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DNA uncovers Antarctic nemertean biodiversity and exposes a decades-old cold case of asymmetric inventory

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Abstract With threats to biodiversity posed by anthropogenic impacts and global climate change, characterization of existing flora and fauna is increasingly important, but continues to focus predominantly on easily studied taxa. In the Southern Ocean, levels of species richness remain relatively unexplored due to remoteness and difficulties of sampling the region. Nemerteans (proboscis worms; ribbon worms) are unusually abundant and occasionally conspicuous in the Antarctic region. Despite being routinely collected, difficulties in preserving voucher material, morphological limitations, and shortage of taxonomic expertise have hindered our understanding of nemertean diversity. To assess patterns of diversity, we examined a fragment of the mitochondrial 16S rRNA gene from larval and adult nemerteans ($n = 192$) from 53 sites along the western Antarctic Peninsula. We found 20 distinct lineages having an uncorrected genetic distance (p) greater than 5% to the nearest sister taxon or group, 19 of which have not been genetically characterized in previous studies. Additionally, the putatively dominant adult species in the region, *Parborlasia corrugatus*, was found to comprise only 4.3% of larvae sampled ($n = 3$ out of 69 samples from 12 locations). Of 47 nemertean species recorded from Antarctic waters, 20 are heteronemerteans and therefore could have a pelagic pilidium larval phase. These results suggest that Antarctic biodiversity is underestimated, and that unknown species of nemerteans await description from Southern Ocean waters.

Keywords Antarctica · Biodiversity · Cryptic species · 16S · Larvae

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Introduction

The Antarctic region harbors approximately 3,000–4,000 described species, many of which are endemic to the region, and estimates suggest that the region may hold as many as 17,000 species (Arntz et al. 1997; Clarke and Johnston 2003; Gutt et al. 2004). The complex glacial history and climate of the Antarctic ecosystem likely have facilitated evolution of numerous lineages in Antarctic waters (see Thatje et al. 2008). Indeed, with application of molecular tools, the number of reported, previously unrecognized, lineages for Antarctic organisms is growing in the literature (e.g., crustaceans, Held 2003; isopods, Held and Wägele 2005; crinoids, Wilson et al. 2007; ophiuroids, Hunter and Halanych 2008; pycnogonids, Mahon et al. 2008).

Nemerteans, commonly referred to as ribbon or proboscis worms, are an important component of Southern Ocean marine benthic fauna. They are found throughout the world's oceans and include approximately 1,275 described species (Kajihara et al. 2008). The defining characteristic of these unsegmented worms is an eversible proboscis in a fluid-filled cavity or rhynchocoel (Turbeville 2002; Thollesson and Norenburg 2003). These ecologically important predators and scavengers range in size from a few millimeters up to 30 m in length (Turbeville 2002). Nemerteans reproduce either via a loosely defined direct development (Paleonemertea and Hoplonemertea) or a distinctive intermediate larval phase, the pilidium, that metamorphoses into adult form (Pilidiophora) (Norenburg and Stricker 2001). A free-swimming pilidium has long been considered typical of Pilidiophora, a group that consists of heteronemertea and the paleonemertean genus *Hubrechtella* (Friedrich 1979; Thollesson and Norenburg 2003). However, actual larval development has been documented for only about 36 pilidiophorans (Friedrich 1979, M. Schwartz, personal communication), with 30 of these having a free-swimming, feeding pilidium, and six having a modified, non-feeding and more or less direct-developing pilidium (Friedrich 1979; Schwartz and Norenburg 2005). The latter may be more common than previously suspected. The phylogenetic relationships among major nemertean clades have been speculated in previous work (see Thollesson and Norenburg 2003). To date, about 42–45 species of benthic and two species of pelagic nemerteans have been reported from the Southern Ocean—approximately 14 from along the Antarctic Peninsula, about 28 extending easterly from the tip of South America to the Scotia Arc (South Georgia and the South Sandwich Islands), with six reported from both regions (compiled from Gibson 1995).

Nemertean classification has been based primarily on internal anatomy as revealed by histology. However, internal anatomy is inadequately documented or unknown for about half of the described species (Gibson 1995). Conversely, species with more or less well-documented histology are often poorly characterized for external appearance, especially from life. As is the case for many nemerteans, but especially common for most of those collected from the Southern Ocean region, voucher specimens, when available, have been inadequately processed and preserved (Gibson 1985a; Thornhill et al. 2008). This situation makes comparative histology and morphology unreliable and species identification of specimens problematic. Furthermore, vouchers are not available for many species. Hence, as is common for nemertean taxonomy, there is uncertainty and debate about the validity and synonymies of Antarctic nemertean species (e.g., Gibson 1985b; Gibson and Crandall 1989; Thornhill et al. 2008).

Fortunately, in taxa where diagnostic morphological characters are problematic, DNA sequence data have become increasingly useful to elucidate species boundaries (Singh 2002). These data can define operational taxonomic units (OTUs) for a variety of organisms, including those that are morphologically indistinguishable, microscopic, meiofaunal, or those from bulk ecosystem DNA samples (see Blaxter et al. 2005). Whereas, recent molecular phylogenetic work has elucidated some key relationships within the Nemertea (e.g., Sundberg et al. 2001; Thollesson and Norenburg 2003; Strand and Sundberg 2005), published sequence data, prior to this study, were available for only about 100 nemertean species, of which only *Parborlasia corrugatus* is from the Southern Ocean (Thollesson and Norenburg 2003; Thornhill et al. 2008). This species has been reported from throughout the range of this study and circum-Antarctic waters (Gibson 1985b, Thornhill et al. 2008).

The Antarctic Peninsula has experienced a ~3.4°C temperature increase in the last century (Vaughan et al. 2003); this climatic shift undoubtedly will induce habitat and resource changes that will likely threaten biodiversity in the region (Mayhew et al. 2008). If we are to understand changes in biodiversity, we must first have an accurate assessment of the levels of biodiversity in this remote region. DNA-barcoding has been used to diagnose species-level relationships, as divergence values within a species are typically lower than those between species or within a genus (Moore 1995; Avise and Walker 1999; Floyd et al. 2002; Hebert et al. 2003, 2004). In this study, our goal was to characterize genetically distinct lineages in adult and larval nemerteans from waters around the Antarctic Peninsula and estimate nemertean diversity by using molecular barcodes in the form of 16S rRNA gene sequences.

Materials and methods

Sample collection

Samples were collected in November–December 2004 and May–June 2006 aboard the *ASRV Laurence M. Gould.* Adult specimens were taken with a Blake trawl, wire dredge, or epibenthic sled and larval specimens were collected using a 0.75 m conical net with a 250 μm mesh and towed for 20 min in an oblique decent to a depth of approximately 180 m and a then returned to the surface. Additionally, H. W. Deitrich (via S. J. Lockhart) provided adult samples from an expedition on the *R/V Nathanial Palmer* in 2004. Nemertean worms were preserved either in >70% ethanol or were frozen at −80°C and transported to Auburn University. For this investigation, 192 individuals (123 adults; 69 larvae) were collected from 53 sampling locations (41 adult sampling locations; 12 larval sampling locations) along the Western Antarctic Peninsula and sub-Antarctic islands (Fig. 1). Supplementary Table 1 lists all samples used in this study, locality information, depth and GenBank accession numbers. When possible, adult specimen vouchers for individuals with novel sequences reported by this study have been deposited at the Smithsonian Institution National Museum of Natural History (see Genbank accession numbers). However, morphological identifications were not made *a priori* because of a lack of identifiable characteristics in the preserved materials (see Supplementary Material of Thornhill et al. 2008). Due to their small size, larvae were unavoidably destroyed during the DNA extraction process (see below). Supplementary Table 2 presents sample information for additional nemertean sequences obtained from GenBank (http://www.ncbi.nlm.nih.gov).

Molecular techniques

For adult nemertean samples, DNA extractions were performed using the Qiagen DNeasy® Tissue Kit (Qiagen Inc., Valencia, CA, USA) following the manufacturer's recommendations. For larval samples, single pilidium larvae were subjected to whole genome amplification using a GenomiPhi Kit (GE Healthcare) following the manufacturer's recommendations without prior DNA extraction; the first heating step of the protocol sufficiently lysed cells and liberated DNA. After whole genome amplification, larval DNA was diluted 1:50 with sterile distilled/deionized water.

For both adult and larval samples, a ~500 bp fragment of the mitochondrial 16S rRNA (16S) gene was amplified using the primers 16SarL (Palumbi et al. 1991) and LR-J-12887 (Simon et al. 1994) and a reaction cocktail consisting of 0.75 U Taq polymerase and 10× PCR buffer (Eppendorf), 2.5 mM $Mg(OAc)_2$, 10 nmol of each dNTP, DNA template and water to 25 μl. The PCR cycling program included a 2 min incubation at 95°C, followed by 40 cycles of 94°C for 30 s, 45°C for 1 min, 68°C for 1 min, and a final extension of 68°C for 7 min. Amplified PCR products were gel purified using a Qiagen QIAquick® Gel Extraction Kit (Qiagen Inc., Valencia, CA, USA) following the manufacturer's recommendations.

Purified PCR products were bi-directionally sequenced on a Beckman CEQ8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA, USA). Resulting sequences were assembled and screened using Sequencher 4.6 (Gene Codes Corporation, Ann Arbor, MI, USA). Sequences were then aligned using BioEdit 7.0.1 (Hall 1999) and manually corrected by eye using Se-Al v2.0a11 (http://tree.bio.ed.ac.uk/software/seal/) and MacClade v4.06 software

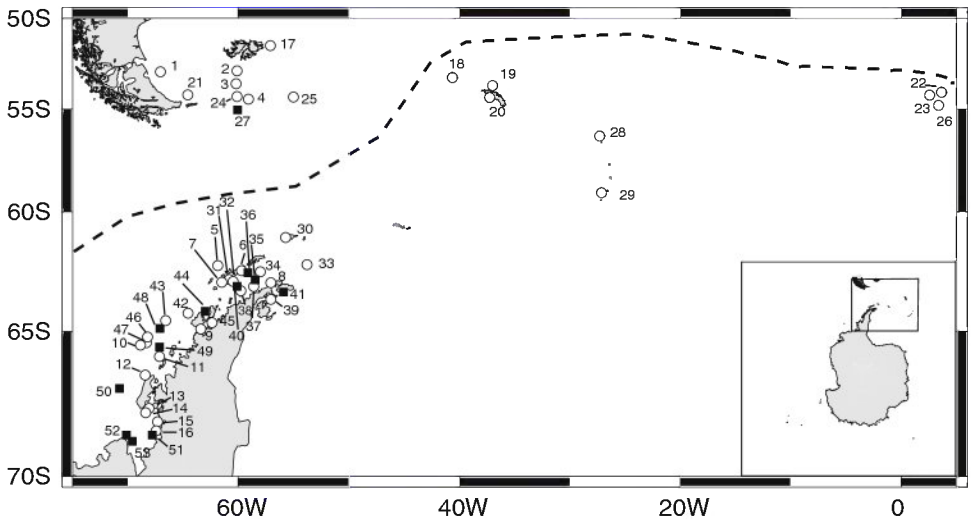

Fig. 1 Map of sampling sites for nemerteans included in this study. *Open circles* represent sites where adults were collected ($n = 41$ stations) and *black squares* indicate sites where pilidium larvae were collected ($n = 12$ stations). Sites are numbered to correspond with information included in Supplementary Table 1. The *dashed line* indicates the approximate position of the Antarctic Polar Front (APF) as denoted by Moore et al. (1999)

(Maddison and Maddison 2000). All novel sequences were deposited in GenBank (Accession numbers EU194791-EU194801, EU718358-EU718469). Alignments used in this study are also available at Treebase (http://www.treebase.org).

Data analyses

Nucleotide content was calculated in MEGA 3.1 (Tamura et al. 2007) and uncorrected genetic distances (p) were calculated using PAUP*4.0 (Swofford 2003) for the 16S dataset. Estimates of genetically distinct lineages of Antarctic nemerteans were calculated by first identifying the average genetic distance for 16S rDNA between species within a given genus previously reported in GenBank. We then calculated percent sequence divergence for each novel larval and adult Antarctic nemertean 16S sequence in the dataset relative to other samples. The use of uncorrected distances is conservative, as a correction model will only increase the percent divergence.

To confirm such groupings were monophyletic, we reconstructed the evolutionary history of all nemerteans with available 16S rDNA sequence data. Topologies were produced with Bayesian inference using MRBAYES 3.1.2 (Huelsenbeck and Ronquist 2001) implementing the nucleotide substitution model GTR + I + Γ as indicated by MRMODELTEST 2.2 (Nylander 2004). Two sets of four chains (3 hot, 1 cold) were run for 2×10^6 generations and sampled every 1,000 generations. Unalignable characters, 140 in total, were excluded from analysis (listed in TreeBase file). Stationarity was evaluated by examining log-likelihood values per generation and the first 5.0×10^4 generations were discarded as *burn-in*. A majority-rule consensus (50%) tree was calculated from the remaining 1951 trees and nodal support values (posterior probabilities) were obtained to assess reliability of the recovered nodes.

Results

The final dataset for this study consisted of 295 16S rRNA gene sequences, including 69 larval sequences and 123 adult sequences newly collected from the Antarctic Peninsula and 103 sequences derived from GenBank (Supplementary Tables 1 and 2). The dataset consisted of 510 characters, of which 369 were variable and 315 were parsimony informative. Mean sequence composition (nucleotide content) for the entire dataset was A = 30.4%, T = 36.8%, G = 20.2%, C = 12.7%.

For previously published nemertean 16S sequence data in GenBank, the mean uncorrected genetic distance for 16S rDNA between species within a described genus was found to be ~4.8% ($p = 0.048$). Based on this information, an uncorrected genetic distance of 0.05 (5%) was used as an approximate estimate of 'species' level designations for Antarctic nemerteans collected herein. Although previous work has shown 16S mtDNA sufficiently variable to assign taxonomic status in some groups of organisms (e.g., Vences et al. 2005; Moura et al. 2007), it is noted to be more conserved in nemerteans and other taxa than in the commonly used barcoding gene cytochrome c oxidase I (COI) (Thornhill et al. 2008; Govindarajan et al. 2005; Hebert et al. 2003; Vences et al. 2005; Wilson et al. 2009), resulting in more conservative diversity estimates than COI data.

Sequence comparisons yielded 19 distinct, previously uncharacterized lineages of Antarctic nemerteans with an uncorrected $p > 0.05$ to their nearest sister lineage (collection information of these lineages is provided in Table 1). These results were corroborated by the Bayesian inference topology (Fig. 2). Of the 19 clades recovered, 11 were restricted to single collection locations, whereas three clades were found at four or more locations (Table 1; Fig. 1; Supplementary Table 1). Adult nemerteans sequenced here were found in 16 distinct clades (including *P. corrugatus*) and larval samples were comprised of five clades. Only *P. corrugatus* was found to contain both larval and adult nemertean samples. Previously uncharacterized genetic lineages are designated as A–S on the Bayesian topology (Fig. 2)—six lineages are within Hoplonemertea and 13 within heteronemertea. Only pilidium larvae were sampled from the plankton, and, as expected, these are all heteronemerteans.

Only three of 69 total larval samples were designated *P. corrugatus* larvae, because they were <1% different from adult *P. corrugatus* 16S rDNA sequences. Samples for the four remaining larval clades did not correspond to any adult samples. A total of 15 genetically distinct lineages of adult nemerteans, four lineages of larval nemerteans, and one of both (*P. corrugatus*) were detected, indicating a disparity between the abundance of adult and larval pools sampled in this investigation. As *P. corrugatus* is the only nemertean from Antarctic waters with previously published sequence data, the unidentified lineages either correspond to those not previously reported in GenBank and/or represent newly discovered species.

Discussion

Nemerteans appear to be considerably more diverse in the Southern Ocean (Dawson 1969, 1971; Gibson 1985b) than previously recognized. Mitochondrial 16S rRNA genes uncovered 19 distinct and previously uncharacterized molecular lineages of nemerteans. By comparison, previous studies of the regions sampled for this investigation yielded

Table 1 Clades recovered in the Bayesian analysis

Clade	# Individuals	# Sites collected	Depth(s) (m)	Adults/larvae
A	1	1	400	A
B	1	1	368	A
C	1	1	261	A
D	7	4	125–238	A
E	1	1	192	A
F	1	1	192	A
G	1	1	188	A
H	3	2	125–334	A
I	5	2	334–400	A
J	1	1	334	A
K	1	1	–	A
L	2	2	170–490	A
M	4	3	0–200	L
N	1	1	0–200	L
O	2	2	117–192	A
P	3	1	232	A
Q	9	7	200–334	A
R	1	1	0–200	L
S	60	10	0–200	L
Parborlasia corrugatus	86 (83 adult, 3 larvae)	30, 1	Adult: 5–440, larvae: 0–180	A, L

Number of individuals, adults and larvae, in each clade, the number of sites where each were found, and the depth of the collections are provided

about 19 and 17 named species of hoplo- and heteronemerteans, respectively (calculated from Gibson 1995). Although no formal extrapolation can be made, the likelihood that these 19 genetic clades primarily represent known species, or even a substantial portion of the available diversity, is low. The sampling regime used in this study focused on a limited portion of Antarctic habitats that included benthic habitats roughly 400–1,000 m in depth. Also, sampling of plankton was limited both spatially and temporally. Thus, our sampling and sequencing methods likely represent a minimal estimate of nemertean biodiversity from the region.

This study also finds two clades of nemerteans (H and S; Fig. 2a, b) that bridge the Drake Passage across the Antarctic Polar Front (APF). This differs from the results of Thornhill et al. (2008), who found that for *P. corrugatus* has distinct evolutionary lineages on either side of the Drake Passage with the APF acting as a relatively recent barrier to open-ocean dispersal from South America to Antarctica (and vice versa), although dispersal events have occurred since its establishment over 20 million years ago.

Despite the lack of a focused effort to effectively survey the group in this region, Gibson (1985b) provides the most recent taxonomic understanding for Antarctic nemerteans. He invalidated four species and erected seven new species of heteronemerteans based on specimens collected more or less incidentally in various National Science Foundation (NSF)-sponsored research projects in the United States Antarctic Program (USAP) during the 1960s through 1970s. Additionally, Gibson (1985b) concluded that more than half of the species he accepted as valid could be considered sparsely or poorly known. In fact, vouchers are available for few of these species. About 25 of the 42–45 potentially valid species from the Southern Ocean were described prior to 1915, and 19 species appear to have been recorded only once. Taken together, a robust collecting program is wanting.

Whereas multi-gene phylogenetic analyses of Thollesson and Norenburg (2003) show important topological differences with results presented here based only on 16S rDNA, some results deserve special note. *Baseodiscus* is one of very few species-rich genera that is consistently monophyletic, with membership based on (almost) unambiguous morphology. Many of its species tend to be large in size and to have large geographic distributions. *Baseodiscus* is sister here to a clade comprising three genetically distinct lineages of adults discovered in this study (Fig. 2b). Only one species, *Baseodiscus antarcticus* Baylis, 1915, has been described from the Southern Ocean, and only from western Antarctic waters outside the range of our study. Other topological results for heteronemerteans must be seen in the context of more than 450 species of heteronemerteans, of which about half are allocated to three genera

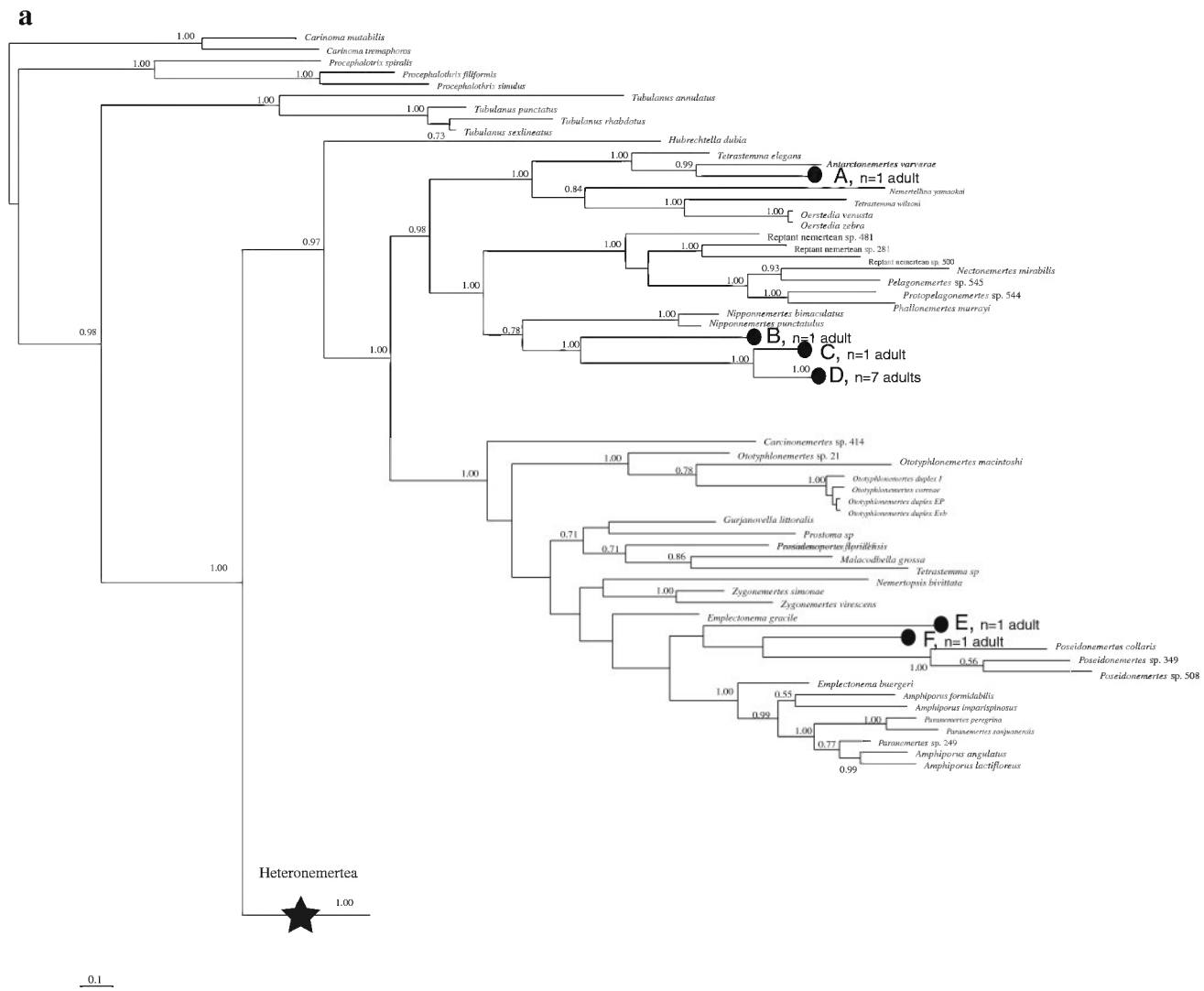

Fig. 2 Bayesian majority rule consensus (50%) tree. *Black dots* represent clades of unidentified Antarctic nemertean larvae and adults, labeled Clades A–S. The clade represented by the *star symbol* consists of all members of the Heteronemertea included in the study and represents the connection point between parts a and b. The *open triangle* represents the *Parborlasia corrugatus* clade that contains three larvae nested within 83 adults

now widely recognized to be para- and polyphyletic and should be investigated in future studies (Schwartz and Norenburg 2001).

In most marine benthic habitats, conspicuous hoplonemerteans tend to be rare. Many species in the hoplonemertean family Cratenemertidae, however, can be relatively conspicuous because of relatively large size. Thus not surprisingly, three of the six hoplonemertean clades discovered in this study likely fall into a cratenemertid clade, as indicated by a sister relationship with *Nipponnemertes* species. Gibson (1995) lists as valid four Southern Ocean species of benthic Cratenemertidae, all *Nipponnemertes*, but Gibson and Crandall (1989) flagged several *Amphiporus* species they suspected to be cratenemertids. This family appears to be unusually diverse in the Southern Ocean. In fact, Crandall (in litt.) has recovered from NSF-USAP hoplonemerteans what he considers to be at least four additional species of benthic Cratenemertidae.

To our knowledge, no Southern Ocean specimens for anatomical study were ever properly relaxed prior to preservation, which makes critical comparative histology very difficult. This considerably clouds validity and synonymy of named species (e.g., Thornhill et al. 2008). Existing taxonomy is further complicated by the unsurprising presence of morphologically cryptic species (Thornhill et al. 2008). Most nemerteans are modestly to highly cryptic in nature, commonly living in various substrata or hiding among encrusting fauna and flora, and much diversity is likely to be overlooked in general surveys unless a nemertean specialist is present. No nemertean specialist participated in the

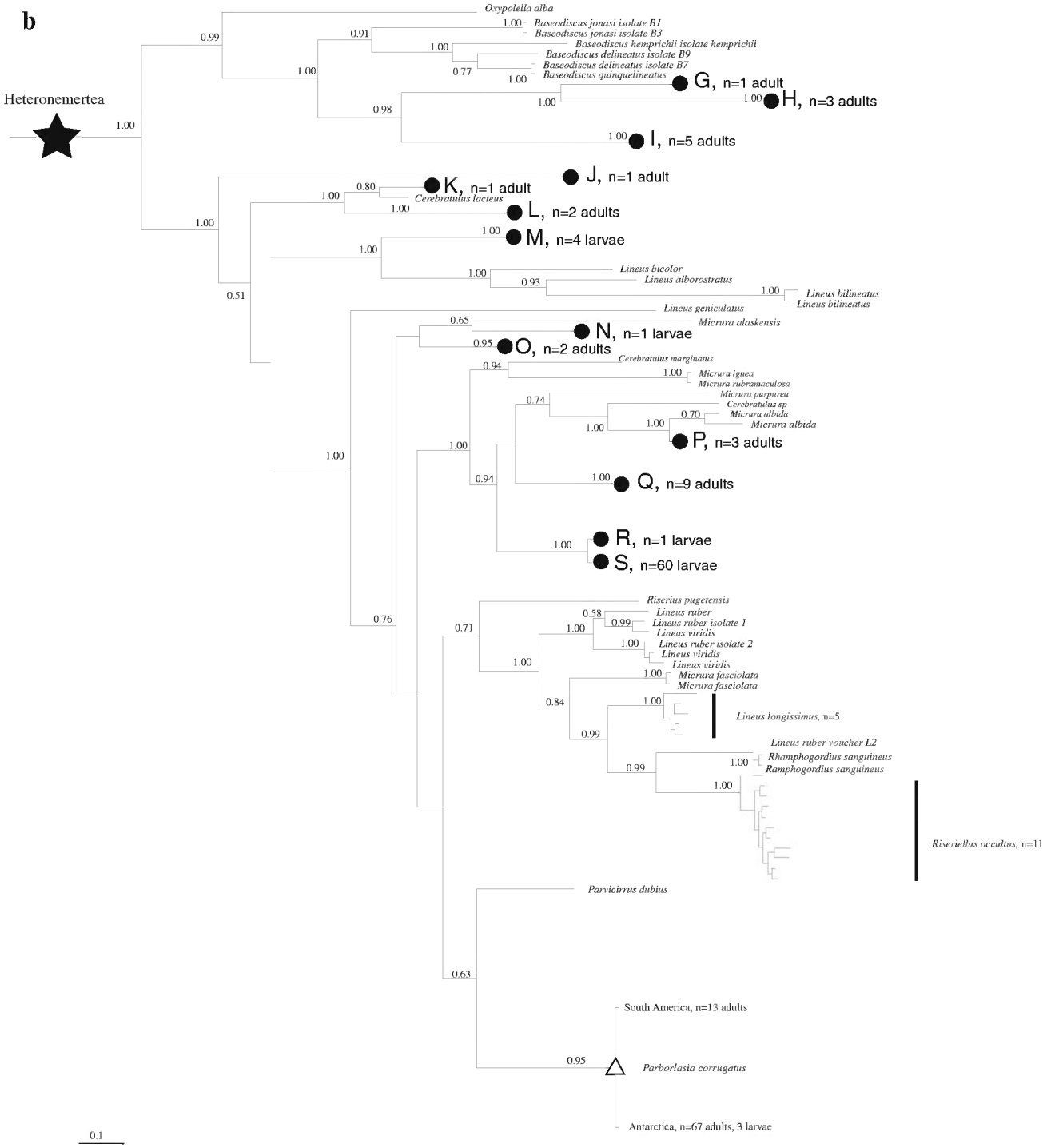

Fig. 2 continued

collections for this study or those for previous taxonomic work derived from Southern Ocean nemerteans. These factors impose unknown limits on assessing the diversity of Southern Ocean nemerteans based on existing collections and morphology, but the relatively superficial nature of sampling suggests that the current number of named species is a severe underestimate. Morphological vouchers (when available) for named species and vouchers from this study, regrettably, lack suitable diagnostic, external species-level characteristics to match them to each other with any useful level of confidence. Histological studies may help identify some specimens, but such studies require funding and long-term commitment. More targeted collection, with proper field-annotation and preservation of

specimens are much more useful for assessing morphological diversity.

Adult versus larval diversity

The reported ecological dominance of *P. corrugatus* in the region (see Rogers et al. 1998) is not reflected in composition of the larval populations sampled in this investigation. Additionally, locations of adults producing larvae collected and analyzed in this investigation are not known. *P. corrugatus* is often one of the most visible and recognized invertebrates in shallow Southern Ocean habitats, a visibility almost unknown for other nemerteans anywhere in the world. For this reason, the highly abundant pilidium larvae in the Southern Ocean plankton were commonly attributed to *P. corrugatus* (e.g., Rogers et al. 1998). Even when seasonal variation is taken into account, the findings of this study suggest that the abundance of *P. corrugatus* larvae has been overestimated in previous studies.

Though limited, our current knowledge suggests that a planktonic pilidium characterizes a large majority of heteronemertean (and some paleonemertean) species (Norenburg and Stricker 2001). Hence, discovering four additional clades of larvae is not surprising and is in keeping with similar results elsewhere in the world (JLN, unpublished observation). The significance of these additional larval clades is their absence among the clades of adult nemerteans collected in this study, and reinforces the suggestion that our sampling of adult nemertean diversity was relatively inefficient. Many hoplo- and paleonemerteans also have planktonic larvae, but these tend to be planuliform (Norenburg and Stricker 2001) and therefore difficult to identify visually as nemerteans in bulk-preserved samples. We would expect them, however, to be recognized in environmental genetic surveys of entire plankton samples.

Collections for this investigation were made during November–December 2004 and May–June 2006. Thus, if some species reproduce at other times of year their larvae may have been overlooked with our sampling regime. However, previous reports have noted that Antarctic nemertean reproduction likely occurs either over prolonged periods or aseasonally throughout the year (Pearse et al. 1991; Schreeve and Peck 1995; Stanwell-Smith et al. 1999). Schreeve and Peck (1995) also note that nemertean samples collected during November–December in the Bellinghausen Sea displayed a wide variety of developmental stages. Furthermore, the planktonic larval collections were from oblique tows sampling a 0–200 m depth range. If nemertean larvae are stratified across different depths in the water column, certain taxa would undoubtedly be missed by our sampling efforts. These limitations, again suggest that more comprehensive surveys from other locations, time periods, and plankton depths may reveal greater species diversity than observed here.

Implications

When considering the effects of climate change on Antarctic fauna, previous studies demonstrated that some Antarctic benthic organisms experienced a 50% failure in essential biological activities (e.g., loss of aerobic capacity) when temperature changed by 2–3°C, whereas a 5°C change causes a complete loss of biological function (Peck et al. 2004). Additionally, studies have shown that small temperature increases can decrease embryonic developmental times in certain Antarctic organisms (e.g., Bosch et al. 1987). Thus, the increasing temperatures along the Antarctic Peninsula and the lack of many organisms' ability to adapt or adjust life history traits could eventually lead to population or species loss (Peck et al. 2004). These changes could dramatically affect levels of nemertean biodiversity in the region.

Furthermore, increasing temperatures along the Antarctic Peninsula could result in removal of physiological barriers that prevent invasions by non-native species (e.g., Aronson et al. 2007; Thatje et al. 2008). Studies in the Southern Ocean have documented increasing numbers of non-indigenous species in recent years, including higher order predators, which could significantly alter the Southern Ocean ecosystem (Aronson et al. 2007). For example, Thatje and Fuentes (2003), Thatje and Arntz (2004) and García Raso et al. (2005) documented the occurrence and/or recent introduction of anomuran and brachyuran crabs, animals that typically occur in more temperate waters, along the Antarctic Peninsula. Increasing the number of top predators in the region could again cause dramatic changes to Antarctic benthic communities. Thus, to understand the impacts of future climate change on the organisms of area, current biodiversity of the Antarctic Peninsula must be thoroughly documented, including undescribed, or hitherto unknown, taxa. Explorations of species diversity in the Southern Ocean is of immediate importance, as climate shifts and invasive species could cause a loss of species before scientists have a chance to discover, describe, and study them.

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