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A new nemertean species: what are the useful characters for ribbon worm descriptions?

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In recent years the Norwegian Taxonomy Initiative started thorough investigations of poorly known organism groups. In this context, several marine inventories have rendered a number of marine invertebrate species new to science. Within the phylum Nemertea (ribbon worms) a characteristic hoplonemertean was encountered on two different occasions. We describe the new species Amphiporus rectangulus *sp. nov. with a combination of histology and DNA data (COI). For the morphological description we use a previously proposed character matrix and, in a context given by the results, also provide a brief discussion on benefits and drawbacks with both methods. We argue that for small animals with soft bodies external characters can be more informative than hitherto expected.*

Keywords: Hoplonemertea, COI, mt-DNA, description, morphology, characters

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INTRODUCTION

The phylum Nemertea currently comprises around 1300 valid species names (Gibson, 1995; Kajihara *et al.*, 2008) of which maybe 30% could be dubious (Gibson, 1985) given the fact that their descriptions lack details that would help to securely identify them. In addition to brief and shallow descriptions, holotypes and paratypes are often either missing or non-informative. The actual number of nemertean species is, therefore, shrouded in mystery and to revise these hundreds of species would demand major efforts from the complete nemertean community. One initiative to stabilize the framework for nemertean taxonomy was provided by Sundberg *et al.* (2009). They provided a matrix of morphological characters that can be used as a basis for descriptions of hoplonemerteans and palaeonemerteans. Several larger and smaller molecular studies published in the last decade (among others: Thollesson & Norenburg, 2003; Sundberg *et al.*, 2010; Kajihara *et al.*, 2011; Andrade *et al.*, 2012) also improve the conditions for more pervasive and comprehensive interventions within the nemertean taxonomy.

Following tradition, much effort and time is put into nemertean histology, but the results are not always conclusive. The soft bodies, the number of preparation artefacts and the scarce number of specimens that can be investigated are all factors that may aggravate precise results (see Sundberg & Strand, 2010). There are many moments of subjective interpretation, and when there are enough numbers of specimens to compare, it has been shown that intraspecific anatomical variation can be in some features that might be used as taxonomic characters (Sundberg, 1979). There are others that can be consistent (Norenburg, 1986). Even so, traditional morphological descriptions continue to be published with varying emphasis on different species characters. An attempt to stress and solve problems in nemertean taxonomy is the DNA-based description of *Pseudomicrura afzelii* (Strand & Sundberg, 2011). In this publication the authors mention both the difficulties with anatomical characters, the value of studies of live specimens and some difficulties that arise when trying to apply the nomenclatural code to the results of molecular analyses. It is sometimes stated that nemertean worms lack external characters and that internal anatomical details are a must for absolute measures (e.g. Gibson, 1985), but there are opposing views on the subject (e.g. Sundberg, 1979). Here, we make an effort to set a structure for testing the functionality of internal and external morphology in combination with a routine molecular analysis. Our results indicate obvious informative values for external characters.

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MATERIALS AND METHODS

Samples

Worms were collected in May 2009 and in July 2012 in Norwegian waters with an RP-sled, from depths of more than 200 m. Specimens were observed alive, and those for histological examination were fixed in 4% paraformaldehyde in filtered seawater or Bouin's fluid, sectioned at 6 μm and stained by the Mallory trichrome technique for histological examination. Additional specimens were preserved in RNAlater and stored for subsequent DNA extraction using

DNeasy (QIAgen, Inc.) following the protocol recommended by the manufacturer.

Morphological studies

To map the morphological characters of the new species we used the character matrix from Sundberg *et al.* (2009). Data for two species (*Raygibsonia bergi* Sundberg *et al.*, 2009 and *Vieitezia luzmurubeae* Junoy *et al.*, 2011, Appendix 2) earlier described with the same matrix were used for comparative studies (Table 1). All characters were treated as neutral.

Molecular studies and analyses

Part of the mitochondrial gene cytochrome oxidase I (COI) was used for molecular analysis (Figure 1). Data were selected with the aim to maximize hoplonemertean diversity, given available data. Outgroup consists of various heteronemertean species (Appendix 3) based on previous results of molecular analyses (Thollesson *et al.*, 2003; Andrade *et al.*, 2012) that show clear distinction between the two clades. The third group Palaeonemertea has a more obscure relationship to both groups, which is a reason to exclude them from this analysis. The PCR was performed according to standard procedures, and sequences were obtained through Macrogen (Korea). Primers used were HCO1490 and LCO2194 (Folmer *et al.*, 1994).

Contigs were assembled in Geneious v.5.6.5. (Biomatters available from http://www.geneious.com). The COI was aligned straightforwardly with the reading frame for amino acids, using the general invertebrate mitochondrial genetic code with Muscle in TranslatorX (Abascal *et al.*, 2010). Models of substitution were tested for each gene alignment with jModelTest 0.1 (Guindon & Gascuel, 2003; Posada, 2008) under the Akaike information criterion (AIC). All datasets were analysed with MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003; Huelsenbeck & Ronquist, 2005) using the freely available Oslo University Bioportal Cluster (see Kumar *et al.*, 2009). Analyses of individual genes were run for 40 million generations. All runs were sampled at every 1000th generation under the six parameter general time reversible (GTR) model with gamma distributed rate variation across sites. Convergence was ascertained by checking the log likelihood graphs, the average standard deviation of split frequencies, and the potential scale reduction factor (PSRF). The first 25% of the sampled trees were regarded as the burn-in period. Since chain mixing was low under the default settings (four MCMC chains for each run and a heating parameter of 0.2) the dataset was analysed using eight MCMC chains in each run with a heating parameter of 0.1 to ensure sufficient chain mixing and a reliable sample from the posterior distribution. Consensus trees were built from two independent runs of 30,000 samples each for the Bayesian analyses. A maximum likelihood analysis was also run on the combined dataset using RAxML v.7.0.3 (Stamatakis, 2006) with 1000 bootstrap replicates to check for congruence with the Bayesian analysis.

TAXONOMY

SYSTEMATICS

Class ENOPLA Schultze, 1851
Order MONOSTILIFERA Brinkmann, 1917
Family AMPHIPORIDAE McIntosh, 1874
Genus *Amphiporus* Ehrenberg, 1831
Amphiporus rectangulus sp. nov.
(Figures 2–5)

TYPE MATERIAL

Holotype: sexually mature male, series of transverse sections (voucher ZMBN 88199, The University Museum of Bergen, Norway). (Skagerrak, Norway; trip 'BIOSKAG 2' with RP-sled; sand; coordinates: 58°57.250′N 10°34.403′E; water depth: 220 m); 18 May 2009. Partial COI mt-DNA sequence of the species has been deposited in GenBank (Accession number KC812587).

Paratype: sexually mature male, series of transverse sections (voucher ZMBN 88200, The University Museum of Bergen, Norway). (Skagerrak, Norway; trip 'BIOSKAG 2' with RP-sled; sand; coordinates: 58°57.250′N 10°34.403′E; water depth: 220 m); 18 May 2009. Partial COI mt-DNA sequences of the paratype and two other specimens are deposited in Genbank (Accession numbers KC812588, KC812589, KC812590).

Additional specimens: sexually mature males and females (Department of Biology and Environmental Sciences, University of Gothenburg). (Frøysjøen, Norway; RP-sled; mud; coordinates: 61°49.417′N 5°13.617′E; water depth: 450 m); 20 July 2012.

DIAGNOSIS

The genus *Amphiporus* is poorly defined in absolute terms and most characters are not genus specific. A compilation of available literature (Bürger, 1904; Gibson & Crandall, 1989; Maslakova *et al.*, 2005) renders the following list of morphological characters for the genus: proboscis well developed, rhynchocoel extends to or almost to posterior end of body, apical organ present, body wall musculature generally well developed without diagonal muscle layer between circular and longitudinal muscles, blood vascular system with three longitudinal vessels, nervous system without neurochords or neurochord cells, intestinal caecum present with a pair of anterior diverticula as well as lateral pouches, cerebral organs anterior to cerebral ganglia, excretory system positioned in the foregut region, sexes separate. None of these

Table 1. Differences in number of characters (internal and external) between *Amphiporus rectangulus* sp. nov., *Raygibsonia bergi* and *Vieitezia luzmurubeae*, the three species described with character matrix.

	Internal characters (90)	External characters (20)	Total characters (116)
Amphiporus rectangulus sp. nov. vs *Raygibsonia bergi*	31 (34.5% of 90)	16 (80% of 20)	47 (40.5% of 116)
Amphiporus rectangulus sp. nov. vs *Vieitezia luzmurubeae*	27 (30% of 90)	14 (70% of 20)	41 (35.3% of 116)
Raygibsonia bergi vs *Vieitezia luzmurubeae*	32 (35.6% of 90)	14 (70% of 20)	46 (39.7% of 116)

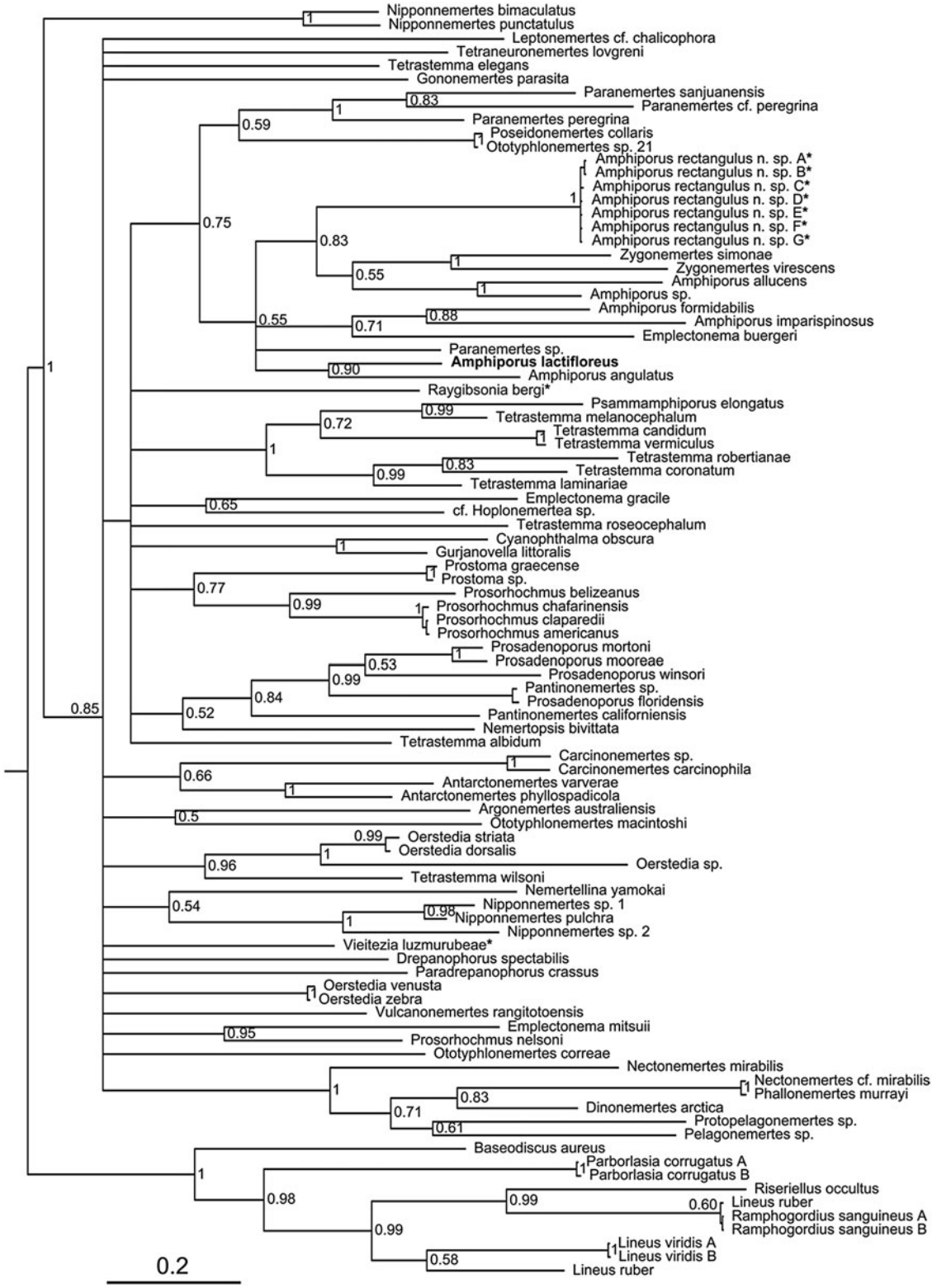

Fig. 1. Phylogenetic relationships of Hoplonemertea inferred from Bayesian analysis of COI mt-DNA. Numbers at nodes refer to posterior probability. The tree is rooted on outgroups represented by members of Heteronemertea. Asterisks mark species that have been coded according to the character matrix provided by Sundberg *et al.* (2009). We have intentionally kept all branches with pp50%, partly to show the different proposed clades, but mainly to show the very weak support for existing genera.

characters contradicts the inclusion of our new species in the genus. The rhynchocoel wall is, for many *Amphiporus* spp., described with separate circular and longitudinal muscle layers. This does not directly apply to *Amphiporus rectangulus* sp. nov., whose rhynchocoel wall musculature is difficult to define (character 43).

Earlier DNA analyses have repeatedly shown the genus *Amphiporus* to be non-monophyletic (see e.g. Sundberg

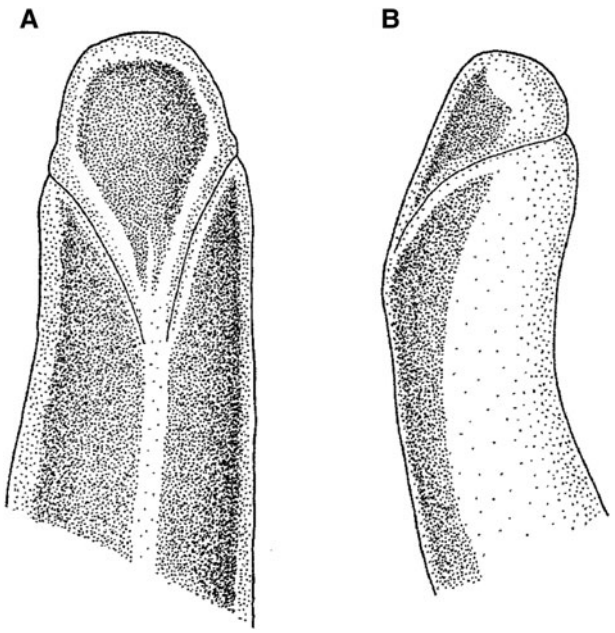

Fig. 2. *Amphiporus rectangulus* sp. nov.: (A) drawing of the anterior part of the body of one specimen to show the 'V' pattern and the cephalic furrows in detail; (B) drawing of the lateral view of the anterior part. Type status: additional specimens. Department of Biology and Environmental Sciences, University of Gothenburg.

et al., 2009; Kajihara *et al.*, 2011). Our analysis supports these results. *Amphiporus rectangulus* sp. nov. nests however (Figure 3) in the same clade as the type species of the genus, *Amphiporus lactifloreus* (Johnston, 1828).

DESCRIPTION

External features. Most of specimens examined alive, before or after anaesthetization, up to 1 cm long and approximately 1 mm wide. Appearance sturdy and chubby. Body soft and movement calm. Body shape very variable, but tapering backwards. Dorsal side brownish, like nougat, with ventral side and lateral margins pale. Furrows pale, deep, and very obvious encircling head meeting in rather steep V-shape dorsally (Figure 2). In some specimens, single pale stripe present, commencing behind V-shape as longitudinal pigmentation reaching backwards full or nearly full body length. Other specimens lacking this paler stripe; coloration obviously variable intra-specifically (Figure 4). No eyes distinguishable. Body wall quite thick, through which rhynchocoel and proboscis difficult to observe.

Body wall, musculature and parenchyma. Epidermis glandular, reaching up to 130 μm in thickness (Figure 5B). Connective tissue dermal layer up to 15 μm, proximally bordering epidermis (Figure 5B). Body wall musculature comprising outer circular and inner longitudinal layers, respectively 10–15 μm and 35–40 μm in thickness (Figure 5F).

Proboscis apparatus. Proboscis pore situated antero-ventrally near tip of the head opening into rhynchodaeum whose epithelium being neither ciliated nor glandular (Figure 5A, E). Rhynchocoel reaching posterior tip of body, its wall lacking distinct musculature, appearing as simple connective membrane (Figures 4A, 5B). Proboscis epithelium well developed into papillae. In everted state, proboscis with outer circular and inner longitudinal muscle layers. Nerve supply consisting of neural ring with ten nerves situated between longitudinal muscle fibres (Figure 4A). Proboscis armature consisting of single central stylet and pair of accessory stylet pouches, containing 1–2 reserve stylets. Several specimens everted proboscis during preservation; no stylets observable in these specimens. Paired accessory stylet pouches not easily observed but clearly visible in sections. Shape of stylet bulb very difficult to determine due to artefacts in preparations (Figure 4B).

Alimentary canal. Oesophagus emerging directly from rhynchodaeum in front of brain, posteriorly reaching cephalic region. Oesophageal epithelium simple, without glands or cilia. Stomach situated under brain, developed into pyloric canal opening into dorsal wall of intestine. Intestinal caecum with two long anterior accessory pouches reaching forward to rear of brain lobes and alongside them (Figure 5F). Intestinal diverticula deeply branched and alternating with gonads in mature specimens (Figure 5G).

Circulatory system. Blood system following basic plan for enoplan nemerteans. Mid-dorsal blood vessel emerging as branch from right lateral vessel in cerebral region. For all body length, the lateral vessels running below lateral nerve cords (Figure 5D); these anastomosing anteriorly and posteriorly by cephalic and caudal loops, respectively. No vascular plug observed.

Nervous system. Brain well developed, with ventral cerebral lobes being much thicker than dorsal ones. Dorsal cerebral commissure longer and narrower than the ventral one (30 μm vs 40 μm in diameter respectively); former positioned anterior to latter. Outer neurilemma enclosing brain, but no inner neurilemma distinguished. Ventral lobes being confluent with lateral nerve cords, which throughout their length containing only single neuropile; no accessory lateral nerves (Figure 5D). Posterior junction of lateral nerve cords subintestinal. Neither neurochords nor neurochord cells distinguished in nervous system. Dorsal cephalic nerves leading forward from anterior borders of brain lobes into cephalic-gland region; two buccal nerves reaching rhynchodaeum pore (Figure 5E).

Frontal organ and cephalic glands. Small, single apical organ opening at very tip of head, 40 μm in diameter (Figure 5C). Cephalic glands scattered between muscle fibres in anterior part of head, forming quite prominent distinct lobes (Figure 5A), opening via apical organ.

Sense organs. No eyes found. Cerebral sensory organs large, located all alongside brain. 65 μm in height, 145 μm in length, each opening laterally by simple, unforked, thick-walled ciliated canal leading inwards from cephalic furrow (Figure 5F). A well-developed cap of acidophilic glands extending a short distance along upper margins of organs.

Excretory system. Well developed, confined to post-cerebral region of body, consisting of two pairs of thick-walled

Fig. 3. *Amphiporus rectangulus* sp. nov.: (A) photograph of a complete specimen with the characteristic 'V' shaped cephalic furrows; (B) photograph of a complete specimen with the papillated proboscis everted; (C) group of specimens together to compare the differences between males (♂) and females (♀). Type status: additional specimens. Department of Biology and Environmental Sciences, University of Gothenburg.

longitudinal collecting tubes running close to lateral nerve cords, and opening by lateral nephridiopores in pyloric region (Figure 5D). No flame cells observed.

Reproductive system. Specimens collected having mature gonads; sexes are separate. No sexual colour dimorphism. Gonads serially disposed along body from pyloric region backwards, lying from dorsal to ventro-lateral margins between intestinal diverticula (Figure 5G). Holotype mature male, with deeply bilobate testes. No gonopore found in two different males. Mature testes is 120 μm in diameter.

ECOLOGY

Specimens were found on both mud bottoms and sandy bottoms. In May 2009 most specimens seem to be sexually mature (Figure 3C). Other organism groups highly represented in the same samples where Sipuncula, Caudofoveata, Bivalvia, Gastropoda, Polychaeta.

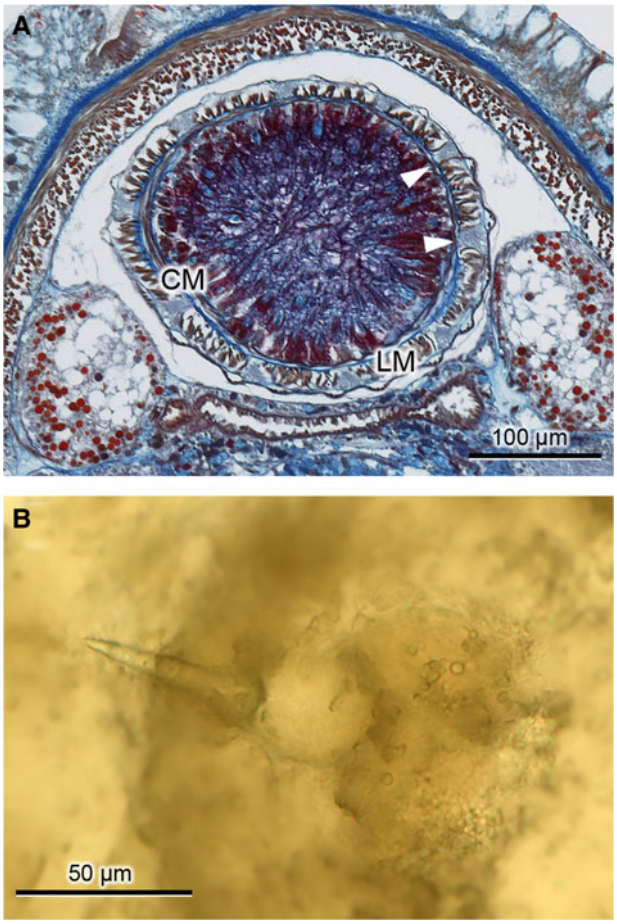

Fig. 4. *Amphiporus rectangulus* sp. nov.: (A) section through pyloric region showing the details of the proboscis and its ten proboscis nerves (arrowheads); (B) microphotograph of a squeezed specimen showing the stylet and its basis. Abbreviations: CM, circular muscle layer; LM, longitudinal muscle layer. Type status: holotype. Voucher ZMBN 88199. The University Museum of Bergen, Norway.

ETYMOLOGY

The name *rectangulus*, a Latin adjective, refers to the shape of the live animal body in transverse section.

RESULTS

Morphology

Complete matrix for *Amphiporus rectangulus* sp. nov. is available (Appendix 1). Between the three hoplonemertean species that have available data we find that the highest number of character differences lies within external morphology. For the comparative matrix see Appendix 2.

The character matrix (see Appendix 1) contains in total 120 characters, 16 of which are only applicable for palaeonemerteans. Of the characters relevant for hoplonemerteans, we were able to code 100, only hesitating on four (regarding gonopores and stylet basis).

Note on matrix correction: character 113 (cephalic gland type) is not only applicable for Palaeonemertea as stated in Sundberg *et al.* (2009). It is also available for Hoplonemertea.

Also, there is a new character added to the matrix with two different states. Character 76a: mid-dorsal blood vessel and lateral blood vessel connection. State 0: from the right lateral blood vessel. State 1: from the left lateral blood vessel.

Molecular analysis

Results from the molecular analysis are consistent with previous studies using COI mt-DNA. Hoplonemerteans form a monophyletic group which inside has very little or no resolution in relation to existing genera.

DISCUSSION

The informative value of a comparative morphology matrix, in terms of relationship or species definitions, with only three hoplonemertean species, is of course extremely low. We need amounts of data of completely other dimensions if we are to evaluate the information potential with the approach. More matrix-based species descriptions will enable the testing of whether histology is helping us towards better phylogenetic resolution and/or to clarify species delimitation issues. In Table 1 we present differences in the number of characters (internal and external). Considering the fact that the three investigated species are obviously different in many aspects—genus placement, appearance, sampling localities and so on—we may expect to find differences of some obvious magnitude regarding morphological characters as well as some genetic distance between the three of them. It is, therefore, interesting to note that even though the listed number of external characters is rather low, and could be improved both in terms of numbers and resolution, most of the counted differences are to be found here. Between the 90 listed internal characters (traditionally said to be of most importance), there are only around 30 characters that show any absolute differences between the three of them. In Appendix 2 there are possibilities for readers to check whether these differing internal characters can be related to, for example, genus diagnosis. We found no such systematic or taxonomic correlations within our set of data. This is consistent with the discussion (Strand & Sundberg, 2011) provided when describing *Pseudomicrura afzelii* with external morphology in combination with DNA. So, what are the useful characters for describing a ribbon worm species? For nemertean taxonomy, species descriptions and systematics, the difficult internal anatomical characters that we can come up with, may not be the most informative, even if they are of high biological interest. To recognize a live and fresh specimen and identify it to species level can be fairly straightforward with a description carrying a combination of notes on habitat, ecology, behaviour and external features. With a barcoding sequence attached, the species determination can be made in a more absolute sense. The molecular analyses (see Figure 1) that were performed in this study are consistent with all previous studies on COI and nemerteans. The resolution of the phylogenetic tree is in parts poor, which reflects the fact that COI is relatively unconserved, and carries many neutral mutations. More genes from both the nuclear and mitochondrial genomes would be ideal for better resolution, but it is difficult to reach the taxonomic breadth represented by the species for which COI sequences are available. However, hoplonemerteans form a monophyletic group. Within this group there are some clades that verify a generic context, but for many clades it is obvious that

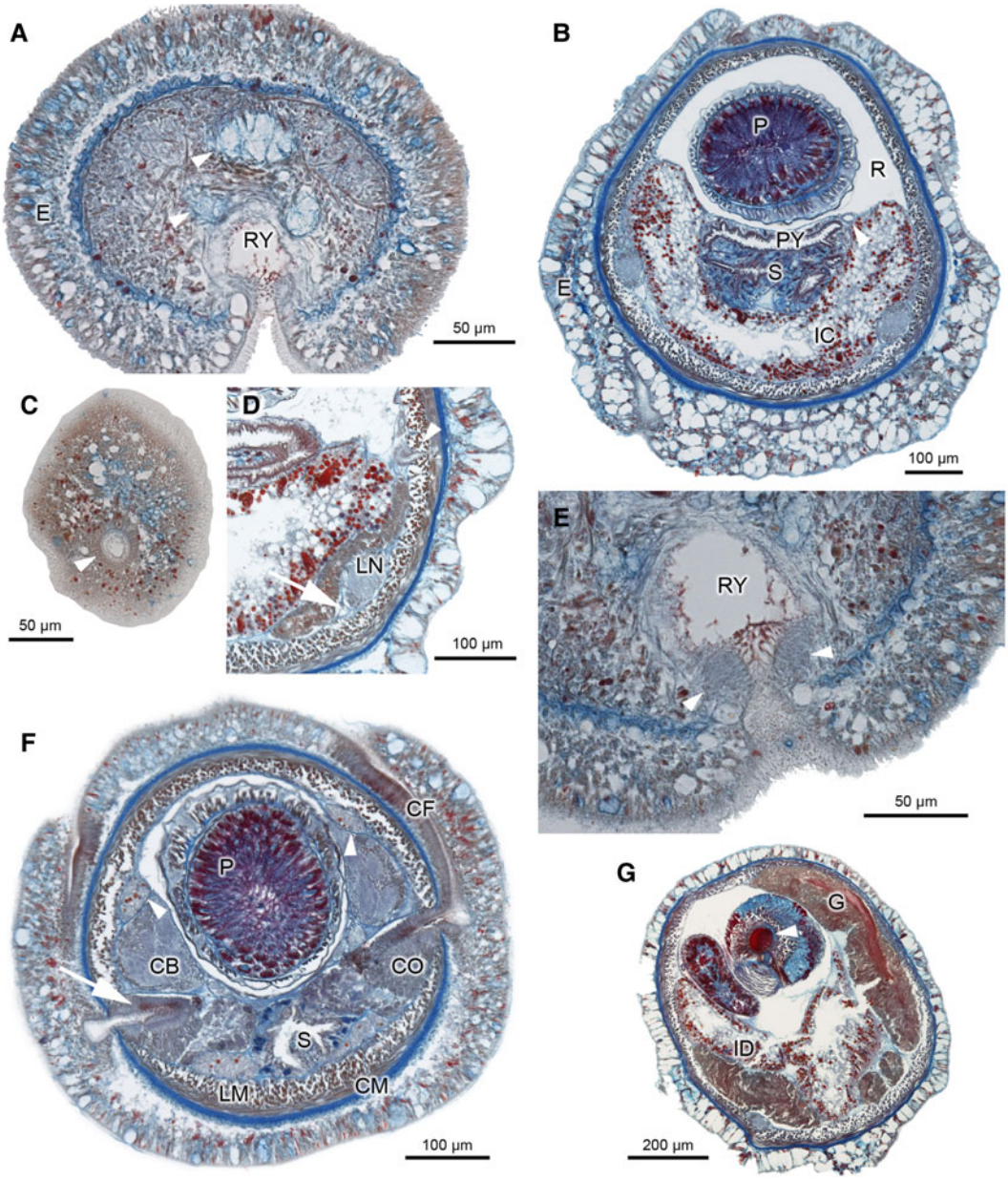

Fig. 5. *Amphiporus rectangulus* sp. nov.: (A) section through the anterior-most part of body showing the cephalic glands (arrowheads); (B) section through the pyloric region showing the parts of the alimentary system; note a mid-dorsal blood vessel (arrowhead); (C) section through the apical region of the body showing the apical organ (arrowhead); (D) detail of a section through the pyloric region showing the lateral nerve cord, the lateral blood vessel (arrow) and one excretory tubule (arrowhead); (E) detail of a section through the cephalic region showing the buccal nerves (arrowheads); (F) section through cephalic region showing the ciliated canals (arrow) that connect the cephalic furrows with the cerebral organs; note the anterior accessory pouches of the intestinal caecum (arrowheads); (G) section through the proboscis armature region of body showing the stylet apparatus (arrowhead), the deeply branched intestinal diverticula and the gonads. Abbreviations: CB, cerebral ganglion; CF, cephalic furrow; CM, circular muscle layer; CO, cerebral organ; E, epidermis; G, gonad (testis); IC, intestinal caecum; ID, intestinal diverticulum; LM, longitudinal muscle layer; LN, lateral nerve cord; P, proboscis; PY, pylorus; R, rhynchocoel; RY, rhynchodaeum; S, stomach. Type status: holotype. Voucher ZMBN 88199. The University Museum of Bergen, Norway.

taxonomy does not reflect natural groups, even with poor resolution. Judging from the tree it can also be said that the barcoding approach has possibilities. For example, 'regular' members of the genus *Tetrastemma* gather in clades, while a few species of the genus separate from these. Going back to morphology, the separated species are also the ones with deviant morphological diagnosis and appearance. Our study opens new approaches for tidying up the taxonomic waste basket that most nemertean species currently dwell in. The nemertean systematics and the relationship between different taxonomic entities is a complicated issue. More than 60% of all valid nemertean genera are monotypic, while a handful of genera carry hundreds of species. To our knowledge, this has nothing to do with reality, but rather reflects historical inconsistencies and the pitfalls of taxonomic rules and traditions. Of the more species-numerous genera, very few have exclusive diagnoses (see Diagnosis part in the description above), and none of them hitherto tested with genetics have been shown monophyletic (see Results above). Resolving phylogeny, and have taxonomy reflect this, is a work of major effort in the phylum Nemertea. Our results are likely to be valid for more groups with similar complications—that is,

benthic animals with soft bodies, small size and rarely found in numbers high enough to declare intraspecific variation (e.g. certain nudibranchs or sipunculans).

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APPENDICES

Appendix 1. Character matrix for *Amphiporus rectangulus,* sp. nov. character states and code from Sundberg *et al.* (2009). N/A: no applicable character.

	Character	Character state	Code
1.	Biology	Free-living	0
2.	Habitat	Marine	0
3.	Benthic divisions	Bathyal 250–453 m	3
4.	Pelagic divisions	Benthic	N/A
5.	Substrate	Sand	1
6.	Behaviour when mechanically disturbed	Contracts without coiling into a spiral	0
	External morphology		
7.	Cephalic furrows	One pair	1
8.	Distribution of anterior cephalic furrows	Ventral and dorsal	4
9.	Shape of anterior (dorsal) cephalic furrows	V-shape	0
10.	Shape of posterior (dorsal) cephalic furrows	Not applicable	N/A
11.	Head demarcated from body	No	0
12.	Position of cephalic furrows (H)	Single pair (ring) both in front and behind the brain lobes	0/1
13.	Shape of head/cephalic lobe	Bluntly rounded	1
14.	Head viewed laterally	Without extensions	0
15.	Shape of posterior tip	Bluntly pointed	1
16.	Eyes	Absent	0
17.	Eye morphology	Not applicable	N/A
18.	Relative eye size	Not applicable	N/A
19.	Eye distinctiveness	Not applicable	N/A
20.	Eye position relative to brain lobes	Not applicable	N/A
21.	Colour pattern	Extending full body length on dorsal surface	2
22.	Primary dorsal body colour	Brown	5
23.	Body colour hue/tint	Without hue/tint	0
24.	Internal organs visible through dorsal epidermis	No	0
25.	Lateral margins	Lateral margins appear paler than dorsal body surface	0
26.	Distribution of bristles/cirri	Not seen	0
	Internal morphology		
	Body wall		
27.	Epidermis non-cellular inclusions	Absent	0
28.	Epidermis of anterior body (P)	Not applicable	N/A
29.	Ratio thickness of epidermis/lateral body diameter in brain region (P)	Not applicable	N/A
30.	Dermis	Forming a distinct zone between epidermis and circular muscle layer	0
31.	Thickness of dermis	Approximately the same thickness as circular muscle layer	3
32.	Muscle processes from dermis into epidermis (P)	Not applicable	N/A
33.	Muscle layers	Outer circular, inner longitudinal	0
34.	Muscle crosses between body wall and circular muscle layers (P)	Not applicable	N/A
35.	Body wall longitudinal muscle layers just behind brain (H)	Not anteriorly divided	0
36.	Precerebral septum (H)	Not available	3
37.	Central (medial) muscle plate (P)	Not applicable	N/A
38.	Parenchyma	Barely distinguishable as a membrane enclosing various body organ systems	0
39.	Muscle fibres in mouth/foregut region (P)	Not applicable	N/A
	Proboscis apparatus		
40.	Proboscis pore	Subterminal, ventral	1
41.	Mouth and proboscis pore connection (H)	Open into atrium/rhynchodaeum	1
42.	Gland cells of rhynchodaeum	Absent	0
43.	Rhynchocoel musculature	Without distinct musculature, appearing as a simple membrane	0
44.	Rhynchocoel musculature in posterior end (P)	Not applicable	N/A
45.	Rhynchocoel length	Extending to or almost to posterior region of body	2
46.	Rhynchocoelic caeca	Absent	0
47.	Size of posterior third of proboscis region	Small, less than 50% of body diameter in retracted position	0
48.	Musculature of proboscis (everted state)	Outer circular and inner longitudinal muscle layers	0
49.	Musculature of posterior proboscis region (everted state)	Outer circular and inner longitudinal muscle layers	2
50.	Epithelium of anterior proboscis region (everted state)	Papillae	1
51.	Number of proboscis nerves	Ten	3
52.	Proboscis nerve arrangement	Peripheral neural sheath absent or indistinct	0

Continued

Appendix 1. Continued.

	Character	Character state	Code
53.	Secondary proboscis nerves (H)	Absent	0
54.	Proboscis armature	With central and accessory stylets	2
55.	Number of accessory stylet pouches (H)	Two	0
56.	Number of stylets in each accessory stylet pouch (H)	One or two	0
57.	Stylet: basis/stylet ratio (H)	Not observed	–
58.	Stylet shaft	Smooth and straight	0
59.	Shape of stylet basis	Not observed	–
60.	Median waist of stylet basis	Not observed	–
61.	Proboscis used for locomotion	Unknown	0
	Alimentary system		
62.	Position of mouth	Under brain	0
63.	Oesophagus	Present	1
64.	Oesophagus epithelium	Unciliated without glands	1
65.	Stomach	Not regionally differentiated	0
66.	Stomach connection with intestine (H)	Posterior stomach developing into pyloric canal which opens into dorsal wall of intestine	1
67.	Length of pyloric canal (H)	As long as stomach	2
68.	Intestinal caecum	Present, ventral	1
69.	Anterior pouches on intestinal caecum (H)	Long, reach forward to rear of brain lobes or alongside them	1
70.	Lateral diverticula on intestinal caecum (H)	Absent	0
71.	Intestinal diverticula	Deeply branched pouches	3
	Circulatory system		
72.	Cephalic vasculature	Arranged as a simple cephalic loop	0
73.	Vascular plugs (H)	Absent	0
74.	Rhynchocoelic villus (P)	Not applicable	N/A
75.	Position of lateral blood vessels (P)	Not applicable	N/A
76.	Mid-dorsal blood vessel	Does not divide in brain region	1
76a.	Mid-dorsal blood vessel and lateral blood vessel connection	From the right lateral blood vessel	0
77.	Length of mid-dorsal blood vessel	Extends to posterior end of the body	0
78.	Extra vascular pouches/valves	Present	1
79.	Pseudometameric transverse connectives linking mid-dorsal and lateral blood vessels in intestinal region (H)	Absent	0
80.	Vascular plexus in foregut region (P)	Not applicable	N/A
	Nervous system		
81.	Location of cerebral ganglia and lateral nerve cords (P)	Not applicable	N/A
82.	Number of dorsal cerebral commisures	One	1
83.	Distinct outer neurilemma of cerebral ganglion	Present	1
84.	Inner neurilemma of cerebral ganglion	Absent	0
85.	Statocysts in brain tissue	Absent	0
86.	Lateral nerve cords (H)	Without accessory lateral nerve	0
87.	Accessory lateral nerve (H)	Not applicable	N/A
88.	Four large nerves in head region (P)	Not applicable	N/A
89.	Number of dorsal nerves (P)	Not applicable	N/A
90.	Posterior junction of lateral nerve cords	Subintestinal	1
91.	Neurochord cells in brain	Absent	0
92.	Neurochords in lateral nerve cords	Absent	0
93.	Myofibrillae in lateral nerve cords	Absent	0
94.	Position of myofibrillae in lateral nerve cords (H)	Not applicable	N/A
95.	Buccal nerves	Paired	1
	Excretory system		
96.	Excretory system	Present	1
97.	Extent of system	Confined to foregut region of body	0
98.	Excretory canal (P)	Not applicable	N/A
99.	Nephridial gland (P)	Not applicable	N/A
100.	Flame cells (H)	No flame cells distinguished	0
101.	Glandular components in excretory tubules	Absent	0
102.	Number of nephridiopores	Limited to one or two on each side of body	0
103.	Position of nephridiopores	Posterior, at or near posterior region of excretory system	2
	Reproductive system		
104.	Nature of sexes	Separate sexes	0
105.	Gonad arrangement in heterogamous taxa	Single gonad alternating with intestinal diverticula	0
106.	Gonad arrangement in hermaphroditic taxa (H)	Not applicable	N/A

Continued

Appendix 1. Continued.

	Character	Character state	Code
107.	Testes	Bilobate	1
108.	Sexual colour dimorphism (H)	Absent	0
109.	Gonoduct position	Not observed	–
110.	Nature of reproduction	Oviparous	0
	Sensory organs		
111.	Apical organ	Present	1
112.	Typical cephalic glands	Confined to anterior half of head	1
113.#	Cephalic gland type	Scattered between muscle fibres in anterior part of head, forming distinct lobules further back	2
114.	Opening of cephalic glands	Via apical organ	0
115.	Position of cerebral sensory organs in relation to brain	Alongside brain	3
116.	Position of cerebral sensory organs in relation to epidermis	Separate from blood vessels under body wall muscle layers	2
117.	Size of cerebral sensory organs	More than half the size of brain lobes	1
118.	Ciliated cerebral canal (H)	Unforked	0
119.	Side organs (P)	Not applicable	N/A
120.	Sensory pits in head region (P)	Not applicable	N/A

*New character in the matrix:
76a. Mid-dorsal blood vessel and lateral blood vessel connection
From the right lateral blood vessel 0
From the left lateral blood vessel 1
This character (cephalic gland type) is not only applicable for Palaeonemertea as stated in Sundberg *et al.* (2009). It is also available for Hoplonemertea.

Appendix 2. Comparative matrix for characters of *Amphiporus rectangulus,* sp. nov., *Raygibsonia bergi* and *Vieitezia luzmurubeae*. Character states and code from Sundberg *et al.* (2009). N/A, no applicable character, N/O, not observed character. Characters with the same state and palaeonemertean characters are excluded.

	Character	*Amphiporus rectangulus* sp.nov.	*Raygibsonia bergi*	*Vieitezia luzmurubeae*
Habitat	1.	0	0	0/2
	3.	3	2	2
	5.	1	0/2	3/4/5
	6.	0	N/O	0
External morphology	7.	1	2	1
	8.	4	0	3
	10.	N/A	0	N/A
	11.	0	1	2
	12.	0/1	2	1
	13.	1	0	2
	14.	0	1	0
	15.	1	1	0
	16.	0	9	3
	17.	N/A	0	0
	18.	N/A	1	0
	19.	N/A	1	0
	20.	N/A	0	0
	21.	2	3	2
	23.	0	2	2
	24.	0	1	1
	25.	0	0	1
	26.	0	2	1
Body wall	27.	0	N/O	2
	36.	3	0	0
Proboscis	40.	1	0	1
	42.	0	1	0
	43.	0	2	2
	47.	0	1	0
	52.	0	1	1
	56.	0	1	1
	57.	–	0	0
	59.	–	1	1
	60.	–	1	1

Continued

Appendix 2. Continued.

	Character	*Amphiporus rectangulus* sp.nov.	*Raygibsonia bergi*	*Vieitezia luzmurubeae*
Alimentary system	62.	0	0	N/A
	64.	1	2	1
	67.	2	2	3
	69.	1	2	2
	70.	0	1	1
	71.	3	1 ?	1
Circulatory system	73.	0	1	0
	74.	N/A	N/A	0
	76.	1	1	2
	77.	0	1	N/O
	78.	1	0	1
Nervous system	86.	0	1	0
	87.	N/A	2	N/A
	90.	1	N/O	1
	93.	0	1	0
	94.	N/A	0	N/A
	95.	1	0	0
Excretory system	100.	0	N/O	0
	101.	0	N/O	0
	102.	0	N/O	0
	103.	2	N/O	0
Reproductive system	104.	0	0?	2
	105.	0	0	N/A
	106.	N/A	N/A	2
	107.	1	N/A ?	0
	108.	0	N/O	1
	109.	N/O	2	1
	110.	0	N/O	0
Sensory organs	112.	1	2	3
	113.	2	2	0
	115.	3	2	2
	117.	1	1	0

Appendix 3. List of nemertean species included in the phylogenetic analysis, together with collection site and GenBank Accession number. Six species of heteronemerteans are used as the outgroup.

Taxon	Accession number	Collection site
Hoplonemertea		
Acteonemertidae		
Argonemertes australiensis (Dendy, 1892)	HQ848601	North-West Tasmania
Leptonemertes cf. *Chalicophora* (Graff, 1879)	HQ848596	Link Port, Fort Pierce, FL, USA
Amphiporidae		
Amphiporus allucens Bürger, 1895	KC812591	Humlesäcken, Sweden
Amphiporus angulatus (Müller, 1774)	AJ436896	Cobscook, ME, USA
Amphiporus formidabilis Griffin, 1898	AJ436897	San Juan Island, WA, USA
Amphiporus imparispinosus Griffin, 1898	HQ848612	Cattle Point, San Juan Island, WA, USA
Amphiporus lactifloreus (Johnston, 1828)	HQ848611	Penmon, Isle of Anglesey, Wales, UK
Amphiporus rectangulus sp. nov. Strand *et al.*, 2013	KC812587	Skagerack, Sweden–Norway
Amphiporus rectangulus sp. nov. Strand *et al.*, 2013	KC812588	Skagerack, Sweden–Norway
Amphiporus rectangulus sp. nov. Strand *et al.*, 2013	KC812589	Skagerack, Sweden–Norway
Amphiporus rectangulus sp. nov. Strand *et al.*, 2013	KC812590	Skagerack, Sweden–Norway
Amphiporus rectangulus sp. nov. Strand *et al.*, 2013	KC812603	Skagerack, Sweden–Norway
Amphiporus rectangulus sp. nov. Strand *et al.*, 2013	KC812604	Skagerack, Sweden–Norway
Amphiporus rectangulus sp. nov. Strand *et al.*, 2013	KC812605	Skagerack, Sweden–Norway
Amphiporus sp.	EU255601	Massachusetts, USA
Gurjanovella littoralis Ushakov, 1926	AJ436904	Kandalaksha Bay, White Sea, Russia
Poseidonemertes collaris Roe & Wickham, 1984	AJ436919	Bodega Bay, CA, USA
Psammamphiporus elongatus (Stephenson, 1911)	HQ848609	Praia de Vilar, Corrubedo, Ribeira, A Coruña, Galicia, Spain
Vulcanonemertes rangitotoensis Gibson & Strand, 2002	AB505828	Takapuna, New Zealand
Zygonemertes simonae Corrêa, 1961	AJ436922	Fort Pierce, FL, USA

Continued

Appendix 3. Continued.

Taxon	Accession number	Collection site
Zygonemertes virescens (Verrill, 1879)	HQ848590	Link Port, Fort Pierce, FL, USA
Carcinonemertidae		
Carcinonemertes carcinophila (Kölliker, 1845)	HQ848619	Beaufort, North Carolina, USA
Carcinonemertes sp.	AJ436901	São Sebastião, Brazil; on *Callinectes danae*
Cratenemertidae		
Nipponnemertes bimaculatus (Coe, 1901)	AJ436909	San Juan Island, WA, USA
Nipponnemertes pulchra (Johnston, 1837)	HQ848597	Tjärnö, Koster Area, Skagerrak, Sweden
Nipponnemertes punctatulus (Coe, 1905)	AJ436910	Oshoro, Hokkaido, Japan
Nipponnemertes sp. 1	HQ848598	Santa Rosa-Cortes Ridge, CA, USA
Nipponnemertes sp. 2	HQ848599	Talcahuano, west Side of Peninsula de Tumbes, Región VIII: Biobío, Chile
Dinonemertidae		
Dinonemertes arctica Korotkevich, 1977	FJ602542	Alaska, Arctic Ocean
Drepanophoridae		
Drepanophorus spectabilis (Quatrefages, 1846)	HQ848610	Punta Santa Anna, Blanes, Girona, Spain
Emplectonemertidae		
Emplectonema buergeri Coe, 1901	HQ848600	Elliott Bay Marina, Dock N, Seattle, Washington, USA
Emplectonema gracile (Johnston, 1837)	NC016952	Unpublished, submitted 29 February 2012
Emplectonema mitsuii Yamaoka, 1947	AB505823	Jeju Island, Korea
Nemertopsis bivittata (Delle Chiaje, 1841)	HQ848608	Pawleys Island, South Carolina, USA
Paranemertes cf. *Peregrina* Coe, 1901	NC016952	Qingdao, China
Paranemertes peregrina Coe, 1901	AJ436915	San Juan Island, WA, USA
Paranemertes sanjuanensis Stricker, 1982	AJ436917	San Juan Island, WA, USA
Paranemertes sp.	AJ436916	San Juan Island, WA, USA
Nectonemertidae		
Nectonemertes cf. *mirabilis* Verrill, 1892	NC017874	Point Conception, CA, USA
Nectonemertes mirabilis Verrill, 1892	AJ436925	160 km off Point Conception, CA, USA
Paradrepanophoridae		
Paradrepanophorus crassus (Quatrefages, 1846)	HQ848603	Ribeira, A Coruña, Galicia, Spain
Oerstediidae		
Oerstedia dorsalis (Abildgaard, 1806)	FJ855363	Rhosneigr, Wales, UK
Oerstedia striata Sundberg, 1988	AY791972	Tjärnö, Sweden
Oerstedia venusta Iwata, 1954	AJ436911	Akkeshi Bay, Japan
Oerstedia zebra (Chernyshev, 1993)	AJ436912	Akkeshi Bay, Japan
Oerstedia sp.	EU489488	Faial, Azores, Portugal
Ototyphlonemertidae		
Ototyphlonemertes correare Envall, 1996	HQ848613	Saltö, Skagerrak, Sweden
Ototyphlonemertes macintoshi Bürger, 1895	HQ848605	Praia do Mindelo, Vila do Conde, Portugal
Ototyphlonemertes sp. 21	AJ436913	Nantucket, MA, USA
Prosorhochmidae		
Gononemertes parasita Bergendal, 1900	HQ848607	Koster Area, Skagerrak, Sweden
Pantinonemertes californiensis Gibson, Moore & Crandall, 1982	EF157597	Tomales Bay, CA, USA
Pantinonemertes sp.	AJ436916	off Carrie Bow Cay, Belize
Prosadenoporus floridensis Maslakova & Norenburg, 2008	EF157596	Link Port, FL, USA
Prosadenoporus mooreae (Gibson, 1982)	EF157595	Picnic Bay and in Cockle Bay, Magnetic Island, Australia
Prosadenoporus mortoni (Gibson, 1990)	EF157593	Nan Ao Island or Xiamen, Fujian Province, China
Prosadenoporus winsori Moore & Gibson, 1981	EF157594	Banks of Ross River, Townsville, Australia
Tetrastemma albidum (Coe, 1905)	EF157598	La Jolla, CA, USA
Prosorhochmus americanus Gibson, Moore, Ruppert & Turbeville, 1986	HQ848595	Rudee Inlet, Virginia, USA
Prosorhochmus belizeanus Maslakova & Norenburg, 2008	EF157591	Reef-crest berm of Carrie Bow Cay, Belize
Prosorhochmus chafarinensis Frutos, Montalvo & Junoy, 1998	EF157587	Savudrija and Zambratija, Adriatic Sea, Croatia
Prosorhochmus claparedii Keferstein, 1862	EF157589	Armintza, Bizkaia, Spain
Prosorhochmus nelsoni Sánchez, 1973	HQ848606	Coquimbo, Coquimbo Region, Chile
Pelagonemertidae		
Pelagonemertes sp.	AJ436924	160 km off Point Conception, CA, USA
Phallonemertidae		

Continued

Appendix 3. Continued.

Taxon	Accession number	Collection site
Phallonemertes murrayi (Brinkmann, 1912)	AJ436926	160 km off Point Conception, CA, USA
Protopelagonemertidae		
Protopelagonemertes sp.	AJ436927	160 km off Point Conception, CA, USA
Tetrastemmatidae		
Antarctonemertes phyllospadicola (Stricker, 1985)	FJ594418	Cattle Point, San Juan Island, Washington, USA
Antarctonemertes varvarae Chernyshev, 1999	AJ436900	Ussuri Bay, Sea of Japan, Russia
Cyanophthalma obscura (Schultze, 1851)	EF208980	Sweden
Nemertellina yamaokai Kajihara, Gibson & Mawatari, 2000	AJ436907	Akkeshi Bay, Japan
Tetrastemma candidum (Müller, 1774)	AY791973	Anglesey, Wales, UK
Tetrastemma coronatum (Quatrefages, 1846)	AY791976	Faial, Azores
Tetrastemma elegans (Girard, 1952)	AJ436920	Nahant, MA, USA
Tetrastemma laminarie Ushakov, 1928	AY791980	Trondheim, Norway
Tetrastemma melanocephalum Johnston, 1837	AY791988	Tjärnö, Sweden
Tetrastemma robertianae McIntosh, 1874	AY791994	Tjärnö, Sweden
Tetrastemma roseocephalum Yamaoka, 1947	KC812592	Jeju Island (South), Korea
Tetrastemma vermiculus (Quatrefages, 1846)	AY791996	Anglesey, Wales, UK
Tetrastemma wilsoni Coe, 1943	AJ436921	Edgewater, MD, USA
Family not assigned		
Prostoma graecense (Böhmig, 1892)	JX017298	Unpublished, submitted 4 May 2012
Prostoma sp.	HQ848594	Concord, Eastbrook Woods, MA, USA
Raygibsonia bergi Sundberg, Chernyshev, Kajihara, Kånneby & Strand, 2009	AY928351	Tjärnö, Sweden
Tetraneuronemertes lovgreni Sundberg, Gibson & Strand, 2007	EF208982	Humlesäcken, Sweden
Vieitezia luzmurubeae Junoy, Andrade & Giribet, 2011	HQ443426	Ría de Arousa, Pontevedra, Galicia, Spain (inside *Ciona intestinalis*)
Outgroup		
Heteronemertea		
Cerebratulidae		
Parborlasia corrugatus (McIntosh, 1876)	KC812593	Huinay, Chile
Parborlasia corrugatus (McIntosh, 1876)	KC812594	Huinay, Chile
Lineidae		
Lineus ruber (Müller, 1774)	KC812595	Punta Tumbes, Caleta Canteras, Chile
Lineus ruber (Müller, 1774)	KC812602	Crosby, England, UK
Lineus viridis (Müller, 1774)	KC812596	Penmon, Wales, UK
Lineus viridis (Müller, 1774)	KC812597	Penmon, Wales, UK
Ramphogordius sanguineus (Rathke, 1799)	KC812598	Rhos-on-Sea, Wales, UK
Ramphogordius sanguineus (Rathke, 1799)	KC812599	Penmon, Wales, UK
Riseriellus occultus Rogers, Junoy, Gibson & Thorpe, 1993	KC812600	Crosby, England, UK
Valenciniidae		
Baseodiscus aureus (Bürger, 1896)	KC812601	Punta Tumbes, Caleta Canteras, Chile