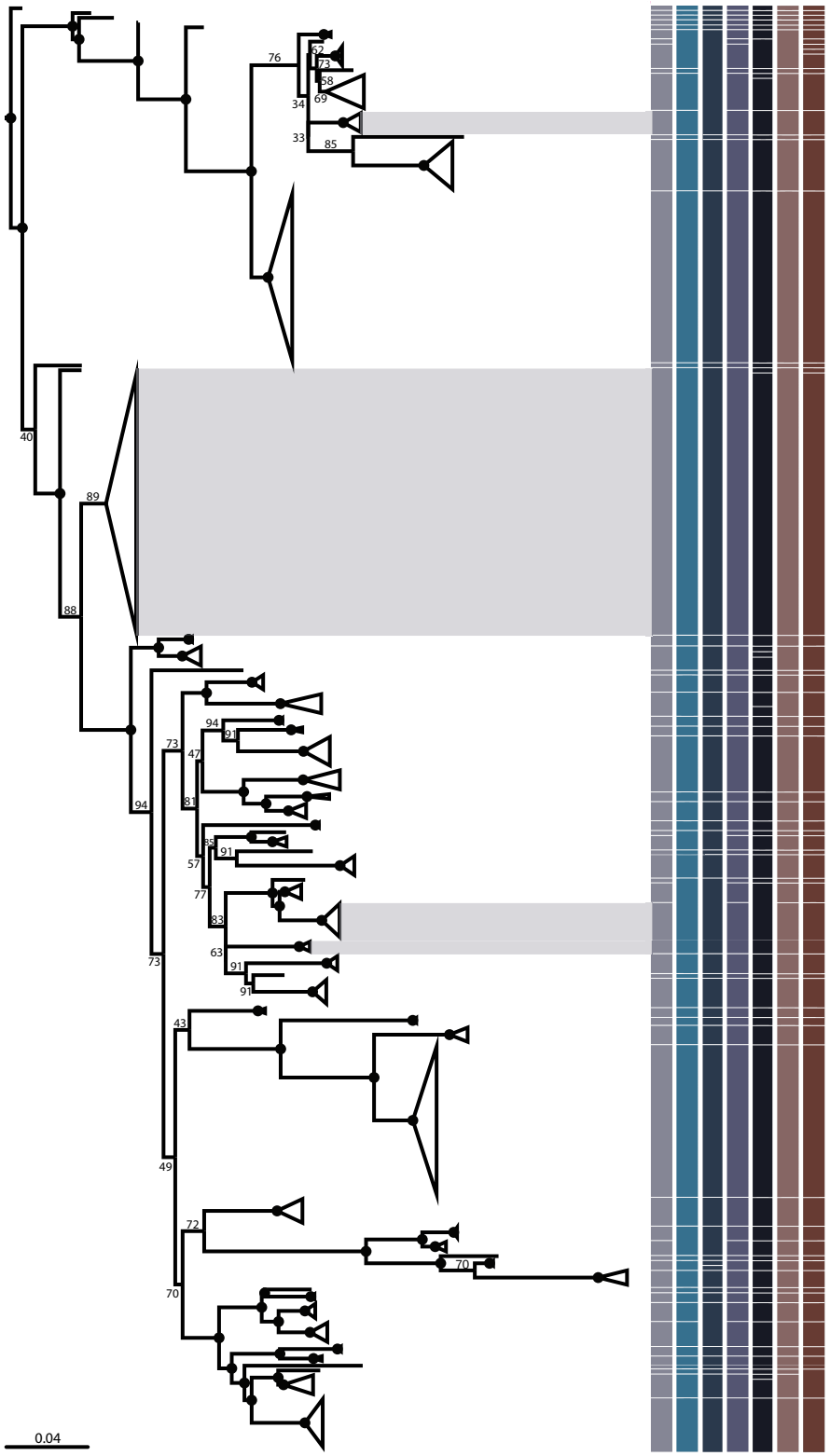

Fig. 2. Maximum likelihood (ML) phylogeny of the unique *COI* haplotypes dataset, including 320 *Doris* sequences and 21 outgroups (not shown) using a TN + F + I + G4 best-fit model. Nodes with ultrafast bootstrap support values of 95 or higher have been denoted by a circular node shape. Triangles represent collapsed clades. Boxes represent the Primary Species Hypothesis (PSH) followed by the partition results for species delimitation analyses (ABGD P2, ABGD P6, ASAP 1st [all input files except ASAP K80 uncorrected], ASAP 1st K80 uncorrected, TCS and mPTP). Grey boxes indicate species with samples collected from both sides of the Antarctic Polar Front (southern South America and Antarctica).

from the ABGD algorithm outputs (following Puillandre *et al.* 2021). In ASAP, the default parameters were also used.

When assessing ABGD and ASAP partitions, we examined a range of partitioning definitions. This was done to encapsulate all inter- and intraspecific variability and accommodate for over-splitting or coalescing of recently radiated taxa, rather than only selecting a single partition to represent species boundaries. ABGD initially divides the

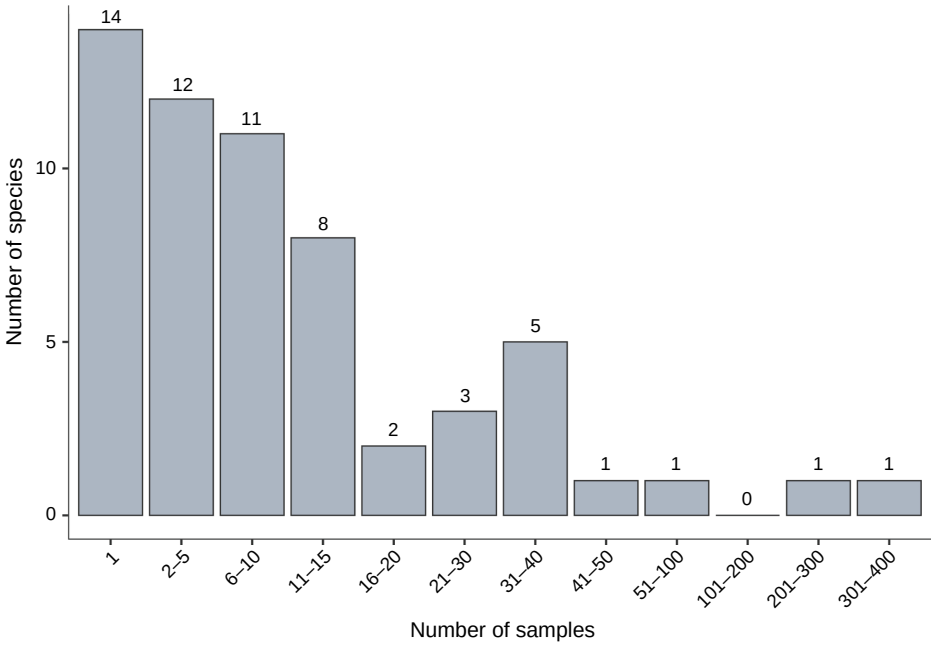

Fig. 3. Frequency plot depicting the number of samples present within species of *Doris kerguelenensis*. For example, 14 species are represented only by singletons.

data into groups based on a range of prior intraspecific divergences and a statistically inferred barcode gap, and subsequently recursively applies the same procedure to the initial groups (Puillandre *et al.* 2012). For this reason, the initial partitions generally represent species or operational taxonomic units (OTUs) whereas the recursive partitions generally reflect population level diversity. Here we only examined the initial partition outputs and only considered ABGD partition 2 ($P = 0.001$; to highlight the intraspecific genetic variability) and partition 6 ($P = 0.01$), as defining species based on less than 1% sequence divergence is not appropriate. Partition 1 was omitted from reports as the primary partition assumes that a single gap can be defined for the entire dataset however the gap distances are highly likely to differ between groups within a dataset (Puillandre *et al.* 2012). We considered these two partitions from both substitution model outputs (JC69 and K80) (Supplementary Fig. S3). ASAP-scores are the average of the *P*-value and relative barcode gap width of the partitions. In terms of the ASAP partition outputs, three input files were examined. The original, uncorrected alignment file was entered into the webserver (https://bioinfo.mnhn.fr/abi/public/asap/asapweb.html#) and both substitution models were applied to the file (=uncorrected *p*-distances). The Tamura and Nei (1993) corrected MEGA X CSV genetic distance file was imported and tested against both JC69 and K80. A MEGA X CSV TN93 corrected distance file with the gamma shape α (0.692) and base frequencies ($A = 0.245$, $C = 0.174$, $G = 0.189$ and $t = 0.391$) specified, was also tested with both substitution models. ASAP 1st and ASAP 2nd partitions were considered due to the low (and thus better supported) ASAP scores of between 5.00 and 8.00 for all input files and substitution models (see Supplementary Fig. S4).

Species diversity estimates

Considering the large percentage of species clades consisting of single specimens (Fig. 3), we assessed whether further diversity remained hidden due to undersampling, by generating species rarefaction–extrapolation (R–E) curves and the associated 95% confidence intervals, using iNEXT (ver. 2.0.2, see https://cran.r-project.org/web/packages/iNEXT/index.html; Hsieh *et al.* 2016). Through iNEXT (interpolation and extrapolation), an R.cran package (Hsieh *et al.* 2016), our diversity estimates used abundance-based data (Gotelli and Colwell 2011) that tally the abundance of each species (SSH results were utilised as input) across predetermined geographic regions (Table 1). A sample-size R–E curve was produced to determine whether the rate of discovery of new species slowed down or reached saturation with an increase in sample size. To avoid discarding data, a sample-size R–E curve that rarefies to smaller sample sizes or extrapolates to larger sample sizes (i.e. plots with respect to sample size) was utilised (Colwell *et al.* 2012; Hsieh *et al.* 2016). We conducted both whole dataset and region-based R–E curves that are represented by the means of repeated resampling (1000 bootstrap replicates). Examining the slope of these curves allowed us to assess the impact of undersampling on estimating diversity.

Results

Species delimitation

A total of 1275 *COI* sequences (433 haplotypes) was generated and the final alignment constituted 658 base pairs (bp) in length. The primary species hypotheses (PSH) generated

with a maximum likelihood (ML) phylogeny recovered 59 species-level clades within the monophyletic *Doris kerguelenensis* species complex, 27 more than previously known. The ML phylogeny generated through IQ-TREE showed low bootstrap support at interior nodes but mostly high support for terminal clusters (ultrafast bootstrap support >95–100), herein referred to as species. The only two species-level clades that were not supported by high bootstrap values were species 15 and 59 (bootstrap support values 69 and 58 respectively) (Fig. 2, Supplementary Fig. S1). The proportion of invariable sites was 0.475, the gamma shape α distribution parameter was 0.692 and the base frequencies were $A = 0.245$, $C = 0.174$, $G = 0.189$, $t = 0.391$ (unequal base frequencies). Rates for the six substitution types estimated from the dataset were AC = 1.0000, AG = 10.6386, AT = 1.0000, CG = 1.0000, CT = 7.1609 and GT = 1.0000 (unequal transition rates, equal transversion rates and unequal purine and pyrimidine rates).

To assess the robustness of the PSH, results were compared to those from four delimitation methods (mPTP, TCS, ABGD and ASAP) (Fig. 2, 4, Supplementary Table S1, Supplementary Fig. S1–S4). The Multi-rate Poisson Tree Process (mPTP) yielded 62 taxonomic units and TCS recovered 56 groups. Species delimitation based on genetic distance using ABGD analysis detected between 49 and 52 groups respectively (initial partitions 6 ($P = 0.01$) and 2 ($P = 0.001$)) using JC69 and K80 models (Supplementary Fig. S3). All ABGD results were identical between both substitution models (JC69 and K80, TS/TV = 2.0) (see Supplementary Fig. S3). The ASAP species hypothesis partition, ranked by best ASAP score (ASAP 1st) delimited 59 (JC69) and 61 (K80) species, and ASAP 2nd delimited 57 (JC69) and 63 (K80) species. When the TN93 corrected genetic distance files were incorporated into analyses and a model subsequently applied, 59 species were delimited (for both JC69 or K80) in ASAP 1st, and 48 or 60 (JC69 and K80 respectively) in ASAP 2nd. When the additional gamma shape α and base frequency parameters were defined, ASAP 1st and 2nd (JC69) delimited 59 and 60 species respectively, and ASAP 1st and 2nd (K80) delimited 59 and 48 species (see all ASAP results: Supplementary Fig. S4).

The species delimitation results for mPTP, TCS, ABGD (initial partitions 2 and 6) and ASAP (ASAP 1st and 2nd (all input file structures)); are plotted against the ML phylogeny (Fig. 2, 4, Supplementary Fig. S1). Overall, for the Secondary Species Hypotheses (SSH), we chose to accept 59 species (Fig. 4), as this number was recovered by the best scoring ASAP partition in five of six conditions. This number of partitions was always recovered by ASAP 1st when the input alignment was adjusted with corrected distances, and for gamma shape and base frequencies.

Intraspecific colour variation (and to a lesser extent, dorsal tubercle exaggeration) was assessed to determine whether colour could be utilised as a taxonomically distinguishing characteristic. Live specimen photos (Supplementary Fig. S5)

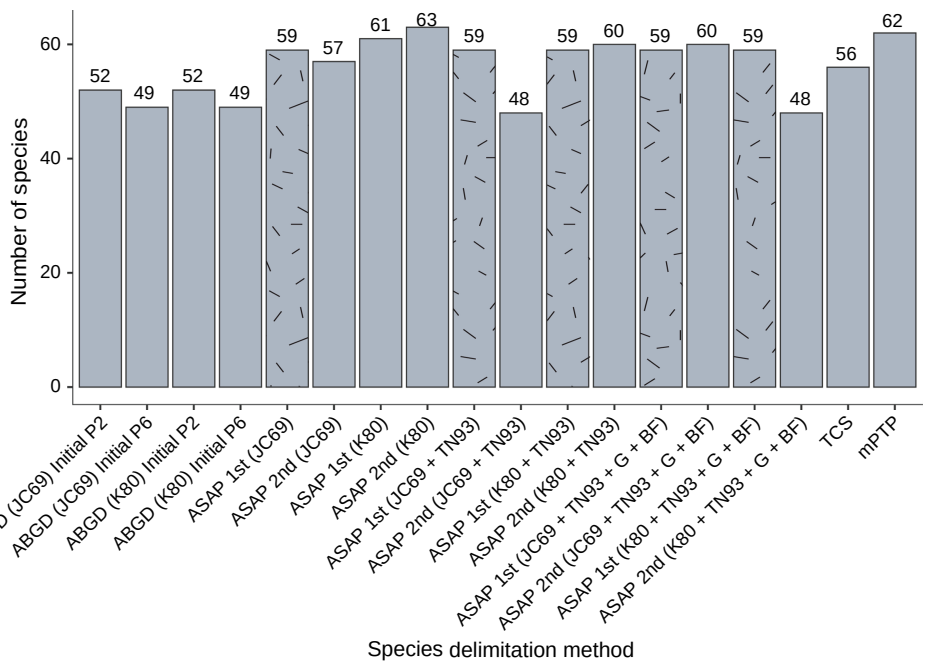

Fig. 4. Comparison of species delimitation results for the Secondary Species Hypotheses (SSH). Results are ordered as shown in Fig. 2 (phylogenetic tree). ASAP 1st (lowest score, stippled for five of the six ASAP 1st results) was chosen as the SSH for this dataset. The Primary Species Hypotheses (PSH) also represented 59 species.

showed substantial colour variation within an example species (24) and was therefore deemed an uninformative trait for taxonomic purposes.

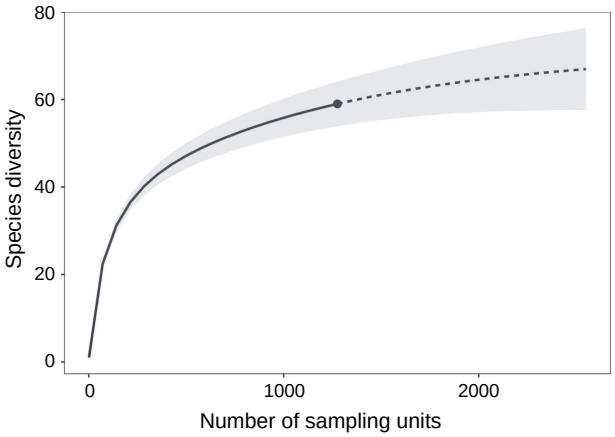

Fig. 5. Estimation of *Doris kerguelenensis* species diversity based on *COI* abundance data. Rarefaction curve for observed samples and the 95% upper and lower confidence intervals based on the abundance-based rarefaction curve.

Sampling effort and geographic distributions

Most clades contained between 1 and 40 samples (mean = 21) and overall, 14 species were represented by single specimens (Fig. 3). The slope of the R–E curve approached saturation (an asymptotic shape) at around 68 species (Fig. 5), indicating that our sampling coverage was relatively high (accurately sampled enough of the landscape to capture species diversity) and we have therefore approached a realistic estimate for the number of species in this complex. The R–E curves reflecting sampling effort for specific locations such as the Palmer Archipelago (PAL), Elephant Island (EL) (Fig. 6*a*), Shag Rocks (SR) and Herdman Bank (HB) (Fig. 6*b*) reached an asymptote. However, the opposite result was detected for regions such as Discovery Bank (DB), the Weddell Sea (WS), Davis Station (DS), Casey Station and others.

One of the most well-sampled species, species 29 (n = 280), has a circum-Antarctic distribution (collected from over 11 000 km) and contains samples collected from Prydz Bay, the Ross Sea and the Antarctic Peninsula. An additional six species have very large distributions (defined here as >2000 km) and data show that at least four species distributions span the Polar Front (clades 14, 24, 26 and 42) (grey highlighted boxes within the PSH, Fig. 3).

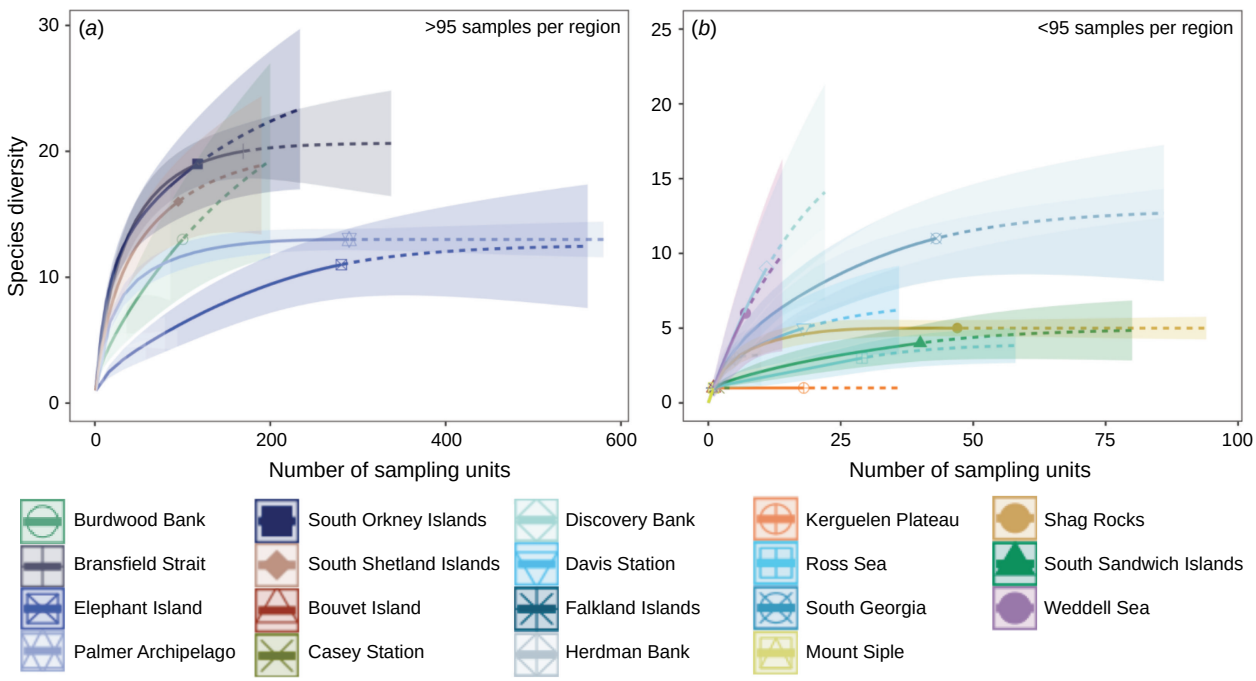

Fig. 6. Rarefaction curves were constructed through a single analysis that was subsequently split for visual ease. (*a*) Estimation of *Doris kerguelenensis* species diversity based on *COI* abundance data separated by geographic sampling region (Burdwood Bank, Bransfield Strait, Elephant Island, Palmer Archipelago, South Orkney Island and South Shetland Island) for regions that include 95 individual specimens or more. Rarefaction curve for observed samples and the 95% upper and lower confidence intervals are based on an abundance-based rarefaction curve. (*b*) Estimation of *Doris kerguelenensis* species diversity based on *COI* abundance data separated by geographic sampling region (Bouvet Island, Casey Station, Discovery Bank, Davis Station, Falkland Island, Herdman Bank, Kerguelen Plateau, Ross Sea, South Georgia, Mount Siple, Shag Rocks, South Sandwich Islands and Weddell Sea) for regions that include less than 95 individual specimens. Rarefaction curves for observed samples within locations and the 95% upper and lower confidence intervals based on the abundance-based rarefaction curve.

Thirteen clades, including the aforementioned four species (1, 2, 12, 28, 35, 44, 47, 48 and 50) have been collected above the Polar Front (Falkland Islands, Burdwood Bank, Bouvet Island and Kerguelen Plateau) and three of these species have an Antarctic continental shelf sister-species (1 with 10 + 55, 2 with 29 and 44 with 30, Supplementary Table S1). The deepest depth at which a specimen was collected was 798 m.

Many species occurred in sympatry. Twenty-one species were collected from the Bransfield Strait, nineteen on the South Orkney microcontinent and thirteen from the Burdwood Bank, Palmer Archipelago and South Shetland Islands (Table 1). Overall, species richness remains a direct reflection of sampling intensity within these results, e.g. seven 'well sampled' regions (each with over 40 samples) had more than 10 species within that corresponding location (Table 1).

Discussion

Doris kerguelenensis was long thought to consist of a single, widespread species (since Wägele 1990). With the application of molecular data, multiple species delimitation algorithms and increased sampling, the species complex is currently recognised to comprise at least 59 geographically overlapping species. Here, we show: (i) 27 previously undiscovered species, (ii) further evidence that more species remain to be discovered within previously sampled regions of the Southern Ocean and (iii) new insights into the distributions of these animals.

Even more new species delimited

The primary species hypotheses (PSH) outlined 59 species within the *Doris kerguelenensis* complex, of which 27 were new species-level clades. Subsequent species delimitation analyses showed highly congruent results (48 – 63 total range), with the best supported ASAP scores converging on 59 species. Our results corroborated the *D. kerguelenensis* clades that were previously detected through mitochondrial, nuclear and metabolomic datasets (Wilson *et al.* 2009, 2013), with one exception (the original clade 12, Wilson *et al.* 2009).

As expected, species delimitation methods based on different underlying assumptions rendered varying species hypotheses. Overall, ASAP 2nd (K80) showed the highest evidence for over-splitting (63 species), relative to the clades recognised in the PSH and all other partition analyses. We suspected that ASAP 2nd (K80) may have been over-splitting clades as mPTP also suggested there were 62 species and mPTP performed poorly when the number of species was small or consisted of a small sample size (Puillandre *et al.* 2021). Also, many of these new *D. kerguelenensis* species comprised one or only a few individuals and this may explain the similar result of 62 species delimited through this method. Our TCS analyses recovered 56 species and this was ultimately identical to the PSH with the single exception that clades 15 + 31 + 46 + 60 were merged into one. The ABGD initial partitions delimited between 49 and 52 species (JC69 and K80). This outcome was generally expected as the initial partitions of ABGD results will delimit a more conservative number of taxa, while recursive have been suggested to represent population level diversity rather than interspecific diversity (Puillandre *et al.* 2012). Overall, ASAP's 2nd partitions delimited between 48 and 63 species. Finally, ASAP 1st partition results (from all input file structures, except K80) all converged on 59 species and this was accepted as the most well-supported delimitation estimation.

Despite some colour variations between specimens collected (ranging from white, yellow, orange, pink, including white with pink colouration around the mouth; N. G. Wilson, pers. obs. and Supplementary Fig. S5), most were morphologically similar. This means that gill or body colour, or tubercle structure could not be used to visually differentiate species. Additionally, when preserved, the colour of specimens disappears and the animals become a uniform off-white colour (first noted in Wägele 1990). Specimens also ranged considerably in length, from ~1 to 20 cm. Morphological studies mention that external traits such as the size and density of the dorsal papillae should only be tentatively relied on for species determination, because these change in appearance when preserved (Wägele 1990; Cattaneo-Vietti 1991). Wägele (1990) highlighted considerable morphological variation in the nervous system, the digestive system, and the colour and shape of the radula. None of these variations were consistent within those species concepts, therefore all animals examined were considered to be one species. Morphology was, therefore, not further considered in species delimitation efforts throughout this study. A phylogenetic framework does offer an opportunity to re-examine morpho-anatomical systems for additional characters, to correlate traits with molecular clades. However, given the relatively uninformative radular and reproductive systems, finding enough informative characters that could act as synapomorphies to resolve more than 59 species is unlikely (Fišer *et al.* 2018). Also, although synonyms proposed by Wägele (1990) may represent genetically distinguishable units, these are mostly preserved in ways that are not conducive to connecting specimens to these numbered species clades. Possibilities may however, still lie in sequencing holotypes and fixing those names to clades.

Still more species to be discovered

To date, expansion in either sampling or geographic scope (Wilson *et al.* 2009, 2013) has increased the number of species known in this complex. Here, we assessed the impact of potential undersampling by extrapolating known species records and found that the true number of cryptic species within this complex may be even greater, as the R–E curves illustrate undersampling of genetic diversity and helped

identify geographic regions that require more investigation. The R–E curve results did not reach an asymptotic shape, but ~90% of *D. kerguelenensis* species expected in the sampled areas appeared to be recovered for this study. At a smaller scale, the Palmer Archipelago R–E curve reaches an asymptote at ~12 species and 250 sampling units. Other well-sampled locations that are approaching an asymptote include the Bransfield Strait and Elephant Island. Even locations such as Shag Rocks, Herdman Bank and the South Sandwich Islands, that consist of 50 specimens or less, still approach asymptotic levels, and likely reflect lower diversity in these areas, perhaps due to size or oceanic isolation (separated from other shallow subsea land masses by deep sea or abyssal plains).

This is interesting compared to regions that are well sampled (<100 specimens collected) but that do not approach an asymptotic shape within the R–E curves, such as Burdwood Bank, the South Orkney Islands or the South Shetland Islands. This likely indicates that high levels of *D. kerguelenensis* species occur in these three regions. Owing to the location (and ancient supercontinent link), the Burdwood Bank plays an important role in diverting circumpolar ocean flow (Fraysse *et al.* 2018), and is associated with the uplift of nutrient rich bottom water that in turn supports an abundant production of phytoplankton and subsequently rich levels of biodiversity (Schejter *et al.* 2020). The South Orkney Islands are bordered by two current regimes (the ACC and Weddell Sea Gyre) and have been recorded to host approximately one-fifth (Barnes *et al.* 2009; Brasier *et al.* 2018) of all benthic marine species recorded for the entire Southern Ocean (as estimated by Clarke and Johnston 2003). The South Shetland Islands are considered a transitional ecosystem from which new species are still regularly recorded despite being documented as one of the most well-sampled regions across the Antarctic (Barnes *et al.* 2008). Given the indications that further *Doris* species diversity is to be found in these areas, these are likely to also be very rich in general benthic marine fauna and should be considered for future biodiversity work (e.g. Barnes *et al.* 2009). For *D. kerguelenensis*, areas that should be of interest for future exploration (due to the low sample numbers but high levels of species diversity detected) include Herdman Bank, the Falkland Islands, Bouvet Island, Kerguelen Plateau, Prydz Bay, and the areas surrounding Davis and Casey stations. Totally unexplored locations for this species also include the south-west of South America where the Humboldt Current comes into contact with this coast upon breaking from the northern ACC.

Large distributions for a directly developing slug

Some of the cryptic species in the *D. kerguelenensis* complex documented here spanned thousands of kilometres. The Southern Ocean, while connected by major currents, still contains numerous potential dispersal barriers for benthic invertebrates, even for those that do have planktonic larvae. Barriers can include temperature and salinity changes, plankton availability, potentially uninhabitable deep sea areas, and the strength and direction of currents and gyres. These large-scale distributions therefore raise many questions regarding the mechanisms of dispersal in *D. kerguelenensis*.

In aquatic ecosystems, connectivity can be related to dispersal ability between populations (Jablonski 1991; Palumbi 1994; Hellberg *et al.* 2002; Shanks *et al.* 2003; Cowen and Sponaugle 2009; González-Wevar *et al.* 2021). Broadcast spawners and species with pelagic larval development should exhibit higher levels of connectivity and less genetic structure when compared to benthic or direct developing organisms (Ronce 2007; Gillespie *et al.* 2012). However, there are examples that contradict this prediction (e.g. Marko 2004; Weersing and Toonen 2009; Mercier *et al.* 2013; Segovia *et al.* 2017). There are also many benthic Southern Ocean species with large distributions and benthic development (typically showing small-scale and geographically structured distributions) that have been subject to phylogeographic studies. These include gastropods (Nikula *et al.* 2011*a*; Cumming *et al.* 2014; González-Wevar *et al.* 2021), chitons (Nikula *et al.* 2011*b*) and crustaceans (Nikula *et al.* 2010).

Dayton *et al.* (1970) proposed several dispersal mechanisms for benthic organisms including (i) adults rafting on detached benthic organisms, (ii) rafting on mobile organisms, either as egg masses or adults, or (iii) anchor ice removing organisms from the benthos and depositing these elsewhere. Alternatively, egg masses could be laid on sessile organisms that could be subsequently dislodged (see Wilson *et al.* 2009). All of these aforementioned examples are based on dispersal by forms of rafting and rafting appears to be unlikely to be the major method of dispersal for adult individuals in the *D. kerguelenensis* complex due to weak adhesive ability of the foot (N. G. Wilson, pers. obs.). *Doris kerguelenensis* species are direct developers (embryonic period recorded up to 21 months; Hain 1989; Moles *et al.* 2017*a*) that feed on sedentary organisms such as demosponges and hexactinellid sponges (reviewed by McDonald and Nybakken 1997; Iken *et al.* 2002). Although there is a small possibility that some species in the *D. kerguelenensis* complex have planktonic larvae, there is very little evidence in support of this. If rafting is the method of dispersal, this most likely involves egg masses or juveniles.

Current geological evidence shows that small, transient, ice-free areas with reduced levels of primary production did persist, even through glacial maxima across the Southern Ocean continental shelves (including during the Last Glacial Maxima, Poulin *et al.* 2002; Thatje *et al.* 2005; Convey *et al.* 2009; Pearse *et al.* 2009). Direct development appears to have experienced strong positive selection within these refugia due to low food conditions, long developmental times and speciation driven by allopatry (Poulin *et al.* 2002; Pearse *et al.* 2009; Lau *et al.* 2020). As ice-free areas on the continental shelf could have enabled *in situ* survival of benthic fauna, populations may have experienced demographic bottlenecks within

these refugia instead of complete eradication (Allcock and Strugnell 2012). If secondary contact of populations occurred before reproductive isolation and post glacial expansion, species would demonstrate widespread distributions (Allcock and Strugnell 2012).

In addition to the exentsive geographic distributions, these nudibranchs have been widely reported from depths of up to 1550 m (Iken *et al.* 2002). The morphologically identified sample from the deepest depth was collected from Halley Bay, in 1998 from 1549 m (Avila *et al.* 1999). This sample is stored at the University of Barcelona and was fixed in formalin (C. Avila, pers. obs.) and was therefore not used in this study. Here we provide the deepest barcoded record of *D. kerguelenensis* (798 m). Wägele (1987) similarly reports specimens from 788 m from the Weddell Sea. Although one *D. kerguelenensis* specimen was reported from New Caledonia from a depth of 680 m (Valdes 2001), given the large geographic gap between this single specimen and all other collected records of this species complex, this specimen likely represents a different taxon. The general absence of *D. kerguelenensis* from the deep sea areas surrounding Antarctica is worth noting; throughout the ANDEEP expeditions (I, II and III: Brandt *et al.* 2004, 2007), no *D. kerguelenensis* were collected from abyssal areas. The ANDEEP project set out to understand the abyssal diversity and faunal exchange between South America and Antarctica (Brandt *et al.* 2004). This question remains unanswered for this group of organisms and how these benthic organisms disperse across the abyssal plains of the Southern Ocean is currently unknown.

Evidence for dispersal across the Polar Front

Despite all records of *D. kerguelenensis* being restricted to the continental shelf (see above), some species within this complex have clearly dispersed long distances, including across the APF. These trans-APF species appear in our *COI* tree in four separate places and nine species were restricted to the Southern American continental shelf. The Antarctic Polar Front represents a strong geographic and oceanographic divide that across time, has split evolutionary lineages between the Antarctic and northern oceans (Page and Linse 2002; Lee *et al.* 2004; Hunter and Halanych 2008; Thornhill *et al.* 2008; Wilson *et al.* 2009, 2013; Krabbe *et al.* 2010). Very few benthic marine taxa have been recorded to span this region except a sea star (Moore *et al.* 2018), a brittle star (Galaska *et al.* 2017), sea spiders (e.g. Linse *et al.* 2006; Munilla and Membrives 2009; Dietz *et al.* 2019), an isopod species (Leese *et al.* 2010) and a tritoniid nudibranch (Moles *et al.* 2021). Most of these species have a dispersive larval stage except for the isopod that has had long-distance dispersal linked to rafting. Additionally, the only directly developing nudibranch example listed is *Tritonia vorax*, a sub-Antarctic species that has been recorded from the Southern South American continental shelf and South Georgia in the Scotia Arc (Moles *et al.* 2021). All of these individuals or populations, unless already in deep water, also overcame the temperature gradient at the Antarctic Polar Front (APF) (3–4°C) that differentiates the SO from more northerly oceans (see Park *et al.* 2014). The APF is one of the world's strongest open ocean barriers that is known to isolate the Southern Ocean from warmer waters at lower latitudes and was formed after the opening of a deep-water passage (the Drake Passage) between South America and Antarctica *c*. 35 Ma (Livermore *et al.* 2004; Pfuhl and McCave 2005; Barker *et al.* 2007). The Antarctic Circumpolar Current (ACC) and major Antarctic gyres (in the Weddell and Ross Seas), however, can act as large-scale dispersal vectors that either jet eastward around Antarctica and meet the southernmost region of Southern America (ACC) or rotate clockwise (gyres) due to the interaction between the APF and ACC. In principle, these currents may have driven the dispersal of *D. kerguelenensis* (Barker and Thomas 2004), however this mechanism remains unclear.

Future directions

Antarctica has long been associated with the diversification and speciation of many benthic marine taxa (Briggs 2003; Rogers 2007; Strugnell *et al.* 2008), including heterobranch gastropods (Wägele *et al.* 2008; Martynov and Schrödl 2009; Moles *et al.* 2017*b*). In support of this, we found that there is a multitude of undescribed species within the Antarctic species complex *D. kerguelenensis* (Bergh, 1884) (59 detected and more than 68 estimated). Although the monophyly of the complex is well supported, the interspecific relationships remain unresolved. Genetic barcoding of the complex, that is characterised by intra- and interspecific morphological similarities, appears effective in delimiting species and highlights how molecular phylogenetics can uncover cryptic diversity. To gain phylogenetic resolution within the complex, we now need large quantities of sequence data from across the genome. Future work could incorporate transcriptomes, exon capture or ultra-conserved elements (UCEs). Several recent studies have shown the power of these methods for marine invertebrate taxa (e.g. Horowitz *et al.* 2020; Layton *et al.* 2020; Richards *et al.* 2020). Better knowledge of this species complex will help us to understand the evolutionary dynamics of benthic taxa in Antarctica and provide a phylogenetic scaffold for tracing the evolution of secondary metabolites (chemical defence compounds) through time and across species.

Also, very few Antarctic marine studies have sampled widely enough to assess whether benthic Antarctic invertebrates are truly circumpolar (but see Allcock *et al.* 2011; Arango *et al.* 2011; Hemery *et al.* 2012; Moore *et al.* 2018; Moles *et al.* 2021). Most studies are restricted by the logistical challenges that occur when sampling such a vast and remote ecosystem. Future research should have a strategic focus on exploring distributions and understudied regions, specifically

the Kerguelen Plateau and East Antarctica as these are likely to contain additional undiscovered diversity. Understanding the extent of diversity is especially important because marine systems worldwide are being affected by climate change, pollution, biological invasions and a host of other anthropogenic disturbances. If no immediate action is taken to address the increasing loss to biodiversity, Antarctic marine systems, along with other unique marine environments spanning the globe, will be at risk of degradation and homogenisation.

Supplementary material

Supplementary material is available online.

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Data availability. Supplementary data for this article can be accessed in the Supplementary material. *COI* sequences are available through GenBank (https://www.ncbi.nlm.nih.gov/): ON419127-ON419135.

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