On the identity of two Antarctic brooding nemerteans: redescription of *Antarctonemertes valida* (Bürger, 1893) and description of a new species in the genus *Antarctonemertes* Friedrich, 1955 (Nemertea, Hoplonemertea)

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Abstract Nemerteans (ribbon worms) constitute an abundant and occasionally conspicuous group of benthic invertebrates in the Southern Ocean. Although recent work has confirmed that this group is far more diverse than previously recognized, the Antarctic nemertean fauna remains poorly understood when compared to other geographic regions. In most cases, the taxonomic information on the known nemertean fauna is incomplete for this region and/or has been inappropriately documented. As a consequence, many of the species described are considered *species inquirendae*. Among the nearly 50 species described so far for the Southern Ocean, two hoplonemerteans are known to brood eggs in cocoons: *Amphiporus incubator* Joubin, 1914 and *Amphiporus michaelseni* Bürger, 1895a. Here, we redescribe *Antarctonemertes valida* (Bürger, 1893), a senior synonym of *A. michaelseni*, and describe a new congeneric species, *Antarctonemertes riesgoae* sp. nov. Both species show a similar reproductive strategy by brooding their cocoons, and similar external appearance, but clearly differ in other aspects of their morphology, such as the cephalic coloration pattern and the number of proboscidial nerves. We provide novel information about their life habitus, reproductive behaviour, internal anatomy, and their phylogenetic placement within hoplonemerteans using one nuclear (28S rRNA) and two mitochondrial [cytochrome *c* oxidase subunit I (COI) and 16S rRNA] markers. We also provide a parsimony haplotype network using 16S rRNA, COI, and the internal transcribed spacer region 2 (ITS-2) showing a clear distinction between individuals of both species. Our results stress the need of combining molecular and morphological information when dealing with closely related species of nemerteans.

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Introduction

Ribbon worms constitute an important component of the Southern Ocean benthic fauna. Although these organisms can be unusually abundant and occasionally conspicuous in the Antarctic region, only ca. 50 species, from the more than 1,200 nemerteans described worldwide (Kajihara et al. 2008), have been described to date. From these 50

Antarctic species, most are endemic or have been recorded just once (Gibson 1995). In spite of this, a recent study by Mahon et al. (2010) using DNA sequence data from larval and adult nemerteans collected in the western Antarctic Peninsula concluded that this phylum is considerably more diverse in Antarctic waters than previously recognized.

As it happens for other geographic regions, the taxonomical information from the Antarctic species described so far is scarce and/or incomplete, with the exception of a few examples (e.g. *Parborlasia corrugatus* (McIntosh, 1876); Gibson 1983). This taxonomic information is mainly based on histology and often lacks or has been inadequately documented for many Antarctic nemerteans. In other cases, species with a well-documented internal anatomy are poorly known with respect to their external appearance, an important aspect in a group where preservation artefacts are often induced after fixation (Gibson 1995). This has contributed to the uncertainty about the validity of many of the currently known Antarctic species, altogether with the fact that many vouchers are not available (e.g. Gibson 1985, 1995; Gibson and Crandall 1989). There is a lack of a desirable integrative approach for most of the species, which may include not only detailed information about the histology (Gibson 1985), but also data using multiple and complementary sources such as comparative morphology, genetics, behaviour, and development, among others (Sundberg et al. 2009a).

Most of the currently known Antarctic nemerteans have been described from shallow waters, with several members belonging to Heteronemertea (e.g. *P. corrugatus*, as one of the most conspicuous and frequent species) and Hoplonemertea. Among hoplonemerteans, there are two brooding species whose females take care of their eggs inside cocoons presumably built by themselves [*A. incubator* Joubin, 1914 and *Antarctonemertes valida* (Bürger, 1893)], a reproductive strategy unique in this phylum (see a review on nemertean reproductive strategies in Thiel and Junoy 2006). It is the aim of this study to contribute to the taxonomy and ecology of two Antarctic brooding hoplonemerteans. Firstly, we redescribe *A. valida*, a species originally described as *Tetrastemma validum* Bürger, 1893 collected in the South Georgia Island (Antarctica) and insufficiently described (Bürger 1893). Later, Joubin (1908, 1914) assigned to *A. michaelseni* Bürger, 1895a, a species originally described from the Chilean waters of Punta Arenas, in the Strait of Magellan, specimens collected in islands along the western Antarctic Peninsula. These specimens are similar in form, coloration, and reproductive behaviour to the specimens here redescribed as *A. valida*. Secondly, we describe a new species, *Antarctonemertes riesgoae* sp. nov., resembling *A. valida* in its reproductive strategy and external appearance, but the species differ in several aspects, including the distinctive cephalic coloration and

the number of proboscidial nerves. For both species, we provide information on their life habitus, reproductive behaviour, internal anatomy, and phylogenetic placement using one nuclear (28S rRNA) and two mitochondrial [cytochrome *c* oxidase subunit I (COI) and 16S rRNA] markers in a phylogenetic tree containing 33 terminal taxa. We also provide a parsimony haplotype network including all samples. Finally, following the standardized approach suggested by Sundberg et al. (2009a), we provide a character matrix for *A. riesgoae* sp. nov.

Materials and methods

Sample collection

Specimens of *A. valida* and *A. riesgoae* sp. nov. were collected on scuba from two areas in Port Foster, Deception Island (South Shetland Islands, Antarctica): (1) in front of the "Gabriel de Castilla" Spanish Antarctic Base, (Fig. 1c; Sta. 1) and (2) in front of Colatinas' area (Fig. 1c; Sta. 2). Additional material of *A. riesgoae* sp. nov. was also collected from intertidal rocks at Fildes Bay, King George Island (South Shetland Islands, Antarctica) (Fig. 1b).

Nemerteans from both species collected in Port Foster were examined alive before and after anaesthetization in 7 % MgCl$_2$ in distilled water, and photographed with a camera (Invenio 5S 5MPixel CMOS) adapted to a stereomicroscope (Zeiss Stemi 2000-C). Prior to preservation, pH was measured in the external secretion in specimens of the two species by using Merck coloured testing stripes. Organisms were preserved for standard morphology and DNA analysis. Material collected from King George Island was photographed alive, and preserved in 70 % EtOH.

Morphological analysis

Organisms for standard morphology were fixed in 10 % buffered formalin in seawater, subsequently dehydrated in a graded series of ethanol, later transferred to toluene, embedded in 56 °C paraffin wax, sectioned at 6 μm, and stained with the Mallory trichrome method for histological examination (Pantin 1960).

Type material and vouchers for the sequencing are deposited in the Department of Invertebrate Zoology, Museum of Comparative Zoology (MCZ), Harvard University (Cambridge, Massachusetts, USA), and are accessible through the MCZbase portal (http://mczbase.mcz.harvard.edu). All animals not deposited as type material are retained in the Centre of Biodiversity Resources (CRBA), University of Barcelona (Spain), and in the first author's collection at the Departament de Biologia Animal, Universitat de Barcelona.
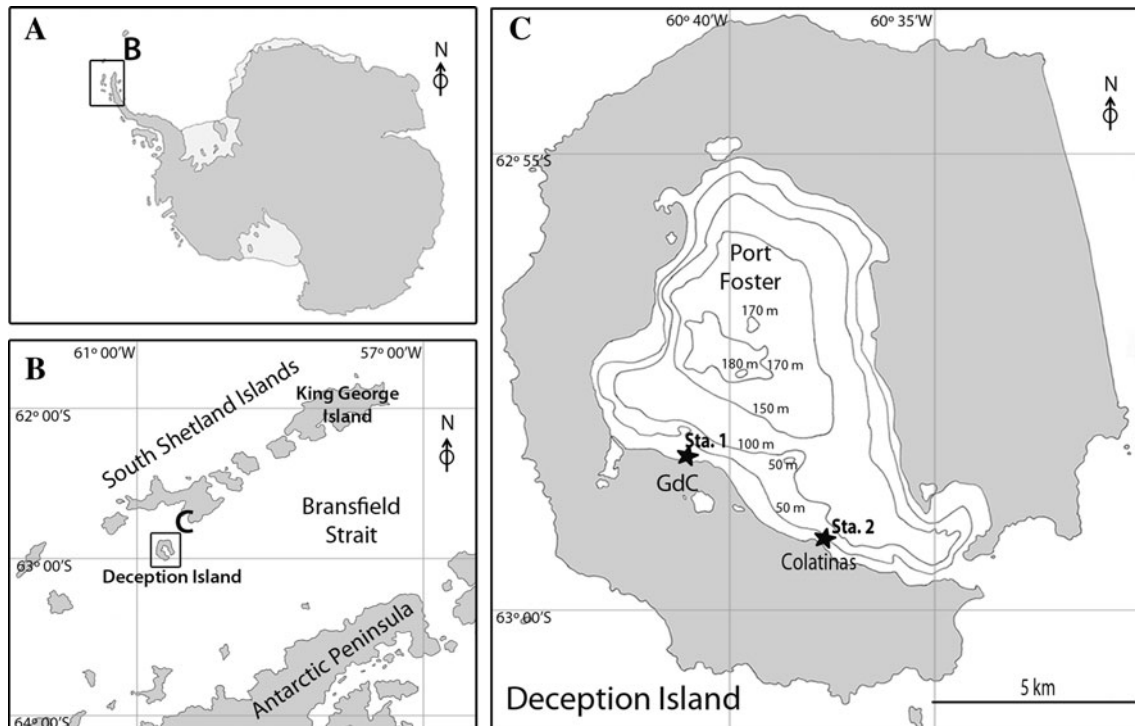
Fig. 1 a Map of Antarctica showing sampling localities, **b** South Shetland Islands area showing location of Deception and King George Islands, **c** deception Island. GdC, "Gabriel de Castilla" Spanish Antarctic Base; *Sta. 1*, station where specimens were collected on rocks; *Sta. 2*, station where specimens were collected on *R. coccocarpa*

DNA analysis

Organisms for DNA sequencing were preserved in 95 % EtOH, and stored at −20 °C. Total genomic DNA was extracted from seven ethanol-fixed specimens for *A. valida* (MCZ IZ-134228; DNA106278), and from seven ethanol-fixed specimens for *A. riesgoae* sp. nov. (MCZ IZ-134229; DNA 106468) using the DNeasy blood and tissue kit (Qiagen, Valencia, CA), following the protocol described by the manufacturer. The mitochondrial protein-encoding gene cytochrome *c* oxidase subunit I (COI) as well as the 16S rRNA, the partial nuclear gene 28S rRNA, and the internal transcribed spacer region 2 (ITS-2) were amplified using the primers listed in Table 1. PCR mixtures and temperature profiles used for each fragment are listed in Table 2. After cycling, the reaction was completed with an extension phase at 72 °C for 10 min, and the reaction products were visualized in a 1 % agarose gel and purified through enzymatic reaction with ExoSAP-IT (USB Corporation, Cleveland, OH).

The purified PCR products were sequenced directly with the same primer pairs used for amplification. Each sequence reaction contained a total volume of 10 µl including 3 µl PCR products, 3.2 µM PCR primer, 0.25 µl ABI BigDye 5× sequencing buffer, and 0.5 µl ABI Big-Dye Terminator v3.0 (Applied Biosystems, Foster City,

CA). The sequencing reactions involved an initial denaturation step for 3 min at 95 °C, and 25 cycles (95 °C for 10 s, 50 °C for 5 s, and 60 °C for 4 min). The BigDye-labelled PCR products were cleaned using Performa DTR Plates (Edge Biosystems, Gaithersburg, MD), and the sequencing reaction products were analysed using an ABI Prism® 3730 Genetic Analyzer (Applied Biosystems).

Chromatograms were edited and overlapping sequence fragments were assembled using Sequencher 4.8 (Gene Codes Corporation, Ann Arbor, MI). Blast searches (Altschul et al. 1997), as implemented in the NCBI website (http://www.ncbi.nlm.nih.gov/), were conducted to check for putative contaminations. In total, four data sets were analysed and MEGA 5.0.3 (Tamura et al. 2011) was used to edit the sequences while Mesquite 2.74 (Maddison and Maddison 2010) was used to concatenate the different nucleotide sequences to form the combined matrix. All new sequences have been deposited in GenBank (see accession numbers in Table 3).

The multiple sequence alignments for each gene were performed using MAFFT v.6 using the strategy G-INS-i (Katoh et al. 2005). The hoplonemerteans *Nectonemertes mirabilis* Verrill, 1892, *Nipponnemertes pulchra* (Johnston, 1837), and *Nipponnemertes* sp. were chosen as putative outgroups in all analyses. The MODELTEST 3.06 (Posada and Crandall 1998) module in HyPhy (Pond et al. 2005)

Table 1 Primers used in this study

Primer	Sequence 5′–3′	References
16S arL	CGCCTGTTTATCAAAAACAT	Palumbi et al. (1991)
16S brH	CCGGTCTGAACTCAGATCACGT	Palumbi et al. (1991)
LCO1490	GGTCAACAAATCATAAAGATATTGG	Folmer et al. (1994)
HCO2498	TAAACTTCAGGGTGACCAAAAAATCA	Folmer et al. (1994)
ITS-2R	ATGCTTAAATTTAGGGGGTAGTC	Nicolas A.N., pers. comm.
ITS-2F	TGTGAACTGCAGGACACATGAA	Nicolas A.N., pers. comm.
28S rd1a	CCCSCGTAAYTTAGGCATAT	Edgecombe and Giribet (2006)
28S rd5b	CCACAGCGCCAGTTCTGCTTAC	Whiting (2002)

Table 2 PCR conditions

Fragment	PCR programme	PCR mixture
16S arL/16S brH	94 °C/180 s − (94 °C/30 s − 40 °C/30 s − 65 °C/180 s) × 49 cycles − 65 °C/7 min	PCRmix1[a]
LCO1490/HCO2198	94 °C/90 s − (94 °C/45 s − 36 °C/60 s − 72 °C/60 s) × 33 cycles − 72 °C/5 min	PCRmix1[a]
ITS-2R/ITS-2F	94 °C/90 s − (94 °C/45 s − 48 °C/60 s − 72 °C/60 s) × 33 cycles − 65 °C/5 min	PCRmix1[a]
28S rD1a/28S rD5b	94 °C/300 s − (94 °C/30 s − 42 °C/30 s − 72 °C/50 s) × 35 cycles − 65 °C/7 min	PCRmix2[b]

[a] 17 μl ddH$_2$O, 0.2 μl each primer (10 μM), 0.1 AmpliTaq® 360 DNA Polymerase (Invitrogen, CA, USA), 2 μl PCR buffer (10×), 0.4 μl dNTP's, 2 μl DNA template

[b] 16 μl ddH$_2$O, 0.2 μl each primer (10 μM), 0.1 AmpliTaq® 360 DNA Polymerase (Invitrogen, CA, USA), 2 μl DNA template (10×), 0.4 μl dNTP's, 1 μl DNA template

was used to select the best-fit model of molecular evolution for our data set under the Akaike information criterion (Akaike 1974). A general time reversible model (GTR) was selected for both nuclear and mitochondrial genes with a discrete Gamma (Γ) distribution to model rate heterogeneity (GTR + Γ). For the phylogenetic approach, we used the mitochondrial genes COI and 16S rRNA and the nuclear 28S rRNA. Due to the lack of hoplonemertean ITS-2 sequences in GenBank, this region was only used for estimating the haplotypic network. The maximum likelihood criterion was performed with Randomized Axelerated Maximum Likelihood (RAxML) v.7.0.4 (Stamatakis 2006; Stamatakis et al. 2008) using the new sequences obtained for *A. valida* and *A. riesgoae* sp. nov. with sequences from other hoplonemerteans obtained from GenBank (Table 3), but largely generated in the Giribet laboratory. The three markers COI, 16S rRNA, and 28S rRNA were concatenated for phylogenetic analysis. The search for the optimal maximum likelihood (ML) trees was performed on the research computing cluster facility from the Faculty of Arts and Sciences located at Harvard University. The ML tree search was conducted by performing 200 distinct runs using the default algorithm of the programme for random trees (option -d) as a starting point for each run and the same parameters were applied for all genes. The final tree was determined by a comparison of likelihood scores under the GTR + Γ model among suboptimal trees obtained per run. One thousand fast bootstrap replicates were conducted to evaluate nodal support. Bootstrap values >70 % were considered to indicate strong support, given that bootstrap values appear to be biased but are conservative measures of phylogenetic support (Felsenstein 2004).

Haplotype networks of the COI, 16S rRNA, and ITS-2 were inferred using statistical parsimony (Templeton et al. 1992), as implemented in the programme TCS v1.21 (Clement et al. 2000). Networks are known to be more appropriate than hierarchical trees for representing intra-specific evolution (Posada and Crandall 2001), which is particularly important when analyzing closely related species. This method links haplotypes with the smallest number of differences as defined by a 95 % confidence criterion. Estimation of the genetic distance among both species was based on the Tajima-Nei distance for all markers separately (Tajima and Nei 1984) calculated in the MEGA 5.0.3 package.

Results

Morphological description

Genus *Antarctonemertes* Friedrich, 1955

Chernyshev (1999) provides a diagnosis for the genus.

Table 3 Taxa and specimens included in the molecular analysis with GenBank accession numbers

Taxa and specimens	28S rRNA	COI	16S rRNA	ITS-2
***Antarctonemertes riesgoae* sp. nov.**	**KC754975**	**KC754998**	**KC754987**	**KC755007**
	–	**KC754999**	**KC754988**	**KC755011**
	–	**KC754995**	**KC754982**	**KC755008**
	–	–	**KC754984**	**KC755012**
	–	–	**KC754981**	–
	–	**KC754996**	**KC754985**	**KC755010**
	–	**KC754997**	**KC754986**	**KC755009**
***Antarctonemertes valida* (Bürger, 1893)**	**KC754974**	**KC754991**	**KC754977**	**KC755002**
	–	**KC754992**	**KC754980**	**KC755004**
	–	–	**KC754978**	**KC755005**
	–	–	**KC754979**	**KC755006**
	–	**KC754990**	**KC754976**	**KC755001**
	–	**KC754993**	**KC754983**	**KC755003**
	–	**KC754994**	**KC754989**	**KC755000**
Amphiporus lactifloreus (Johnston, 1828)	HQ856876	HQ848611	JF277617.1	–
Amphiporus imparispinosus Griffin, 1898	HQ856878	HQ848612	JF277618.1	–
Antarctonemertes phyllospadicola (Stricker, 1985)	–	FJ594418	–	–
Antarctonemertes varvarae Chernyshev, 1999	AJ436845	AJ436900.1	AJ436790.1	–
Cyanophthalma obscura (Schultze, 1851)	–	EF208980.1	–	–
Diplomma polyophthalma (Gibson and Sundberg 2001)	–	AB505816.1	–	–
Diplomma serpentina Stimpson, 1855	AB505817.1	AB505819.1	–	–
Emplectonema gracile (Johnston, 1837)	JF293022	HQ848620	JF277621.1	–
Geonemertes pelaensis Semper, 1863	JF293017	HQ848592	JF277610.1	–
Gononemertes parasita Bergendal, 1900	JF293014	HQ848607	JF277606.1	–
Gurjanovella littoralis Ushakov, 1926	AJ436849.1	AJ436904.1	AJ436794.1	–
Nemertellina yamaokai Kajihara, Gibson and Mawatari, 2000	AJ436852.1	AJ436907.1	AJ436797.1	–
Nemertopsis bivittata (Delle Chiaje, 1841)	JF293021	HQ848608	JF277609.1	–
Oerstedia dorsalis (Abildgaard, 1806)	AY210465.1	AY210465.1	FJ855382.1	–
Oerstedia striata (Sundberg, 1988)	–	AY791972.1	–	–
Oerstedia venusta Iwata, 1954	AJ436856.1	AJ436911.1	AJ436801.1	–
Oerstedia zebra (Chernyshev, 1993)	AJ436857.1	AJ436912.1	AJ436802.1	–
Ototyphlonemertes correae Envall, 1996	JF293025	HQ848613	JF277612.1	–
Ototyphlonemertes macintoshi Bürger, 1895b	JF293024	HQ848605	JF277613.1	–
Paranemertes peregrina Coe, 1901	AJ436860.1	FJ594419.1	AJ436805.1	–
Prosorhochmus americanus Sánchez, 1973	JF293023	HQ848595	JF277619.1	–
Prosorhochmus nelsoni Sánchez, 1973	JF293013	HQ848606	JF277604.1	–
Tetrastemma candidum (Müller, 1774)	AB505827.1	AY791973.1	–	–
Tetrastemma elegans (Girard, 1852)	AJ436865.1	AJ436920.1	AJ436810.1	–
Tetrastemma longissimum Bürger, 1895b	–	AY791981.1	–	–
Tetrastemma peltatum Bürger, 1895b	–	AY791993.1	–	–
Tetrastemma wilsoni Coe, 1943	AJ436866.1	AJ436921.1	AJ436811.1	–
Vieitezia luzmurubeae Junoy, Andrade and Giribet, 2010	HQ443428	HQ443426	JF277607	–
Outgroups				
Nipponnemertes mirabilis Verrill, 1892	AJ436870.1	AJ436925.1	AJ436815.1	–
Nipponnemertes pulchra (Johnston, 1837)	JF293012	HQ848597	JF277625.1	–
Nipponnemertes sp.	JF293019	HQ848599	JF277624.1	–

Accession numbers for the new sequences in bold

– Not available

Antarctonemertes valida (Bürger, 1893) (Figs. 2, 3, 4)

   Tetrastemma validum Bürger, 1893; Wheeler (1934, 1940); Coe (1950)

   Amphiporus michaelseni: Joubin (1908, 1914); not Bürger (1895a)

   Antarctonemertes validum [sic.]: Friedrich (1955)

Sectioned material Female after oviposition, series of transverse sections, anterior part (NEM1D-S14); female after oviposition, series of transverse sections, anterior part (NEM3-SZ); juvenile, series of transverse sections, whole worm (NEM3-SY). NEM1D-S14, NEM3-SZ, and NEM3-SY collected in front of the "Gabriel de Castilla" Spanish Antarctic Base (62°58.587′S; 60°40.580′W), Deception Island (South Shetland Islands, Antarctica) (Fig. 1c; Sta. 1); Leg. S. Taboada, J. Cristobo, and C. Avila, 9 January 2010, under rocks at 1–2 m depth (Fig. 2a, b).
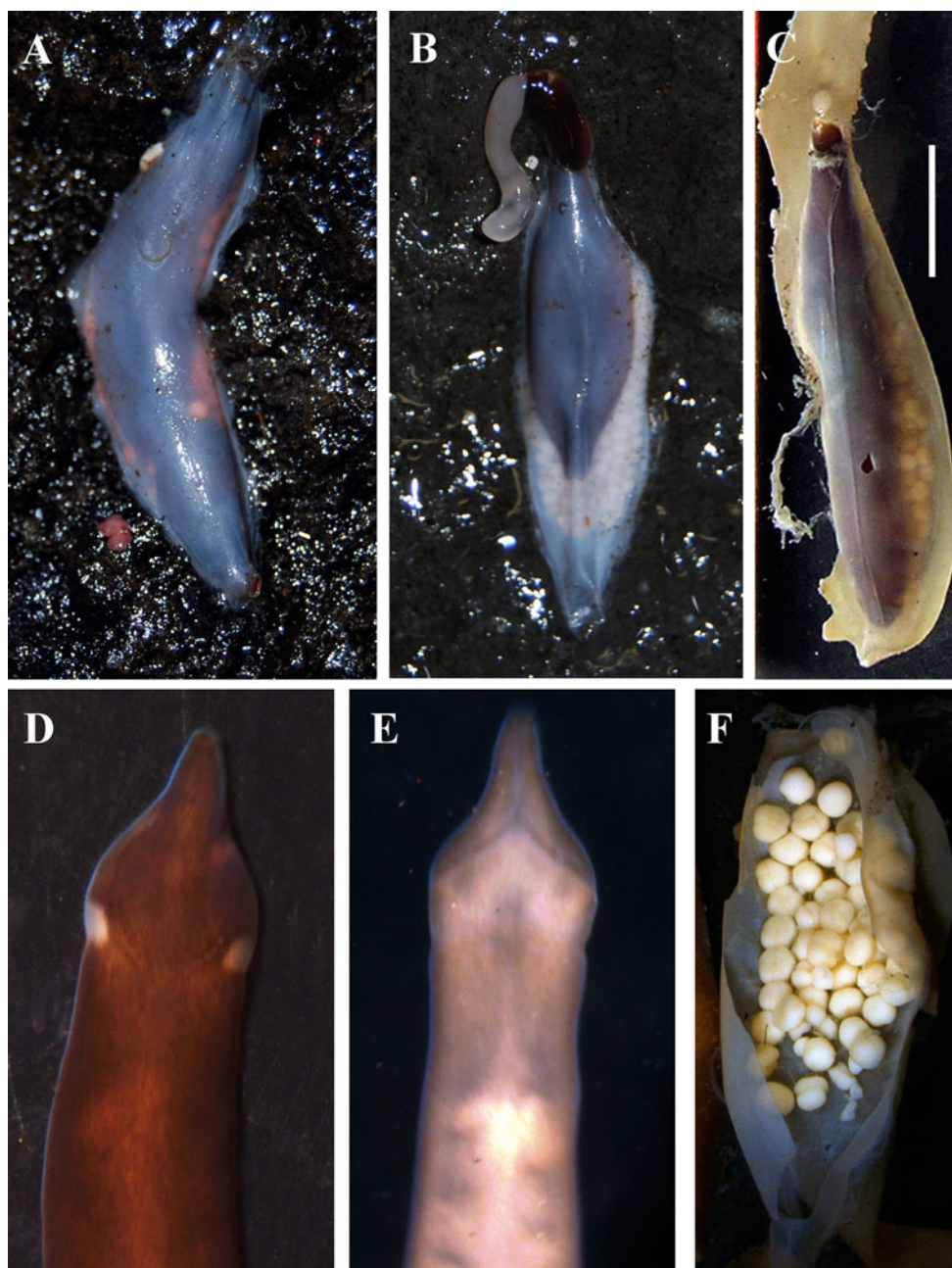
Fig. 2 *Antarctonemertes valida*. **a** Cocoon under a stone, with living female inside guarding the eggs, **b** female everting proboscis after being disturbed, **c** preserved cocoon attached to an alga with female inside, **d** dorsal view of cephalic region of a living female, showing the head shape and the two lateral white patches, **e** ventral view of the cephalic region of a living female, showing cephalic furrows, **f** preserved cocoon opened to show eggs, some of them at 4-cell embryo stage. *Scale bar* **c** 5 mm
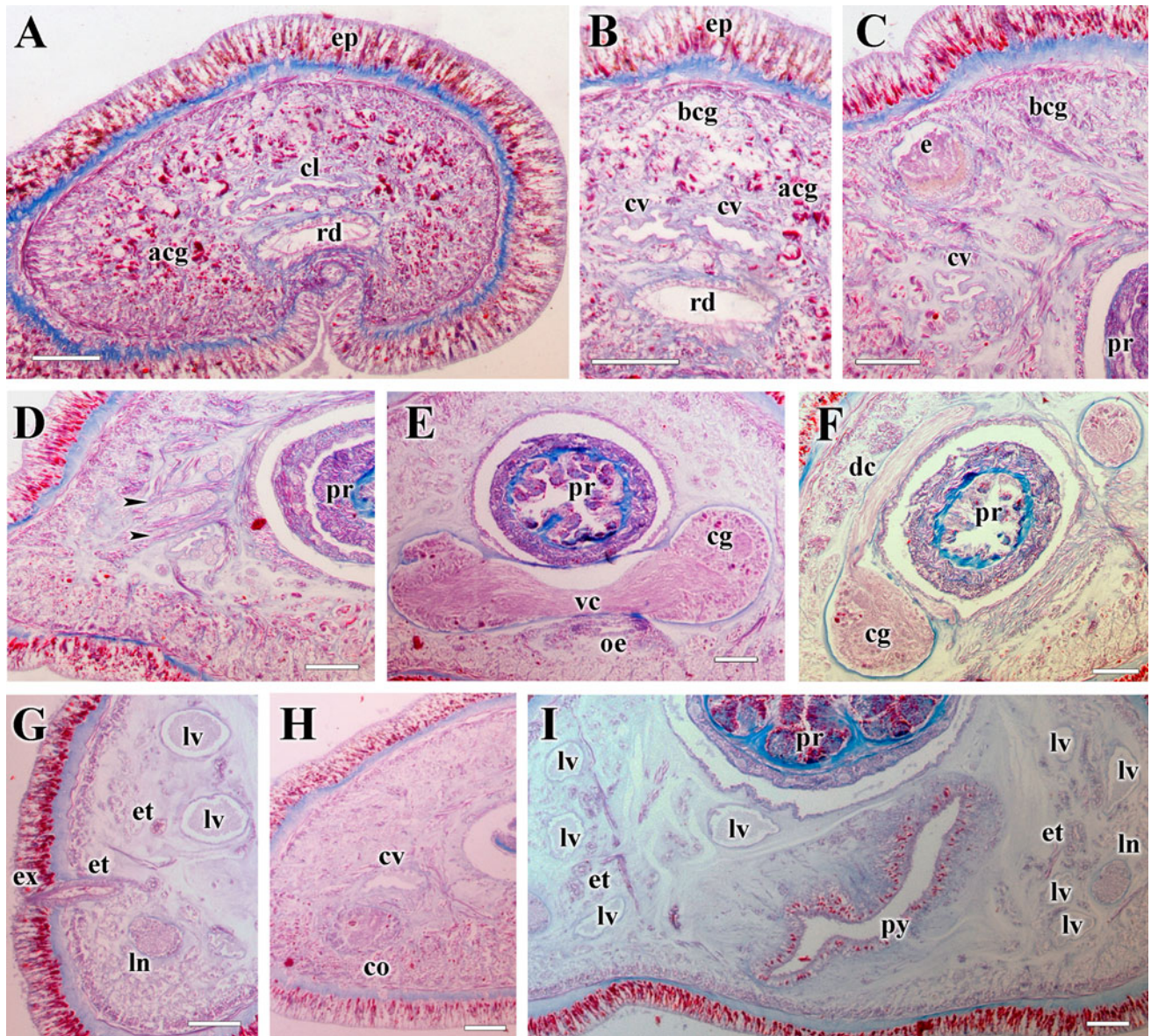
Fig. 3 *Antarctonemertes valida*. **a** anterior cephalic loop, **b** cephalic blood vessels, **c** transverse section showing one eye, **d** proboscis insertion (*arrowheads*), **e** ventral commissure, **f** dorsal commissure, **g** excretory tubule and excretory pore, **h** cerebral sensory organ, **i** multiple longitudinal blood vessels. *Scale bar* 100 μm. *acg* acidophilic cephalic glands, *bcg* basophilic cephalic glands, *cl* cephalic loop, *cg* cephalic ganglion, *co* cerebral sensory organ, *cv* cephalic blood vessel, *dc* dorsal commissure, *e* eye, *ep* epidermis, *et* excretory tubule, *ex* excretory pore, *ln* lateral nerve cord, *lv* longitudinal blood vessel, *oe* oesophagus, *pr* proboscis, *py* pylorus, *rd* rhynchodaeum, *vc* ventral commissure

Female before oviposition, series of transverse sections, whole worm (NEM1B-S1); female in cocoon, series of transverse sections, anterior part (NEM1B-S2); male, series of transverse sections, whole worm (NEM1B-S3). NEM1B-S1 and NEM1B-S3 collected in Colatinas' area (62°59.482′S; 60°37.095′W), Deception Island (Fig. 1c; Sta. 2); Leg. S. Taboada, J. Cristobo, and C. Avila, 11 January 2010, associated with the alga *Rhodymenia coccocarpa*, at 1–2 m depth (Fig. 2c).

Additional material examined Seven free-living specimens, two of them females before oviposition, and eight females in cocoons attached to *R. coccocarpa*, collected from Colatinas' area (Fig. 1c; Sta. 2).

External features Preserved specimens 8–30 mm long, up to 2 mm wide. Body of uniform width throughout, dorsally rounded, ventrally flattened, tapering only near the tail, ending in a blunt tip. Live specimens with triangular
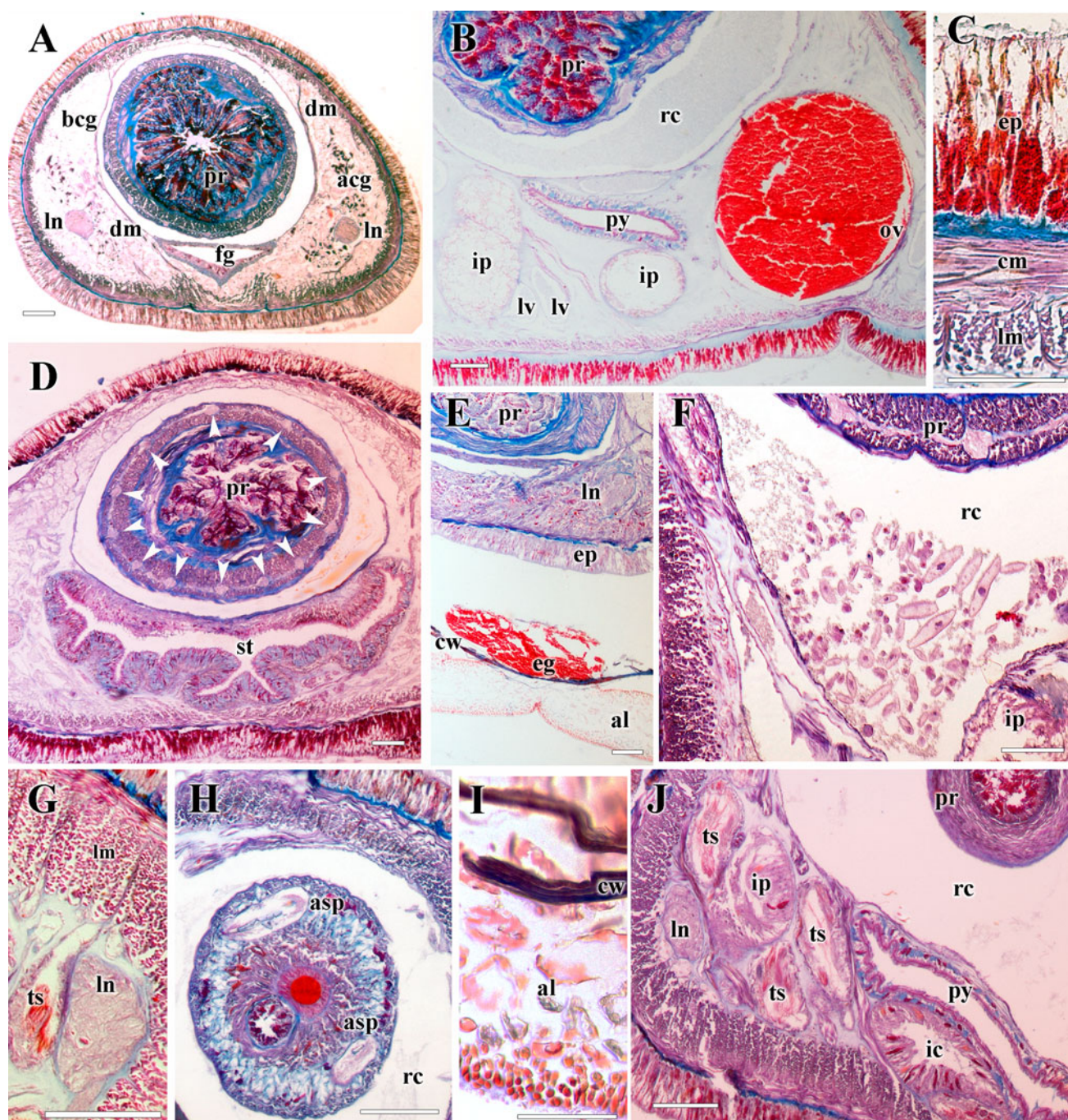
Fig. 4 *Antarctonemertes valida*. **a** transverse section at foregut level, **b** ovary with one oocyte, **c** transverse section of the body wall, **d** transverse section at stomach level showing the twelve proboscidial nerves (*arrowheads*), **e** transverse section of a female inside the cocoon attached to an alga, **f** protozoan parasites in the rhynchocoel, **g** myofibrillae in lateral nerve cord, **h** proboscis bulb region showing two accessory stylet pouches, **i** cocoon wall showing its layers, **j** male showing testes. *Scale bars* **a**, **b**, **d–f**, **h**, **j** 100 μm; **c**, **g**, **i** 50 μm. *al* alga, *asp* accessory stylet pouch, *acg* acidophilic cephalic glands, *bcg*, basophilic cephalic glands, *cw* cocoon wall, *cm* body-wall circular muscle layer, *dm* dorso-ventral muscle, *eg* egg, *ep* epidermis, *fg* foregut, *ic* intestinal caecum, *ip* intestinal pouch, *lm* body-wall longitudinal muscle layer, *ln* lateral nerve cord, *lv* longitudinal blood vessel, *ov* ovary, *pr* proboscis, *py* pylorus, *rc* rhynchocoel, *st* stomach, *ts* testis

head having a prominent median lobe (Fig. 2d, e), resembling the lancet-shaped type represented by Sundberg et al. (2009a); head shape pointed in preserved specimens. Two pairs of cephalic furrows only evident ventrally; anterior pair forming a ventral, anterior directed "V" that continues with a median longitudinal furrow extending forward

almost to proboscis pore (Fig. 2e); shallow posterior cephalic furrow indistinct. In life, dorsal colour brown, ventral surface dirty white or pale yellow; two white lateral patches at the head (Fig. 2d). After preservation body colour and head patches retained. In life and when disturbed, they secrete a very dense transparent mucous that can cause death to other small invertebrates; this secretion has a neutral pH.

Body wall, musculature, and parenchyma Epidermis 50–80 μm thick, with typical hoplonemertean arrangement; dorsal epidermis with dark pigment granules (Fig. 3a–c). Body-wall muscles with outer circular and inner longitudinal layers, 10–15 μm and 30–50 μm thick, respectively (Fig. 4c). Diagonal muscle layer between circular and longitudinal musculature not observed. Longitudinal musculature divided into outer and inner portion along cerebral region with cephalic glands in between; some fibres from inner portion continue into the head as cephalic retractors. In front of cerebral ganglia, bundles of fibres lead inwards from the longitudinal muscle layer to proboscis insertion; these comprise the pre-cerebral septum, which is thus of closed type (Fig. 3d). Longitudinal muscle layer (splanchnic musculature) surrounding oesophagus and stomach. Dorsoventral muscles crossing the body behind brain and between lateral gut diverticula throughout intestinal region (Fig. 4a). Parenchymatous connective tissue extending post-cerebrally, stopping before gonadal region.

Proboscis apparatus Proboscis apparatus resembling that of most other monostiliferan hoplonemerteans. Rhynchopore ventral. Anterior region of proboscis with short epithelial papillae and 12 proboscidial nerves between longitudinal muscle fibres, connected by a peripheral neural sheath; two accessory stylet pouches (Fig. 4d, h). Protozoan parasites observed in rhynchocoel (Fig. 4f).

Alimentary canal Oesophagus epithelium devoid of gland cells; ciliation only detected anteriorly. Stomach with typical hoplonemertean structure, with densely ciliated folded epithelium with basophilic and acidophilic glands (Fig. 4d). Pylorus, longer than stomach, narrowing before opening into intestine (Fig. 3i, 4j). Caecum short; anterior caecal diverticula not reaching brain.

Circulatory system Circulatory system distinct to that of the typical monostiliferous hoplonemerteans, i.e., paired lateral and single mid-dorsal vessels. The two lateral vessels appear transversely joined by a suprarhynchodeal loop (Fig. 3a, b). Mid-dorsal blood vessel emerging as a branch of one of the lateral vessels in cerebral region and not branching along its entire length; no vascular plug observed. Lateral vessels branching behind cerebral ganglia twice or more (Fig. 3g); three to five pairs of spacious

vessels running between gut and body wall (Fig. 3i), reaching the end of pylorus without anastomosis, though their ultimate fate is not observed. Division of lateral blood vessels observed both in females (before and after oviposition) and male.

Nervous system Brain consisting of two dorsal and two ventral ganglia, similar in size (Fig. 3e, f); cephalic nerves lead anteriorly from these ganglia to supply head. A thin outer neurilemma enclosing brain as a whole, though no inner neurilemma separating fibrous and ganglionic cerebral components observed. Lateral nerve cords containing a main fibrous core and a bundle of fibres dorsally, the so-called "upper nerve". In a specimen (NEM1B-S1), a short nerve parallel to right lateral nerve cord was observed. Some myofibrillae situated in middle of lateral nerve cords (Fig. 4g). Neither neurochords nor neurochord cells observed.

Excretory system Well-developed excretory system, confined to post-cerebral region of body, consisting of two pairs of thick-walled longitudinal collecting tubules, running close to lateral vessels opening by ventro-lateral nephridiopores in stomach region (Fig. 3g, i). No flame cells observed.

Frontal organ and cephalic glands Frontal organ observed in one specimen (NEM3-SZ). Typical basophilic lobules forming the most abundant type of cephalic glands; posteriorly extending far behind cerebral ganglia (Figs. 3b, c, 4a). Presumably these glands discharge through frontal organ but also through independent pores. Acidophilic glands irregularly distributed between basophilic lobules in anterior region of the head, mainly ventrally (Fig. 3a, b).

Sensory structures Two pairs of eyes, formed of typical pigmented cup ocellus-type up to 125 μm in diameter, observed in life but not in preserved specimens due to dark colour of worms. Anterior pair of eyes close to the tip of head (about 20 μm beneath epidermal basement layer), posterior pair in front of brain (100 μm beneath basement layer) (Fig. 3c). Only three eyes observed in juvenile specimen sectioned. Cerebral sensory organs 115–150 μm in diameter, situated between anterior and posterior pairs of eyes, in front of brain (Fig. 3h). Each organ opening ventro-laterally from cephalic furrow, via thick-walled ciliated canals.

Reproductive systems Specimens with mature gonads in separate individuals in specimens examined, concluding that species is gonochoristic. Gonads serially arranged along body from back of oesophagus backwards, lying mainly ventrally under intestine. Each ovary typically containing a single oocyte, up to 500 μm in diameter, with a nucleus 30–40 μm across (Fig. 4b). Up to eight oocytes

observed in a single section, six arranged ventrally. Testes also lying ventrally; up to 15 testes observed in transversal sections, ranging 100–125 μm in diameter (Fig. 4j). Spermatozoa with elongated head.

Cocoons Transparent and elongated, 10–23 mm long by 4–6 mm maximum wide, dorsally rounded, firmly attached to substrate by ventrally flattened part (Fig. 2a–c). In some cocoons, different superimposed layers and a longitudinal suture line observed (Fig. 4e, i). Cocoons with two openings; when disturbed, females show a defensive behaviour everting proboscis (Fig. 2b). When removed from cocoon, females remain inactive and barely move, as opposed to non-brooding specimens. About 70–140 eggs per cocoon, about 0.5 mm in diameter, white, yellowish, orange, or pink (occasionally purple) in life becoming white opaque after preservation (Fig. 2a, b, f). A female in a cocoon kept in an aquarium with no oxygenation supply was observed performing peristaltic movements.

Habitat Specimens of *A. valida* were collected in the shallow Antarctic waters of Port Foster, Deception Island (South Shetland Islands). Cocoons with eggs and brooding female were collected attached to the rocks in front of the "Gabriel de Castilla" Spanish Antarctic Base (Fig. 1c; Sta. 1) or attached to the alga *R. coccocarpa* in the Colatinas' area (Fig. 1c; Sta. 2). In this latter area, apart from cocoons, several free-living specimens (including some females before oviposition) were observed aggregating beneath algae as in a pre-mating behaviour.

Gibson (1995) restricts the distribution of this species to the Antarctic and Subantarctic (South Georgia and South Shetland Islands, off Enderby Land, and the Antarctic Peninsula) region.

Remarks Bürger (1893) was the first to formally describe *A. valida* under the name of *T. validum*. This species, originally collected from the South Georgia Island, was later reported by Joubin (1908, 1914) also from Antarctic waters, although as *Amphiporus michaelseni*. In our opinion, Joubin (1908, 1914) erroneously assigned the specimens collected in Petermann and King George Islands to *A. michaelseni*, a species insufficiently described by Bürger (1895a) from the waters of Punta Arenas (Chile). One of the most conspicuous characters of *A. valida* is the presence of two white lateral patches at the head, which are retained even after preservation, as reported previously (Joubin 1908; Wheeler 1934). Although Joubin's (1908, 1914) complementary descriptions of *A. michaelseni* did not report the presence of eyes (observed in the *A. valida* specimens we examined), these structures are usually difficult to distinguish in living or fixed specimens because of the dark coloration of the body (Wheeler 1940). As noted by Wheeler (1934, 1940) and Coe (1950), this species is easily recognizable by its colour and form, having lancet- or "shark-like"-shaped head, as we observed in the living specimens of *A. valida* (Fig. 2d, e). In agreement with these previous descriptions, the specimens we studied have four eyes and share a list of histological features reported in the description of *A. valida*. Besides, we also noticed differences in the morphology of the circulatory system and in the number of proboscidial nerves: Bürger's (1893) single specimen had 10 proboscidial nerves, the same number described by Wheeler (1934), although he also referred to one specimen with 12 proboscidial nerves, the number of nerves observed in our study. Finally, the description of the cocoon given by Joubin (1914) and Wheeler (1934) is coincident with the description we provide.

Antarctonemertes riesgoae sp. nov. (Fig. 5)

Type material holotype (MCZ IZ-128729): Female before oviposition, series of transverse sections, anterior part. *Paratype* (MCZ IZ-128730): Female guarding a cocoon, series of transverse sections, whole animal. Both holotype and paratype collected in front of the "Gabriel de Castilla" Spanish Antarctic Base (62°58.587′S; 60°40.580′W), Deception Island (South Shetland Islands, Antarctica) (Fig. 1c; Sta. 1); Leg. S. Taboada, J. Cristobo, and C. Avila, 9 January 2010, under rocks 1–2 m depth of water. Additional material was collected from intertidal rocks at Fildes Bay, King George Island (South Shetland Islands, Antarctica), 30 January 2006, 62°11.911′S; 58°57.026′W (Fig. 1b); Leg. J. Cristobo, M. Ballesteros, and J.A. Moya.

Diagnosis As for the genus; with a V-shaped white dorsal cephalic band and with 10 proboscidial nerves.

External features Preserved specimens 10–22 mm long, up to 2.5 mm wide. Body tapering at anterior and posterior ends, dorsally rounded, ventrally flattened. Live specimens with triangular head having a prominent median lobe resembling the lancet-shaped type represented by Sundberg et al. (2009a); head shape pointed in living disturbed organisms (Fig. 5a–c) and after preservation. One pair of cephalic furrows evident ventrally forming a semicircular arch (Fig. 5c); posterior cephalic furrow indistinct, only evident in one preserved specimen; rhynchopore ventral (Fig. 5c). In life, dorsal colour dark brown, ventral surface pale brown; V-shaped white band with the apex pointing posteriorly at the head (Fig. 5a). After preservation body colour and head band retained (Fig. 5g). In life and when disturbed, they secrete a very dense transparent mucous that can cause death to other small invertebrates; the mucous secretion has a neutral pH.

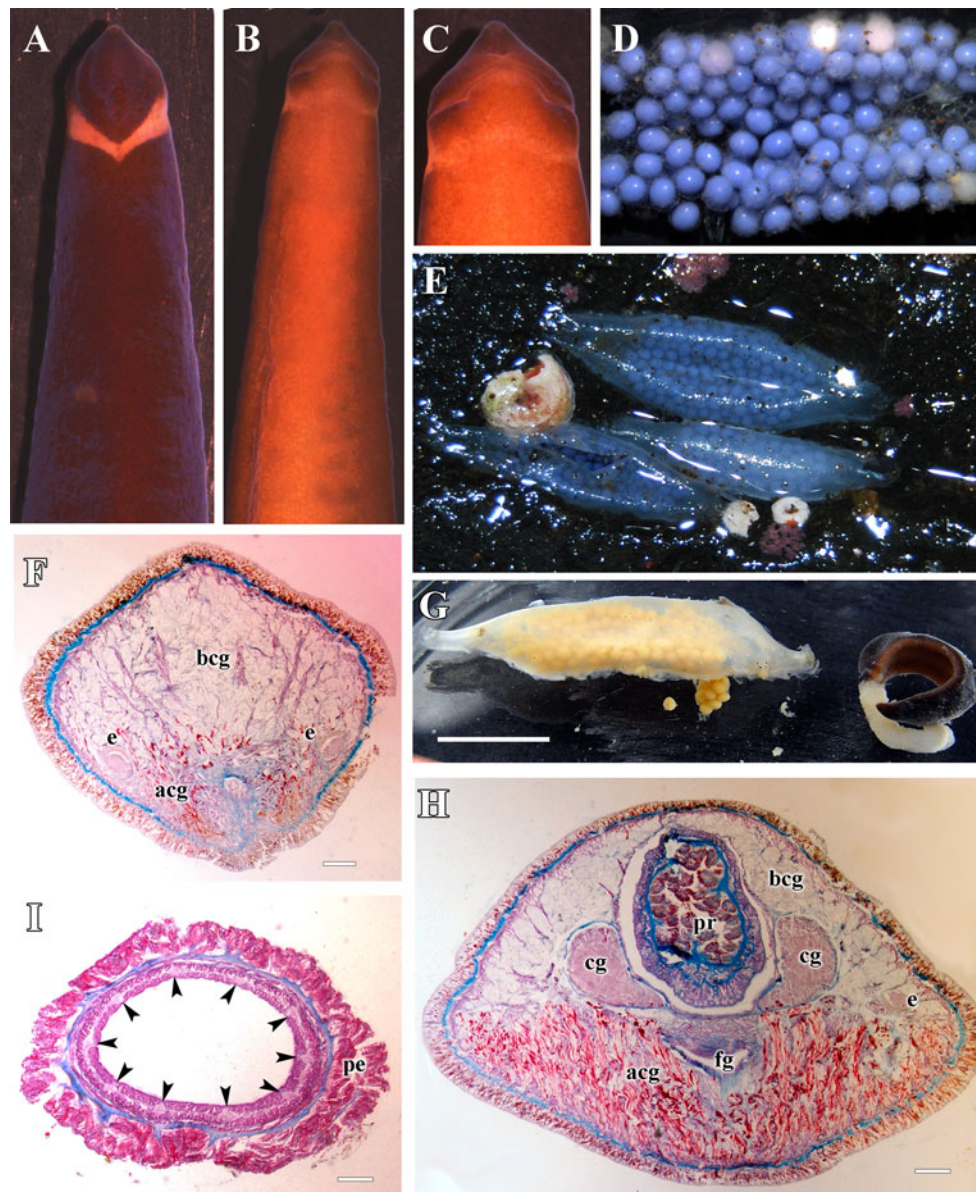*Body wall and musculature* Similar to those described for *A. valida*.

Fig. 5 *Antarctonemertes riesgoae* sp. nov. **a** Anterior region, dorsal view, living specimen after being disturbed, **b** anterior region, ventral view, living specimen, **c** detail of the cephalic region in ventral view to show cephalic furrows, living specimen, **d** detail of the eggs of a cocoon, **e** three cocoons under a stone without females, **f** transverse section, anterior part, showing a pair of eyes (holotype), **g** preserved cocoon and female, **h** transverse section at brain level, showing the ventral distribution of the acidophilic cephalic glands (holotype), **i** everted proboscis showing the ten proboscidial nerves (*arrowheads*) (paratype). *Scale bars* **f**, **i**, **h** 100 μm; **g** = 5 mm. *acg* acidophilic cephalic glands, *bcg* basophilic cephalic glands, *cg* cerebral ganglion, *e* eye, *fg* foregut, *pe* proboscidial epithelium, *pr* proboscis

Proboscis apparatus Similar to those described for *A. valida* except for the number of proboscidial nerves, 10 in this species (Fig. 5i).

Alimentary canal, circulatory system, nervous system, and excretory system Similar to those described for *A. valida*. The new species also presents the division of the lateral vessels and the upper nerve in lateral nerve cords.

Apical organ and cephalic glands Apical organ not observed. Holotype showing a clear division between acidophilic cephalic glands, which occupy anterior ventral half of body and dorsal basophilic cephalic glands (Fig. 5h).

Sensory structures Eyes indistinct due to dark colour of worms. Two pairs of eyes observed in sections of holotype (Fig. 5f).

Reproductive system The two sectioned specimens were mature females, and thus the species is probably gonochoristic. Gonads serially disposed along body from the posterior of oesophagus backwards, lying mainly ventrally under intestine.

Cocoons Transparent and elongated, 15 mm long per 4 mm maximum wide, dorsally rounded, firmly attached to rocks by its ventrally flattened part (Fig. 5e, g). In some cases, up to three cocoons located close to each other (Fig. 5e). Cocoons have two openings; when disturbed, females show a defensive behaviour everting proboscis. When removed from cocoon, females remain inactive and barely move, as opposed to non-brooding specimens. From 50 to 125 eggs per cocoon, each egg about 0.5 mm in diameter, blue to light purple in life becoming white opaque after preservation (Fig. 5d, e, g).

Habitat Specimens of *A. riesgoae* sp. nov. were collected at the shallow Antarctic waters of Port Foster, Deception Island as well as from intertidal rocks at Fildes Bay, King George Island (South Shetland Islands) (Figs. 1b, c; Sta. 1). Cocoons with eggs with or without brooding females were collected attached to rocks. During the ACTIQUIM-II Antarctic cruise (2012–2013), we additionally confirmed the occurrence of *A. riesgoae* sp. nov. under subtidal rocks along the South Shetland Islands (King George, Half Moon, and Livingston Islands) and the Antarctic Peninsula (vicinities of O'Higgins Chilean Antarctic Base and vicinities of Primavera Argentinian Antarctic Base).

Remarks So far, only three brooding nemerteans are known from Antarctic waters: *Amphiporus incubator*, *Antarctonemertes valida*, and *A. riesgoae* sp. nov. Although females of the three species seem to build a cocoon with a similar elongated shape, *A. incubator* differs from the two *Antarctonemertes* in the nature of the cocoon. While *A. incubator* appears to secrete an opaque cocoon with no openings where females brood their eggs, females of *A. valida* and *A. riesgoae* sp. nov. secrete a transparent cocoon with two openings, one at each end. Besides that, no clue of the spongy substance described by Joubin (1914) in *A. incubator* was detected in any of the cocoons examined in both *A. valida* and *A. riesgoae* sp. nov. This substance that fills the cocoons and packs the eggs was postulated to be utilized by recently hatched juveniles as a food resource. Also, no marked morphological change was observed in any of the examined brooding females of *A. valida* and *A. riesgoae* sp. nov., besides a flattening of their transverse section. Conversely, Joubin (1914) thoroughly described the dramatic changes that females undertake once inside the cocoon, including the occurrence of big lobes that surround the mass of eggs or the later rupture of the walls of the intestine and ovary. As for the external appearance of adults, the three species seem to have a similar colour in life (brown to dark brown), although differ in the pattern of cephalic coloration: Joubin's (1914) description does not point to any particular pattern for *A. incubator*, while *A. valida* and *A. riesgoae* sp. nov. have two white lateral patches and a V-shaped white band, respectively. The

number and arrangement of cephalic furrows also distinguish the three species: both *A. incubator* and *A. riesgoae* sp. nov. have a pair of cephalic furrows evident ventrally forming a semicircular arch, while *A. valida* has two pairs of cephalic furrows evident ventrally forming an anterior directed "V". Regarding the histological characters, the most remarkable difference between *A. valida* and *A. riesgoae* sp. nov. is the number of proboscidial nerves (10 and 12, respectively), but this was not mentioned in Joubin's description for *A. incubator* (Joubin 1914).

Etymology Antarctonemertes riesgoae sp. nov. is named after Ana Riesgo, renowned sponge biologist, and esteemed colleague, in recognition of her help to all authors through these years and particularly for the support and friendship to the lead author.

Character matrix Sundberg et al. (2009a) proposed a list of characters and their states to be used when describing nemerteans in order to facilitate subsequent comparative studies. Online Resource 1 shows the data for *A. riesgoae* sp. nov.

Molecular analysis

Phylogenetic relationships

The 28S rRNA data set comprised 27 sequences, which resulted in a multiple sequence alignment of 1,145 bp. MAFFT yielded a 589-bp alignment from the COI region comprising 33 sequences and a 480-bp alignment, comprising 25 sequences, for 16S rRNA. A concatenated data set of 2,214 bp was analysed using the maximum likelihood approach, giving a tree of lnL = −14,847.12 (Fig. 6), with nodal supports of 100 % bootstrap frequency (BF) for the sister group relationship of the Antarctic *A. valida* and *A. riesgoae* sp. nov. *Prosorhochmus nelsoni* Sánchez, 1973 appears as the sister group to the latter clade, although support is below 50 % BF. These form part of a clade containing *Tetrastemma elegans* (Girard, 1852), *Vieitezia luzmurubeae* Junoy, Andrade and Giribet, 2010, *Gononemertes parasita* Bergendal, 1900, and two other *Antarctonemertes*, *Antarctonemertes varvarae* Chernyshev, 1999 and *Antarctonemertes phyllospadicola* (Stricker, 1985); however, support for this large clade is again negligible (Fig. 6). The next node with some bootstrap support includes other *Tetrastemma*, *Oerstedia*, and *Nemertellina yamaokai* Kajihara, Gibson and Mawatari, 2000.

The Tajima-Nei genetic distance estimate and the parsimony network analyses were performed with 10 individuals for COI, 14 for 16S rRNA and 13 for ITS-2, depending on data availability for each gene region (Table 3). The average genetic distance among groups was 5.8, 1.5 and 1.3 % for COI, 16S rRNA and ITS-2, respectively, and in the case of ITS-2, there are diagnostic

Fig. 6 Maximum likelihood phylogenetic tree resulting from the combined analysis of 28S rRNA, 16S rRNA, and COI (ln$L$ = −14,847.12). *Numbers* on nodes indicate bootstrap support values >70 %. Tree rooted on *Nipponemertes* sp., *N. pulchra*, and *Nectonemertes mirabilis*
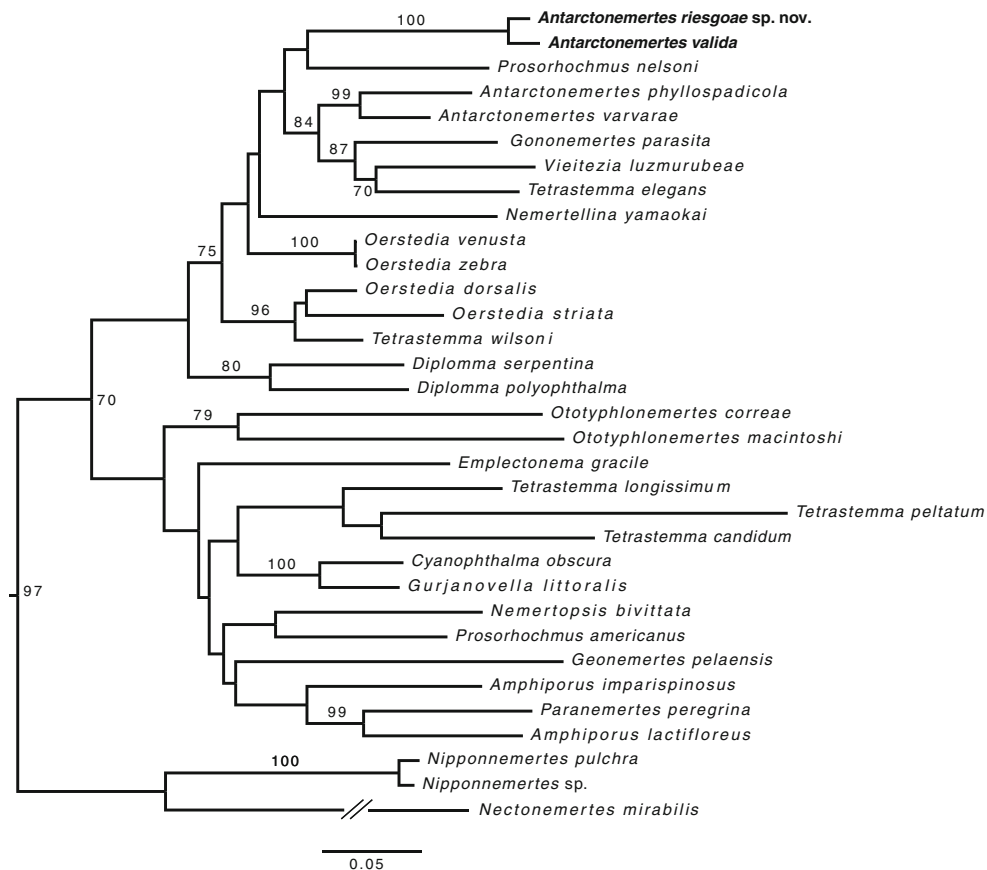
indels not accounted for in the genetic distance. The haplotype networks for each marker are presented in Fig. 7. TCS resulted in two non-connected networks (ten mutational steps at 95 % confidence) for COI with two different haplotypes found for each species and 35 mutational steps between them (Fig. 7a). The 16S rRNA TCS analysis produced a single network (eight mutational steps at 95 % confidence), with three different haplotypes and seven substitution steps among species (Fig. 7b). TCS analysis of the ITS-2 region produced two non-connected networks (with ten mutational steps at 95 % confidence), four different haplotypes, and 25 mutational steps among species (Fig. 7c). All markers clearly showed differentiation of haplotypes among *A. valida* and *A. riesgoae* sp. nov.

Discussion

Brooding nemerteans were first described in Antarctic waters after the discovery of *Amphiporus incubator* by Joubin (1914). The other known Antarctic brooding nemertean, *Amphiporus michaelseni*, was also reported by Joubin (1908, 1914) in the waters of Booth and Petermann Islands. Both species, however, were considered by Gibson and Crandall (1989) as not sufficiently well characterized
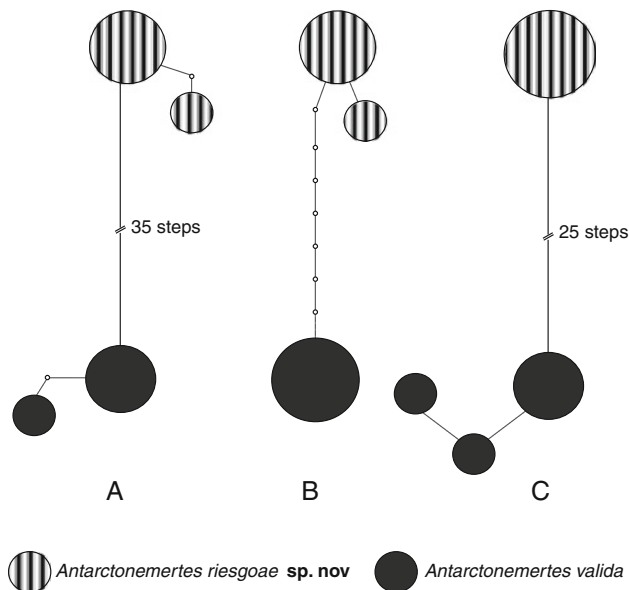

Fig. 7 Statistical parsimony network using **a** COI, **b** 16S rRNA, **c** ITS-2 regions. Species are indicated by alternative colour patterns. *Dots* indicate additional mutational steps between sampled haplotypes

and consequently were considered *species inquirendae*. With our study, combining histological data with data from other sources, we shed light on this group of Antarctic

brooding nemerteans by establishing a new synonymy for *A. michaelseni* sensu Joubin as *A. valida*, giving a detailed redescription of the species. Besides, we describe a new species in the genus *Antarctonemertes*, *A. riesgoae* sp. nov., similar to *A. valida* in external appearance and reproductive strategy, but highly differentiated molecularly based on three different markers (Fig. 7).

First, we had to solve the identity of *A. michaelseni*. Bürger's original description was based on specimens collected in the Strait of Magellan, Punta Arenas (South of Chile), and was brief and lacking illustrations (Bürger 1895a). Further, the species, which type material was lost, was never re-collected in South America even after the extensive work conducted by Friedrich (1970) and other researchers (M. Thiel, pers. comm.). Joubin (1908, 1914) assigned to *A. michaelseni* Antarctic specimens similar in form, coloration, and reproductive behaviour (female inside a cocoon that opens at both ends) to the specimens previously described by Bürger (1893) and the specimens here described as *A. valida.* Among the characters observed in the descriptions of *A. michaelseni*, the number of proboscidial nerves appears to vary between 10 and 12 (Bürger 1893; Wheeler 1934), the latter being the number of nerves we observed. Although the number of proboscidial nerves has traditionally been used as a specific taxonomic character (Chernyshev 1999), intraspecific variation has been reported for some species (e.g. Berg 1972; Norenburg 1986). Another possibility we may consider is that both Bürger (1893) and Wheeler (1934) mixed *A. valida* and *A. riesgoae* sp. nov. during their investigation, since our results confirm they have 12 and 10 proboscidial nerves, respectively. Thus, considering the above-mentioned, we propose to synonymize *A. michaelseni* sensu Joubin (1908) with *A. valida* (Bürger, 1893).

Another question to assess was the identification of *A. incubator*. This species, originally collected in the shallow waters of King George and Petermann Islands, was thoroughly described by Joubin (1914) who gave a plethora of details on its histology and external features, and particularly on the peculiar female's reproductive behaviour. The species is similar to *A. michaelseni* sensu Joubin (1908) (here synonymized with *A. valida*), although Joubin (1914) distinguished both mainly in having differences in the cocoon and the incubating behaviour. The cocoon of *A. incubator* is opaque, closed at both ends, and full of a spongy substance that fills the spaces inside the cocoon and packs the eggs. Further, the behaviour of the female resembles the European medieval myth of the *pelican in her piety*: the enclosed female degenerates to become food for the hatched young. We have not observed this in *A. riesgoae* sp. nov. As opposed to *A. incubator*, *A. riesgoae* sp. nov. clearly differs in having a cocoon opening at both ends and by the presence of a dorsal V-shaped white band not reported in the species described by Joubin (1914). Even if we considered the possibility that *A. incubator* females first build an open cocoon and thereafter sealed both openings, Joubin would have noticed the suture line at both ends.

The use of molecular tools in the phylum Nemertea has helped unravel biodiversity undetected with traditional taxonomic methods (e.g. Strand and Sundberg 2005; Sundberg et al. 2009b, c; Chen et al. 2010; Andrade et al. 2011). Some of these studies, however, have failed to correlate distinct morphological variation with genetic relatedness (e.g. Strand and Sundberg 2005; Sundberg et al. 2009b, c), a fact that can be attributed to the extraordinary phenotypic plasticity depicted in some taxa. Statistical parsimony network analyses have proven a powerful tool for detecting undescribed/cryptic species specially when using non-recombining loci (Hart and Sunday 2007). Our results show that the haplotype networks for the mitochondrial marker COI and the highly variable ITS-2 are distant enough, and present a barcode gap to consider *A. valida* and *A. riesgoae* sp. nov. two separate species (Fig. 7); however, the haplotypes for the slower evolving 16S rRNA are separated by only seven mutational steps for the two species, probably indicating a recent divergence among both species. In the past, both *A. valida* and *A. riesgoae* sp. nov. could have been mixed since both species share habitat, have a similar reproductive strategy, and have a similar external appearance. The genetic analysis, together with the morphological, behavioural, and ecological traits observed, corroborates the species status of *A. valida* and *A. riesgoae* sp. nov.

The molecular phylogenetic analysis places the Antarctic *A. valida* and *A. riesgoae* sp. nov. in a clade with species that share some characteristics usually used to distinguish between monostiliferan genera (Fig. 6). Except for *P. nelsoni*, these species possess a mid-dorsal vessel that does not enter the rhynchocoel. On the other hand, as opposed to *A. valida* and *A. riesgoae* sp. nov., *G. parasita* and *V. luzmurubeae* (commensals of ascidians) lack the upper nerve or accessory nerves, although this character is sometimes difficult to observe. The phylogenetic analysis shows that non-solved relationships in different genera and families seem to be the rule in nemerteans as previously reported in several studies (e.g. Sundberg et al. 2001; Thollesson and Norenburg 2003; Andrade et al. 2012), at least for the markers here employed. A thorough taxonomic revision including morphological and molecular sequence data taking into account a species diversity approach should be conducted to provide a sound classification system based on phylogenetic evidence. Nonetheless, until this revision is achieved, we adopt a conservative decision placing *A. valida* and *A. riesgoae* sp. nov. in the genus *Antarctonemertes*, although the phylogenetic analysis

could suggest other possibilities. The fact that both species could be the result of evolutionary convergence remains a possibility to be further studied.

References

Abildgaard PC (1806) *Planaria dorsalis*. In: Müller OF (ed) Zoologia Danica seu animalium Daniae et Norvegiae rariorum ac minus notorum descriptiones et historia. Havniae, N. Christensen 4, 25

Akaike H (1974) A new look at the statistical model identification. IEEE Trans Autom Control 19:716–723. doi:10.1109/TAC.1974.1100705

Altschul S, Madden T, Schaffer A, Zhang J, Zhang Z, Miller W, Lipman D (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402. doi:10.1093/nar/25.17.3389

Andrade SCS, Norenburg JL, Solferini VN (2011) Worms without borders: genetic diversity patterns in four Brazilian *Ototyphlonemertes* species (Nemertea, Hoplonemertea). Mar Biol 158:2109–2124. doi:10.1007/s00227-011-1718-3

Andrade SCS, Strand M, Schwartz M, Chen H, Kajihara H, von Döhren J, Sun S, Junoy J, Thiel M, Norenburg JL, Turbeville JM, Giribet G, Sundberg P (2012) Disentangling ribbon worm relationships: multi-locus analysis supports traditional classification of the phylum Nemertea. Cladistics 28:141–159

Berg G (1972) Studies on *Nipponnernertes* Friedrich, 1968 (Nemertini, Hoplonemertini). Zool Scr 1:211–225

Bergendal D (1900) Uber ein Paar sehr eigenthümliche nordische Nemertinen. Zool Anz 23:313–328

Bürger O (1893) Südgeorgische und andere exotische Nemertinen. Zoologische Jahrbücher Abteilungen Systematik Ökologie und Geographic der Tiere 7:207–240

Bürger O (1895a) Beiträge zur Anatomie, Systematik und geographischen Verbreitung der Nemertinen. Z Wiss Zool Abt A 61:16–37

Bürger O (1895b) Die Nemertinen des Golfes von Neapel und der angrenzenden Meeres-Abschnitte. Fauna und Flora des Golfes von Neapel 22:1–743

Chen H, Strand M, Norenburg JL, Sun S, Kajihara H, Chernyshev AV, Maslakova SA, Sundberg P (2010) Statistical parsimony networks and species assemblages in cephalotrichid nemerteans (Nemertea). PLoS One 5:e12885

Chernyshev AV (1993) A review of the genera of nemerteans allied to *Oerstedia* (Monostilifera, Tetrastemmatidae) with description of four new species. Zool Zhurnal 72:11–20

Chernyshev AV (1999) Nemerteans of the genus *Antarctonemertes* (Enopla, Monostilifera). Zool Zhurnal 78:939–948

Clement M, Posada D, Crandall KA (2000) TCS: a computer program to estimate gene genealogies. Mol Ecol 9:1657–1659

Coe WR (1901) Papers from the Harriman Alaska Expedition. XX. The nemerteans. Proc Wash Acad Sci 3:1–110

Coe WR (1943) Biology of the nemerteans of the Atlantic coast of North America. Trans Conn Acad Arts Sci 35:129–328

Coe WR (1950) Nemerteans from Antarctica and the Antarctic Ocean. J Wash Acad Sci 40:56–59

Delle Chiaje S (1841) Descrizione e notomia degli animali invertebrati della Sicilia citeriore osservati vivi negli anni 1822–1830, vol 3, Molluschi, Acefali, Bracciopedi, Cirropedi, Crostacei, Anellosi. C. Batelli, Napoli

Edgecombe GD, Giribet G (2006) A century later—a total evidence re-evaluation of the phylogeny of scutigeromorph centipedes (Myriapoda: Chilopoda). Invertebr Syst 20:503–525

Envall M (1996) *Ototyphlonemertes correae* sp. nov. and a redescription of *O. duplex* (Nemertea: Monostilifera: Ototyphlonemertidae), with a phylogenetic consideration of the genus. J Zool 238:53–277

Felsenstein J (2004) Inferring phylogenies. Sinauer Associates, Sunderland, MA

Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek RC (1994) DNA primers for amplification of mitochondrial cytochrome *c* oxidase subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotech 3:294–299

Friedrich H (1955) Beiträge zu einer Synopsis der Gattungen der Nemertini monostilifera nebst Bestimmungsschlüssel. Z Wiss Zool Abt A 158:133–192

Friedrich H (1970) Nemertinen aus Chile. Sarsia 40:1–80

Gibson R (1983) Antarctic nemerteans: the anatomy, distribution and biology of *Parborlasia corrugatus* (McIntosh, 1876) (Heteronemertea: Lineidae). Antar Res S 39:289–316

Gibson R (1985) Antarctic nemerteans: heteronemertea—descriptions of new taxa, reappraisals of the systematic status of existing species and a key to the heteronemerteans recorded south of latitude 50°S. Zool J Linn Soc-Lond 83:95–227

Gibson R (1995) Nemertean genera and species of the world: an annotated checklist of original names and description citations, synonyms, current taxonomic status, habitats and recorded zoogeographic distribution. J Nat Hist 29:271–561. doi:10.1080/00222939500770161

Gibson R, Crandall FB (1989) The genus *Amphiporus* Ehrenberg (Nemertea, Enopla, Monostiliferoidea). Zool Scr 18:453–470

Gibson R, Sundberg P (2001) Some nemerteans (Nemertea) from Queensland and the Great Barrier Reef, Australia. Zool Scr 18:1259–1273

Girard C (1852) Descriptions of two new genera and two species of *Nemertes*. Proc Boston Soc Nat Hist 4:185–186

Griffin BB (1898) Description of some marine nemerteans of Puget Sound and Alaska. Ann NY Acad Sci 11:193–217

Hart MW, Sunday J (2007) Things fall apart: biological species for unconnected parsimony networks. Biol Lett 3:509–512

Iwata F (1954) The fauna of Akkeshi Bay. XX. Nemertini in Hokkaido, Journal of the Faculty of Science, Hokkaido University, Series 6. Zoology 12:1–39

Johnston G (1828) Contributions to the British fauna. Zool J 3:486–491

Johnston G (1837) Miscellanea zoologica. II. A description of some planarian worms. Mag Zool Bot 1:529–538

Joubin L (1908) Némertiens. Expédition Antarctique Française 1903–1905(12):1–16

Joubin L (1914) Némertiens. Deuxième Expédition Antarctique Française 1908–1910(5):1–33

Junoy J, Andrade SCS, Giribet G (2010) Phylogenetic placement of a new hoplonemertean species commensal on ascidians. Invertebr Syst 24:616–629

Kajihara H, Gibson R, Mawatari SF (2000) Redescription and taxonomic reassessment of *Nemertellina minuta* Friedrich, 1935, sensu Yamaoka, 1940 (Nemertea, Hoplonemertea, Monostilifera). Zool Sci 17:265–276

Kajihara H, Chernyshev A, Sun S-C, Sundberg P, Crandall FB (2008) Checklist of nemertean genera and species published between 1995 and 2007. Species Divers 13:245–274

Katoh K, Kuma K, Toh H, Miyata T (2005) MAFFT version 5: improvement in accuracy of multiple sequence alignment. Nucleic Acids Res 33:511–518. doi:10.1093/nar/gki198

Maddison WP, Maddison DR (2010) Mesquite: a modular system for evolutionary analysis, ver. 2.73. Program and documentation available at http://mesquiteproject.org

Mahon AR, Thornhill DJ, Norenburg JL, Halanych KM (2010) DNA uncovers Antarctic nemertean biodiversity and exposes a decades-old cold case of asymmetric inventory. Polar Biol 33:193–202. doi:10.1007/s00300-009-0696-0

McIntosh WC (1876) Descriptions of some new species of Annelida from Kerguelen's Island. Ann Mag Nat Hist 4(17):318–323

Müller OF (1774) Vermium terrestrium et fluviatilium, seu animalium infusoriorum, helminthicorum, et testaceorum, non marinorum, succincta historia, vol 1, Part 2. Heineck and Faber, Havniae et Lipsiae

Norenburg J (1986) Redescription of a brooding nemertine, *Cyanophthalma obscura* (Schultze) gen. et. comb. n., with observations on its biology and discussion of the species of *Prostomatella* and related taxa. Zool Scr 15:275–293

Palumbi S, Martin A, Romano S, McMillan WO, Stice L, Grabowski G (1991) The simple fools guide to PCR, ver. 2.0. Department of Zoology and Kewalo Marine Laboratory. University of Hawaii, Honolulu, HI

Pantin CFA (1960) Notes on microscopical techniques for zoologists. University Press, Cambridge 76 pp

Pond SLK, Frost SDW, Muse SV (2005) HyPhy: hypothesis testing using phylogenies. Bioinformatics 21:676–679. doi:10.1093/bioinformatics/bti079

Posada D, Crandall KA (1998) MODELTEST: testing the model of DNA substitution. Bioinformatics 14:817–818. doi:10.1093/bioinformatics/14.9.817

Posada D, Crandall KA (2001) Evaluation of methods for detecting recombination from DNA sequences using computer simulations. PNAS 98:13757–13762

Sánchez M (1973) Sobre 4 especies de nemertinos de Quintero (Chile). Stud Neotrop Fauna 8:195–214

Schultze MS (1851) Beiträge zur Naturgeschichte der Turbellarien. C.A. Koch, Greifswald

Semper C (1863) Reisebericht. Briefliche Mittheilung an A. Kölliker. Z Wiss Zool Abt A 13:558–570

Stamatakis A (2006) RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics 22:2688–2690. doi:10.1093/bioinformatics/btl446

Stamatakis A, Hoover P, Rougemont J (2008) A fast bootstrapping algorithm for the RAxML web-servers. Syst Biol 57:758–771. doi:10.1080/10635150802429642

Stimpson W (1855) Descriptions of some of the new marine invertebrata from the Chinese and Japanese seas. Proc Acad Natl Sci Phila 7:375–384

Strand M, Sundberg P (2005) Delimiting species in the hoplonemertean genus *Tetrastemma* (phylum Nemertea): morphology is not concordant with phylogeny as evidenced from mtDNA sequences. Biol J Linn Soc 86:201–212

Stricker SA (1985) A new species of *Tetrastemma* (Nemertea, Monostilifera) from San Juan Island, Washington, USA. Can J Zool 63:682–690

Sundberg P (1988) A new monostiliferous hoplonemertean (Nemertea), *Oerstedia striata* sp. n., from the west coast of Sweden. Zool Scr 17:135–139

Sundberg P, Turbeville JM, Lindh S (2001) Phylogenetic relationships among higher nemertean (Nemertea) taxa inferred from 18S rDNA sequences. Mol Phylogenet Evol 20:327–334

Sundberg P, Chernyshev AV, Kajihara H, Kånneby T, Strand M (2009a) Character-matrix based descriptions of two new nemertean (Nemertea) species. Zool J Linn Soc 157:264–294

Sundberg P, Vodoti ET, Strand M (2009b) DNA barcoding should accompany taxonomy—the case of *Cerebratulus* spp. (Nemertea). Mol Ecol Resour 10:274–281. doi:10.1111/j.1755-0998.2009.02774.x

Sundberg P, Vodoti ET, Zhou H, Strand M (2009c) Polymorphism hides cryptic species in *Oerstedia dorsalis* (Nemertea, Hoplonemertea). Biol J Linn Soc 98:556–567

Tajima F, Nei M (1984) Estimation of evolutionary distance between nucleotide sequences. Mol Biol Evol 1:269–285

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28:2731–2739. doi:10.1093/molbev/msr121

Templeton AR, Crandall KA, Sing CF (1992) A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. III. Cladogram estimation. Genetics 132:619–633

Thiel M, Junoy J (2006) Mating behavior of nemerteans: present knowledge and future directions. J Nat Hist 40:1021–1034. doi:10.1080/00222930600834154

Thollesson M, Norenburg JL (2003) Ribbon worms relationship: a phylogeny of the phylum Nemertea. Proc R Soc Lond B Biol 270:407–415. doi:10.1098/rspb.2002.2254

Ushakov P (1926) K faune nemertin belogo morya. Issledovaniya Russkikh Morei 3:47–71

Verrill AE (1892) The marine nemerteans of New England and adjacent waters. Trans Conn Acad Arts Sci 8:382–456

Wheeler JFG (1934) Nemerteans from the South Atlantic and Southern Oceans. Discov Rep 9:215–294

Wheeler JFG (1940) Nemerteans of Kerguelen and the Southern Ocean, Report, B.A.N.Z. Antarctic Research Expedition 1929–1931, Series B, 4:233–256

Whiting MF (2002) Mecoptera is paraphyletic: multiple genes and phylogeny of Mecoptera and Siphonaptera. Zool Scr 31:93–104