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Disentangling ribbon worm relationships: multi-locus analysis supports traditional classification of the phylum Nemertea

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Abstract

The phylogenetic relationships of selected members of the phylum Nemertea are explored by means of six markers amplified from the genomic DNA of freshly collected specimens (the nuclear 18S rRNA and 28S rRNA genes, histones H3 and H4, and the mitochondrial genes 16S rRNA and cytochrome *c* oxidase subunit I). These include all previous markers and regions used in earlier phylogenetic analyses of nemerteans, therefore acting as a scaffold to which one could pinpoint any previously published study. Our results, based on analyses of static and dynamic homology concepts under probabilistic and parsimony frameworks, agree in the non-monophyly of Palaeonemertea and in the monophyly of Heteronemerta and Hoplonemertea. The position of *Hubrechtella* and the Pilidiophora hypothesis are, however, sensitive to analytical method, as is the monophyly of the non-hubrechtiid palaeonemerteans. Our results are, however, consistent with the main division of Hoplonemertea into Polystilifera and Monostilifera, the last named being divided into Cratenemertea and Distromatonemertea, as well as into the main division of Heteronemertea into *Baseodiscus* and the remaining species. The study also continues to highlight the deficient taxonomy at the family and generic level within Nemertea and sheds light on the areas of the tree that require further refinement.

Nemertea (ribbon worms) is a phylum of mostly marine animals with a few species inhabiting limnic environments and is one of the few animal phyla that has successfully colonized the terrestrial environment—the others being one deuterostome phylum (Vertebrata), several ecdysozoans (Arthropoda, Onychophora, Tardigrada, Nematoda, and Nematomorpha) and three spiralian phyla (Annelida, Mollusca, and Platyhelminthes). With about 1280 described species (Gibson, 1995; Kajihara et al., 2008) (see Figs 1 and 2 for the habitus of some key representatives), Nemertea is considered by some to be a "minor" phylum, but it is widespread and also contains the longest metazoan ever recorded, *Lineus longissimus*, which can measure more than 30 m in length (McIntosh, 1873–1874). It also contains a large number of small species, of which many are interstitial and constitute an important component of the meiofauna, such as for example the genera *Ototyphlonemertes* and *Cephalothrix* (Norenburg, 1988),

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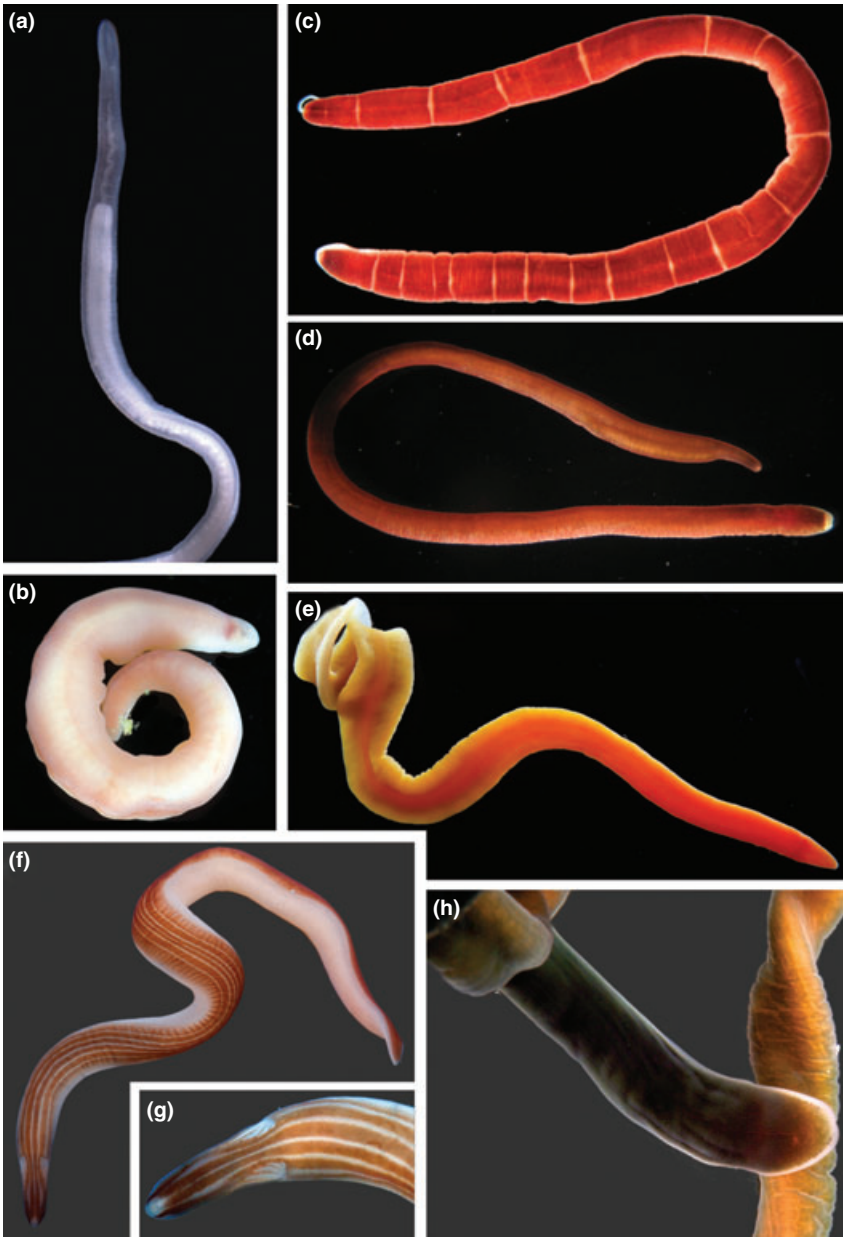

Fig. 1. Habitus of selected species of nemerteans studied in these analyses. (a) *Cephalothrix filiformis* from Sylt (Germany). (b) *Nipponnemertes* sp. 1 (MCZ DNA105622) from the Santa Rosa-Cortes Ridge, California (USA). (c) *Micrura fasciolata* from Tjärnö, Koster area, Skagerak (Sweden). (d) *Micrura purpurea* from Tjärnö (Sweden). (e) *Micrura ignea* from Isla Cristóbal, Archipiélago de Bocas del Toro (Panama). (f, g) *Drepanophorus spectabilis* from Punta Santa Anna, Blanes, Girona (Spain). (h) *Riseriellus occultus* from Crosby, Liverpool (UK). Photographs by J. v. Döhren (a), G. Rouse (c, d) and G. Giribet (b, e–h).

and a wide range of sizes between these two extremes. Most nemerteans are carnivores or scavengers. They use a protrusible, eversible proboscis to capture their prey, which sometimes is much larger than the nemertean itself. The proboscis is contained within a coelomic cavity (rhynchocoel), and together with the rhynchodeum forms the synapomorphic proboscis apparatus unique to the phylum. The position of the mouth relative to the proboscis pore is an important taxonomic character distinguishing the main classes of nemerteans—in palaeo- and heteronemerteans the mouth and the proboscis pore are separate, but they share an opening in most monostiliferan hoplonemerteans (with exceptions such as *Duosnemertes*).

The classification of nemerteans has been in constant flux, both at the intra-phylum level and with respect to the position of the phylum among metazoans. Schultze (1851) was the first to correctly understand the structure

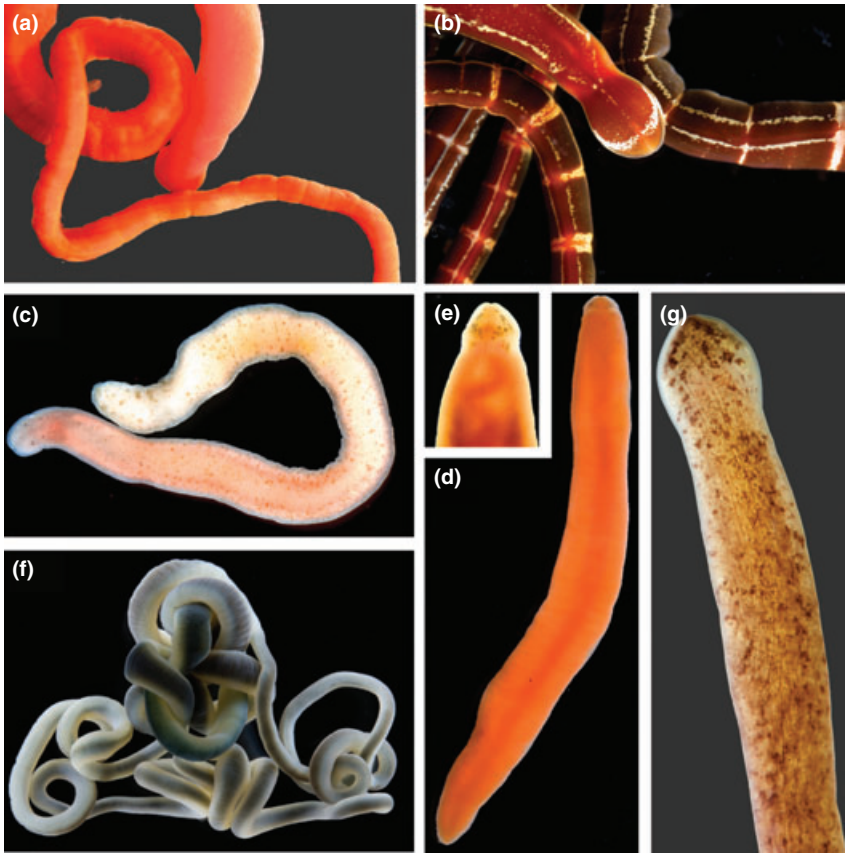

Fig. 2. Habitus of selected species of nemerteans studied in these analyses. (a) *Tubulanus polymorphus* from Cattle Point, San Juan Island, Washington (USA). (b) *Tubulanus sexlineatus* from Elliott Bay Marina, Dock N, Seattle, Washington (USA). (c) *Prostoma* cf. *eilhardi* from Concord, Eastbrook Woods, Massachusetts (USA). (d, e) *Nipponnemertes pulchra* from Tjärnö, Koster area, Skagerak (Sweden). (f) *Emplectonema gracile* from Crosby, Liverpool (UK). (g) *Emplectonema buergeri* from Elliott Bay Marina, Dock N, Seattle, Washington (USA). Photographs by G. Rouse (d,e) and G. Giribet (a–c, f–h).

and function of the proboscis complex, coining the term Rhynchocoela for the group. Later, Schultze (1852 (for 1853)) named the suborders Anopla and Enopla, following Johnston's (1837) grouping based on the absence or presence of a stylet apparatus in the proboscis, respectively. Schultze (and many before and after him) regarded nemerteans as turbellarians with a proboscis, and the view of a close relationship with Platyhelminthes prevailed into the late 20th century. It was not until the mid-1900s that the taxon was discussed as a phylum (e.g. Coe, 1943; Hyman, 1951). Stiasny-Wijnhoff (1923, 1936) proposed a classification of the more inclusive groups that has been mostly followed by subsequent authors (e.g. Coe, 1943; Gibson, 1994) and in textbooks (e.g. Ax, 1996; Brusca and Brusca, 2003). Stiasny-Wijnhoff (1936) used a system with two classes, dividing Anopla into two orders, Palaeonemertea and Heteronemertea, and Enopla into the two orders Hoplonemertea and Bdellonemertea. Hoplonemertea was further subdivided into the suborders Monostilifera and Polystilifera, the latter further divided into the tribes Reptantia and Pelagica. Although the ranking of these taxa has remained, the rank-naming has changed over time (Sundberg, 1991). Iwata (1960) proposed a new anoplan order, Archinemertea, to accommodate the cephalothricid palaeonemerteans, but subsequent analyses have shown it to be paraphyletic (e.g. Sundberg and Hylbom, 1994; Thollesson and Norenburg, 2003) (see Results below) and it is generally not used or recognized in more recent publications.

From the 1980s to the early 2000s, several numerical analyses of nemertean internal relationships appeared (e.g. Sundberg, 1985, 1990; Sundberg and Hylbom, 1994; Sundberg and Svensson, 1994; Härlin and Sundberg, 1995; Crandall, 2001; Härlin and Härlin, 2001; Maslakova and Norenburg, 2001; Schwartz and Norenburg, 2001; Sundberg et al., 2003) in a time when the phylogenetic placement of nemerteans within Bilateria was addressed with detailed ultrastructural analyses (Norenburg, 1985; Turbeville and Ruppert, 1985; Turbeville, 1986) and with the first cladistic analyses of

the metazoan phyla (e.g. Schram, 1991; Eernisse et al., 1992; Nielsen et al., 1996).

However, relationships among nemertean species were difficult to recover based on morphology alone due to their soft-bodied anatomy, prone to fixation artefacts, and the large degree of homoplasy observed within the phylum (Sundberg and Svensson, 1994; Schwartz and Norenburg, 2001; Sundberg et al., 2009). With the arrival of molecular systematics, nemertean workers rapidly tested the coelomate phylogenetic affinities of the phylum (e.g. Turbeville et al., 1992; Winnepenninckx et al., 1995; Giribet et al., 1996) and explored relationships among selected species. A series of articles focused on the relationships or population genetics of closely related taxa (Envall, 1997; Envall and Sundberg, 1998; Sundberg and Saur, 1998; Strand and Sundberg, 2005a,b; Mateos and Giribet, 2008; Chen et al., 2010), while others used molecular data in studies of descriptive taxonomy (e.g. Sundberg et al., 2003; Junoy et al., 2010; Puerta et al., 2010; Strand and Sundberg, 2011), often using fragments of one or two markers. A few studies focused on the higher taxonomy of nemerteans.

Sundberg et al. (2001) analysed the nuclear small ribosomal subunit RNA gene (18S rRNA) for 15 nemertean species representing the major nemertean clades to find paraphyly of the class Anopla, polyphyly of the order Palaeonemertea (Archinemertea were separated from Palaeonemertea *sensu* Gibson, 1994), and a sister-group relationship of Bdellonemertea and Hoplonemertea. Basal support and stability was low for most relationships, with the exception of the Bdellonemertea–Hoplonemertea clade (sometimes Bdellonemertea was nested within Hoplonemertea).

Thollesson and Norenburg (2003) published the most comprehensive account of nemertean relationships to date, using fragments of four molecular markers (28S rRNA, histone H3 and the mitochondrial markers 16S rRNA and cytochrome *c* oxidase subunit I) of 55 nemertean species representing all major clades. Their tree showed paraphyly of Anopla with respect to a monophyletic Enopla. Within Anopla, Palaeonemertea was also paraphyletic, with *Tubulanus* + *Procephalothrix* forming a clade sister to all other nemerteans, followed by *Carinoma*, and with *Hubrechtella* sister to Heteronemertea, the latter clade named Pilidiophora due to the shared presence of a pilidium larva. *Malacobdella* (formerly in the enoplan order Bdellonemertea) appeared nested deep inside the monostiliferan Hoplonemertea and therefore the order Bdellonemertea was abandoned, making Hoplonemertea a synonym of Enopla. The new Monostilifera showed a sister-group relationship between *Nipponnemertes* (representing Cratenemertidae, for which they proposed the new name Cratenemertea) and the remaining species, a clade they named Distromatonemertea (after Distromatorhynchocoelomia of Gibson, 1988), with roughly the same composition. Polystilifera was also monophyletic. They also introduced the name Neonemertea for Pilidiophora + Enopla.

Sundberg and Strand (2007) analysed the 18S rRNA gene of 22 nemerteans with the aim of placing the annulated hoplonemertean *Annulonemertes minusculus*, also finding paraphyly of Anopla and Palaeonemertea, but the study was more limited in non-hoplonemertean samples.

In an unpublished dissertation, Schwartz (2009) analysed fragments of the nuclear 28S rRNA gene, the mitochondrial genes 16S rRNA and cytochrome *c* oxidase subunit I, together with over 100 morphological characters, for a total of 62 nemerteans. The analyses focused on the clade Pilidiophora, as defined in Thollesson and Norenburg (2003), i.e. Heteronemertea plus *Hubrechtella* spp. with a pilidium larva. Her results did not support the monophyletic status of Pilidiophora, but low clade support values make the results somewhat inconclusive. There is furthermore little correlation between her results and the generic and familial taxonomy of the group.

While these studies agree in some fundamental points (monophyly of Hoplonemertea, including *Malacobdella*; paraphyly of Anopla and Palaeonemertea; discordance with low-level taxa), published data sets are based on different markers and non-overlapping taxa. For these reasons, we combined efforts to obtain fresh tissues from a wide array of nemertean species and sequenced six markers, including all fragments used in prior nemertean analyses, with the aim of making our data combinable with those of all previous studies. We therefore used the complete 18S rRNA gene, approximately 3 kb of 28S rRNA, histones H3 and H4, and the mitochondrial markers 16S rRNA gene and cytochrome *c* oxidase subunit I in order to obtain a well-supported intra-phylum phylogeny based on exemplar taxa covering all main groups. We furthermore aimed to test the composition of the clade names proposed by Thollesson and Norenburg (2003) (i.e. Pilidiophora, Neonemertea, Distromatonemertea).

Materials and methods

Specimens

This study is based mostly on freshly collected specimens by the authors (see Appendix 1 for collection sites and voucher numbers; Figs 1 and 2 for some represented species), including samples from Japan, China, USA, Central and South America, the European Atlantic and the Mediterranean coasts, among other locations. Fifty-seven taxa, of which seven remain undescribed or could not be reliably identified to species

level, were analysed (Appendix 1). Most specimens were preserved in RNA*later* (Ambion, Inc., Austin, TX) and shipped to Harvard University for nucleic acid extraction, and others were sent alive or preserved in high-grade EtOH for subsequent molecular work. Some of the specimens used in this study were also used for high-throughput sequencing using 454 and Illumina sequencing (our unpublished data). Some specimens were also fixed for ultrastructural work. The list of the 66 specimens used and their respective GenBank accession numbers are provided in Table 1.

Outgroup selection

In a recent study, Dunn et al. (2008) placed the phylum Nemertea in a clade with Nemertea and Brachiopoda, later called Kryptrochozoa (Giribet et al., 2009). This clade is grouped with Annelida, Sipuncula, and Mollusca in a larger Trochozoa clade (Hejnol et al., 2009). Based on previous evidence (e.g. Giribet et al., 2000; Dunn et al., 2008; Struck and Fisse, 2008; Hejnol et al., 2009; Paps et al., 2009a,b), the following 13 representatives were selected as outgroups: two brachiopods (*Terebratalia transversa* and *Novocrania anomala*), two phoronids (*Phoronis ijimai* and *P. hippocrepia*), three annelids (*Capitella teleta*, *Paranerilla limicola*, and *Urechis caupo*), two sipunculans (*Sipunculus nudus* and *Phascolion strombi*) and four molluscs (*Antalis entalis*, *Crepidula fornicata*, *Laevipilina hyalina*, and *Yoldia limatula*).

Nucleic acid purification

Total genomic DNA was extracted from the specimens using the DNeasy kit (Qiagen Inc., Valencia, CA), following the manufacturer's protocol.

PCR amplification

Six markers were amplified from the genomic DNA. The nuclear 18S rRNA gene was amplified with primer pairs 1F/5R, 3F/18Sbi and 18Sa2.0/9R (Giribet et al., 1996; Whiting et al., 1997). The nuclear 28S rRNA gene was amplified using the following set of primers: LSU3 and LSU5 (Littlewood, 1994); 28Srd1a and 28Srd4b (Edgecombe and Giribet, 2006); 28Sa (Whiting et al., 1997); 28Srd5b, 28Srd7b1, and 28Srd4.8a (Schwendinger and Giribet, 2005); and 28SF2762 and 28SR2012 (Giribet et al., 2010). The mitochondrial 16S rRNA gene fragment was amplified using the primer pair 16Sar-L/16Sbr-H (Palumbi et al., 1991). A stretch of the mitochondrial protein-encoding gene cytochrome *c* oxidase subunit I (COI) was amplified using the primer pair LCO1490/HCO2198 (Folmer et al., 1994). The nuclear genes histone H3 and H4 were amplified, respectively, using primer pairs H3aF and H3aR (Colgan et al., 1998) and H4-2S and H4-2ER (Pineau et al., 2005). The oligonucleotide sequences of all the primers are presented in Appendix 2.

PCR reactions were performed using AmpliTaq DNA polymerase (Perkin-Elmer, Waltham, MA). Thermal cycling was initiated with 2 min of denaturation at 94 °C followed by 35 cycles of 30 s at 94 °C, annealing (between 40 and 46 °C) for 1 min, and extension at 72 °C for 1 min. 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 Corp., 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 1.5 μL PCR product, 1 μM PCR primer, 0.25 μL ABI BigDye 5× sequencing buffer, and 0.5 μL ABI BigDye Terminator ver. 3.0 (Applied Biosystems, Foster City, CA). The sequencing reactions consisted of an initial denaturation step for 3 min at 95 °C, followed by 25 cycles of 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).

Sequence analysis

Chromatograms were edited and overlapping sequence fragments were assembled using Sequencher 4.8 (Gene Codes Corp., 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 contamination. In total, six data sets were analysed and MEGA 4.0.1 (Tamura et al., 2007) 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 are deposited in GenBank (accession numbers in Table 1).

Alignment and phylogenetic analyses

Multiple sequence alignment of all markers was performed with MAFFT ver. 6 using the strategy G-INS-i (Katoh et al., 2005), with the following parameters: gap penalty of 1.53 for COI and 16S rRNA and histones, 3 for 18S rRNA and 28S rRNA; scoring matrix for nucleotide sequences of 200PAM/K2; offset value of 0.0. We then ran two sets of analyses, one using the alignment originally obtained by MAFFT and a second set after removing uncertain positions in the ribosomal genes, identified with GBlocks ver. 0.91b (Castresana, 2000). For this, 60% was used as the minimum number of sequences for a conserved position

Table 1
List of species (and taxonomy) with MCZ voucher numbers and GenBank accession numbers for the amplified fragments

Exemplar	MCZ voucher	18S rRNA	28S rRNA	Histone H3	Histone H4	16S rRNA	COI
Baseodiscus sp.	DNA105581	JF293052	HQ856862	–	JF277689	JF277568	HQ848588
Baseodiscus sp.	DNA105588	JF293046	HQ856866	JF277749	JF277667	JF277569	HQ848589
Cerebratulus lacteus	DNA100912	JF293044	HQ856857	JF277728	JF277653	JF277575	HQ848576
Cerebratulus marginatus	DNA105590	JF293042	HQ856858	JF277729	JF277652	JF277576	HQ848575
Parborlasia corrugata	DNA105584	JF293037	HQ856851	JF277732	JF277662	JF277578	–
Lineus acutifrons	DNA104799	JF304778	HQ856855	JF277727	JF277681	JF277573	GU590937*
Lineus bilineatus	DNA105620	JF293041	HQ856844	JF277731	JF277682	JF277571	DQ280014*
Lineus torquatus	DNA105828	JF293035	HQ856856	JF277730	JF277683	JF277572	HQ848574
Lineus viridis	DNA106128	JF293032	HQ856854	JF277719	JF277654	JF277582	HQ848579
Micrura fasciolata	DNA105591	JF293038	HQ856846	JF277721	JF277660	JF277585	HQ848577
Micrura fasciolata	DNA105621	JF293039	HQ856847	JF277720	JF277659	JF277586	HQ848578
Micrura ignea	DNA103892	JF293043	HQ856859	JF277734	JF277664	JF277588	HQ848587
Micrura purpurea	DNA105619	JF293036	HQ856845	JF277726	JF277663	JF277577	HQ848586
Ramphogordius lacteus	DNA106129	JF293065	HQ856850	JF277725	JF277656	JF277584	HQ848583
Ramphogordius sanguineus	DNA103903	JF293040	HQ856853	JF277718	JF277655	JF277583	HQ848580
Riseriullus occultus	DNA105612	JF293031	HQ856848	JF277724	JF277679	JF277581	HQ848581
Riseriullus occultus	DNA105611	JF293033	HQ856849	JF277723	JF277657	JF277580	HQ848582
Riseriullus occultus	DNA106140	JF293034	HQ856852	JF277722	JF277658	JF277579	HQ848633
Zygeupolia rubens	DNA105580	JF293045	HQ856861	JF277735	JF277661	JF277574	HQ848585
Freshwater heteronemertean	DNA106130	JF29303	HQ856860	JF277733	JF277666	JF277587	HQ848584
Argonemertes australiensis	DNA105574	JF293010	HQ856892	JF277750	–	JF277605	HQ848601
Leptonemertes cf. *chalicophora*	DNA106131	JF293011	HQ856898	–	–	JF277608	HQ848596
Amphiporus imparispinosus	DNA106137	JF293029	HQ856878	JF277696	JF277671	JF277618	HQ848612
Amphiporus lactifloreus	DNA103901	JF293018	HQ856876	–	JF277672	JF277617	HQ848611
Psammamphiporus elongatus	DNA106136	JF293026	HQ856874	JF277702	JF277638	JF277622	HQ848609
Zygonemertes virescens	DNA105575	JF293016	HQ856885	JF277694	JF277675	JF277615	HQ848590
Carcinonemertes carcinophila	DNA105576	JF293007	HQ856893	JF277693	JF277636	JF277603	HQ848619
Nipponnemertes pulchra	DNA105577	JF293012	HQ856871	JF277704	JF277632	JF277625	HQ848597
Nipponnemertes sp.	DNA105589	JF293019	HQ856870	JF277705	JF277634	JF277623	HQ848599
Nipponnemertes sp.	DNA105622	JF293020	HQ856872	JF277703	JF277633	JF277624	HQ848598
Emplectonema buergeri	DNA10567	JF293066	HQ856880	JF277697	JF277685	JF277616	HQ848600
Emplectonema gracile	DNA10615	JF293022	HQ856883	JF277751	JF277680	JF277621	HQ848620
Nemertopsis bivittata	DNA106135	JF293021	HQ856877	JF277701	JF277640	JF277609	HQ848608
Malacobdella grossa	DNA105592	JF293015	HQ856882	JF277700	JF277670	JF277614	HQ848591
Ototyphlonemertes correae	DNA106134	JF293025	HQ856884	JF277706	JF277637	JF277612	HQ848613
Ototyphlonemertes macintoshi	DNA106133	JF293024	HQ856886	JF277707	JF277635	JF277613	HQ848605
Paradrepanophorus crassus	DNA1048000	JF293008	HQ856867	JF277711	JF277646	JF277628	HQ848603
Geonemertes pelaensis	DNA102574	JF293017	HQ856887	JF277736	JF277668	JF277610	HQ848592
Geonemertes pelaensis	DNA105582	JF304779	HQ856888	JF277737	JF277669	JF277611	HQ848593
Gononemertes parasita	DNA105583	JF293014	HQ856889	JF277745	JF277651	JF277606	HQ848607
Prosorhochmus americanus	DNA105665	JF293023	HQ856879	JF277698	JF277641	JF277619	HQ848595
Prosorhochmus nelsoni	DNA105586	JF293013	HQ856891	JF277744	JF277647	JF277604	HQ848606
Prostoma cf. *eilhardi*	DNA103928	JF293027	HQ856875	JF277695	JF277639	JF277620	HQ848594
Vieitezia luzmurubeae	DNA104801	HQ443428*	HQ856890	JF277746	JF277650	JF277607	HQ443426*
Drepanophorus spectabilis	DNA105587	JF293009	HQ856868	JF277710	JF277645	JF277627	HQ848610
Polystilifera sp.	DNA100544	JF293055	HQ856869	JF277712	JF277644	JF277626	HQ848632
Protopelagonemertes beebei	DNA10632	JF293028	HQ856873	JF277752	JF277665	JF277629	HQ848602
Carinina ochracea	DNA105601	JF293050	HQ856896	JF277753	JF277684	JF277631	HQ848627
Carinoma hamanako	DNA105597	JF293047	HQ856863	JF277714	JF277673	JF277600	HQ848628
Carinoma hamanako	DNA105597	JF293048	HQ856864	JF277715	JF277674	JF277601	HQ848629
Carinoma tremaphoros	DNA105579	JF293049	HQ856865	JF277713	JF277642	JF277602	HQ848630
Cephalothrix filiformis	DNA105614	JF293054	HQ856842	JF277743	JF277687	JF277594	HQ848616
Cephalothrix filiformis	DNA106138	JF293053	HQ856843	JF277742	JF277686	JF277593	HQ848617
Cephalothrix rufifrons	DNA105613	JF293056	HQ856841	JF277741	JF277688	JF277592	HQ848604
Cephalothrix hongkongiensis	DNA106145	JF293057	HQ856839	JF277739	JF277648	JF277591	HQ848614
Cephalothrix hongkongiensis	DNA106145	JF293058	HQ856840	JF277740	JF277649	JF277590	HQ848615
Interstitial cephalotricid	DNA106139	JF293059	HQ856838	JF277738	–	JF277589	HQ848618
Hubrechtella dubia	DNA105599	JF293051	HQ856897	JF277699	JF277692	JF277630	HQ848631
Callinera grandis	DNA105600	JF293067	HQ856881	JF277709	JF277643	JF277570	HQ848626
Tubulanus annulatus	DNA105593	JF293060	HQ856901	JF277717	JF277691	JF277599	HQ848622
Tubulanus pellucidus	DNA105594	JF293062	HQ856900	JF277708	JF277676	JF277595	HQ848625
Tubulanus polymorphus	DNA105595	JF293061	HQ856899	JF277716	JF277690	JF277598	HQ848621

Table 1
(Continued)

Exemplar	MCZ voucher	18S rRNA	28S rRNA	Histone H3	Histone H4	16S rRNA	COI
Tubulanus punctatus	DNA105596	JF293063	AY210473*	JF277748	JF277677	JF277597	HQ848624
Tubulanus sexlineatus	DNA105628	JF293064	HQ856895	JF277747	JF277678	JF277596	HQ848623
Outgroups							
Novocrania anomala	AToL000049	DQ279934*	DQ279949*	JF509710	–	DQ280024*	JF509716
Terebratalia transversa	AToL000135	JF509725	JF509729	JF509711	–	JF509720	JF509715
Phoronis ijimai	GenBank	AY210450*	AF342797*	–	–	–	–
Phoronis hippocrepia	AToL000022	JF509726	JF509730	–	–	–	JF509717
Capitella teleta	AToL000007	JF509728	JF509732	JF509713	–	JF509722	–
Paranerilla limicola	AToL000019	–	DQ279948*	JF509714	–	–	–
Urechis caupo	AToL000328	JF509727	JF509731	JF509712	JF509708	JF509721	JF509718
Phascolion strombi	AToL000106	AF519248*	JF509733	DQ279998*	–	–	–
Sipunculus nudus	AToL000255	DQ300008*	–	DQ300091*	JF509709	JF509723	–
Antalis entalis	AToL000061	DQ279936*	JF509734	DQ280000*	–	DQ280027*	DQ280016*
Crepidula fornicata	AToL000306	AY377660*	JF509736	AY377778*	–	JF509724	JF509719
Laevipilina hyalina	DNA102581	FJ445774*	FJ445777*	FJ445778*	–	FJ445782*	FJ445781*
Yoldia limatula	DNA101158	AF120528*	JF509735	AY070149*	–	–	AF120642*

Asterisks indicate sequences obtained from GenBank. Dashes indicate missing sequence for this particular fragment. Voucher numbers for outgroups refer only to new sequences.

and as the minimum number of sequences for a blank position, eight as the maximum number of contiguous non-conserved positions, ten as the minimum length of a block, with half allowed gap positions and using a similarity matrix. Nevertheless, we put more weight on the unedited alignment including variable positions, as suggested by Lindgren and Daly (2007). Alternatively, direct optimization (Wheeler, 1996) was also used as a dynamic criterion to assign homology (see below).

Maximum-likelihood (ML) analysis was performed using the GTR model of sequence evolution with corrections for a discrete gamma distribution (GTR + Γ). Analyses were performed with RAxML ver. 7.0.4 (Stamatakis, 2006; Stamatakis et al., 2008). The search for the optimal ML trees was performed on the cluster computing facility from the Faculty of Arts and Sciences at Harvard University. The ML tree search was conducted by performing 300 independent runs using the default algorithm of the program for random trees (option −d) as a starting tree for each run. The final tree was determined by a comparison of likelihood scores under the GTR + Γ model among suboptimal trees obtained for each 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 conservative measures of phylogenetic accuracy (Felsenstein, 2004).

The same data set was also analysed under parsimony (static homology) in TNT (Goloboff et al., 2008) and under Clade-Bayes (see Wheeler and Pickett, 2008) in MrBayes (Huelsenbeck and Ronquist, 2001). For TNT we used a driven search with sectorial searches, ratcheting, and tree fusing (Goloboff, 1999; Nixon, 1999; Giribet, 2007), specifying to find trees of minimum length 10 times. Nodal support was evaluated with 1000 replicates of parsimony jackknifing, with a probability of deletion of e^{-1} (Farris et al., 1996; Farris, 1997).

Bayesian inference was carried out using MrBayes ver. 3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003), with a GTR + Γ model and using the same data. Each Markov chain initiated from a random tree and was run for 10^6 generations, sampling every 1000 generations from the chain. Each run comprised one cold chain and three heated chains (temperature parameter = 0.2). After burn in, where 250 000 samples were discarded, trees were combined in a single majority consensus topology, and the percentage for a node recovered in the consensus was taken as the posterior probability for that node.

For the dynamic homology analyses under direct optimization the program POY ver. 4.1.2 (Varón et al., 2010) was run on a subcluster of 20 processors in the same cluster described above. Timed searches (multiple Wagner trees followed by SPR + TBR + ratchet and tree fusing) of 2–4 h each were run for four partitions (nuclear ribosomal genes, COI, 16S rRNA, histones) and for the combined analyses of all molecules under six analytical parameter sets (see below). Several additional rounds of sensitivity analysis tree fusing (SATF) (Giribet, 2007), taking all input trees from the previous round of analyses, and alternating *auto sequence partition* were conducted for the combined analysis of molecules under the multiple parameter sets evaluated. These were also 2–4-h timed searches, and the results of these were plotted to check for stability in the results. Once a parameter set stabilized and the optimal result was found multiple times, we stopped that inquiry, but continued with additional rounds of searches for those parameter sets that continued

improving or that found the optimal solution only once.

To avoid excessive computation time, we restricted the dynamic homology analyses to six parameter sets, named 111, 121, 211, 221, 3221 and 3211. Parameter set 3221 (indel opening cost = 3; indel extension cost = 1; transversions = 2; transitions = 2) has been favoured in many analyses and some authors have argued that philosophically it is the best way of analysing data under direct optimization (De Laet, 2005). In addition, we explored a parameter set, named 3211, where transversions and transitions receive different costs (indel opening cost = 3; indel extension cost = 1; transversion cost = 2; transition cost = 1). For other parameter sets, we tried limiting the difference between indel costs and transformation costs (Spagna and Álvarez-Padilla, 2008). As in previous studies, the $_{W}$ILD (Wheeler, 1995; Sharma et al., 2011) was used to select the tree that minimized overall incongruence among all partitions as our best hypothesis. In addition, Navajo rugs (sensitivity plots) were generated for the relationship of the most-basal nodes of the tree (Giribet, 2003).

A jackknife resampling analysis (Farris et al., 1996) with 1000 replicates and a probability of deletion of each character of 0.36 was applied to assess nodal support. As resampling techniques may be meaningless under dynamic homology, different strategies can be applied. Dynamic characters can be converted to a static set, but this tends to inflate support values, as it is based on the implied alignment that favours the topology. Instead, we resample characters that were static *a priori* (e.g. morphology and pre-aligned protein-coding genes), as well as fragments of the dynamic characters by using both the number of fragments (21 fragments for 18S rRNA and 18 fragments for 28S rRNA; one fragment for all other genes) as well as the command *auto_sequence_partition*, which evaluates each predetermined fragment. If a long region appears to have no indels, then the fragment is automatically broken inside that region.

To confirm the placement of the genus *Hubrechetella*, a RaxML analysis was performed using the same parameters and including the only available sequence from *Hubrechetella kimuraorum* at GenBank (18S rRNA fragment, accession number EU495308). We decided not to include additional GenBank information. First, we cannot check all identifications of specimens with sequences in GenBank while all our specimens have been identified by experts, and we have kept vouchers of all of them for subsequent analyses. Second, the goal of our study was to test nemertean higher-level phylogenetics by using a complete data set. Much effort was put into ensuring that every major lineage of nemertean was represented by at least one taxon with complete data. Adding fragmentary data to this data set will defy the purpose of the study, as any instability in the results would be difficult to tease apart.

Results

The data set used in the ML analysis consisted of five aligned subsets: the combined histones H3 and H4 (487 bp), COI (657 bp), 16S rRNA (607 bp), 18S rRNA (2017 bp) and 28S rRNA (3515 bp). The combination of all six markers produced a tree of ln $L = -122853.16$ (Fig. 3). The resulting tree shows the monophyly of nemerteans [96% bootstrap frequency (BF)], where Monostilifera is monophyletic with 100% BF, as well as Polystilifera (100% BF), forming the clade Hoplonemertea (100% BF). Hoplonemerteans are here a sister group to a clade comprising *Hubrechtella* + Heteronemertea (100% BF), where the group classified as lineids (Gibson, 1985) is paraphyletic. Palaeonemertea, as observed in previous studies, is not monophyletic, with *Hubrechtella dubia* forming a clade with Heteronemertea. However, in the combined tree, the remaining Palaeonemertea do form a clade (71% BF), which includes: *Cephalothrix* + the interstitial cephalothricid (100% BF), *Tubulanus* + *Callinera* (100% BF), cephalothricids = "tubulanids" (74% BF), and *Carinoma* + *Carinina* (72% BF).

After removing ambiguous sites from alignments of the ribosomal markers, we obtained 369, 1452 and 1776 bp for 16S rRNA 18S rRNA and 28S rRNA, respectively. The markers were combined and analysed using the same settings applied to the complete data set. The resulting tree (ln $L = -73\,987.08$) produced a similar topology as the previous ML tree, were *Hubrechtella* + heteronemerteans and hoplonemerteans are sister groups, and palaeonemerteans are not monophyletic (BFs in italics on Fig. 3), because *Hubrechtella* is excluded.

The Bayesian tree topology of the consensus tree is identical to the ML tree, and the posterior probabilities equal to 1 are shown on the nodes in Fig. 3. The tree including *H. kimuraorum* (not shown) has a clade with both hubrechtids with 100%BF. This clade is sister to the Heteronemertea with a BF of 65%.

The direct optimization analyses for all combined data sets stabilized after five rounds of sensitivity analysis tree fusing using *auto_sequence_partition* in the second round. For some parameter sets, results remained stable throughout the rounds of SATF (e.g. parameter set 111). The $_{W}$ILD analysis indicated that parameter set 3211 was the optimal one, followed by 3221 (Table 2). The phylogenetic hypothesis under the optimal parameter set is presented in Fig. 4.

While under two parameter sets an outgroup taxon appeared nested within the ingroup (these two parameter sets represent the lowest $_{W}$ILD values), all other parameter sets supported nemertean monophyly as well as the monophyly of the following clades: (i) *Cephalothrix* + the interstitial cephalothricid, (ii) *Carinina* + *Carinoma*, (iii) *Tubulanus* + *Callinera*, (iv)

Fig. 3. Phylogenetic hypothesis resulting from the maximum-likelihood analysis of all genes combined with GTR + Γ (ln L = −122 853.16). Numbers at nodes indicate bootstrap support values ≥ 50%. Numbers in italics indicate bootstrap support values obtained from the analysis after the alignment was edited with Gblocks (ln L = −73 987.08). Asterisks indicate posterior probability = 1.0 obtained with Bayesian analysis using the model GTR + Γ.

Table 2
Tree lengths for the individual and combined data sets at different parameter values, with incongruence length difference (ILD) values

	RIB	COI	16S	HIS	MOL	ILD
111	15 001	4643	4265	2206	26 854	0.02752
121	23 802	6852	6808	3166	41 786	0.02771
211	18 895	4643	4934	2209	31 624	0.02982
221	30 988	6855	8006	3173	50 597	0.03113
3211	23 974	6836	6984	3150	42 030	0.02584
3221	30 416	9286	8804	4407	54 357	0.02657

Data sets: RIB, 28S rRNA and 18S rRNA; COI, cytochrome *c* oxidase subunit I; 16S, 16S rRNA; HIS, histones H3 and H4; MOL, combined data set (28S + 18S + COI + 16S + H3 + H4).

Heteronemertea, and (v) Hoplonemertea. The interrelationships among these five clades and the hubrechtiid *H. dubia* varied with different parameter sets, some suggesting monophyly of Palaeonemertea (minus *Hubrechtella*) (parameter sets 121, 3211) and some suggesting its para- (e.g. 111) or polyphyly (e.g. 211, 3221). In one case, under the next optimal parameter set, *Hubrechtella* was the sister group to Heteronemertea (parameter set 3221). Palaeonemertea, as in all previous studies, is not strictly monophyletic, given the position of *Hubrechtella*, nor is there strong support or stability for the monophyly of Palaeonemertea minus *Hubrechtella*.

Other results that appear under every parameter set and analytical methods are a basal division of Heteronemertea into *Baseodiscus* and the rest, as suggested in several traditional classifications (see Discussion below). There is little resolution within the lineid clade, but high support for a few (heterospecific) terminal duets and for two deeper nodes within the lineids that segregate three *Micrura* species (Fig. 4). Hoplonemertea is a well-supported clade with a basal dichotomy between Polystilifera and Monostilifera; Monostilifera shows a well-supported split between *Nipponnemertes* (Cratenemertea of Thollesson and Norenburg, 2003) and the remaining species (Distromatonemertea of Thollesson and Norenburg, 2003), including *Malacobdella grossa*, which is supported as the sister species of the terrestrial *Geonemertes pelaensis*. Within Distromatonemertea, *Carcinonemertes* appears as the sister to all other species in some of the POY analyses, but there is little bootstrap support for this hypothesis.

The parsimony static homology analysis in TNT yielded four optimal trees at 27 344 steps (tree not shown). This tree agrees with the other analyses in the monophyly of Hoplonemertea, Polystilifera, Monostilifera (divided into *Nipponemertes* and the rest), Heteronemertea (divided into *Baseodiscus* and the rest), and a clade of Palaeonemertea that excluded *Carinoma* + *Carinina*. This tree also finds monophyly of Pilidiophora [60% jackknife frequency (JF)], which is sister to the clade containing *Carinoma* + *Carinina*, although with low nodal support, while the remaining palaeonemerteans are the sister group to Hoplonemertea, but again with low nodal support. Monophyly of Heteronemertea and Hoplonemertea receive 100% JF each.

Discussion

Molecular data have been used in recent studies of nemertean systematics, a group notorious for its morphological homoplasy (but see novel data on promising character systems by Bartolomaeus and von Döhren, 2010; von Döhren et al., 2010), and a classification system that in many parts does not reflect monophyletic groups. Our new phylogeny based on molecular data is not immune to error, but adds support to several previously proposed clades, including Heteronemertea, Enopla (= Hoplonemertea), Polystilifera, Monostilifera, Cratenemertea, and Distromatonemertea, and the basal division between *Baseodiscus* and the remaining heteronemerteans (e.g. Thollesson and Norenburg, 2003). This phylogeny serves as a scaffold to which one can now pinpoint any previously published nemertean sequence, although most of the named families and genera still need to be tested further, especially due to the large number of monotypic genera erected without sound phylogenetic testing. This task will require very dense sampling within each of the main clades here obtained, and we hope our results can form the phylogenetic scaffold for future choice of taxa. Our results thus support most clades corroborated or proposed by Thollesson and Norenburg (2003), but we remain cautious about the validity of Pilidiophora and Neonemertea, considering the instability of such clades among all the sound analytical methods employed here. One could get distracted in discussing the pros and cons of each phylogenetic method and approach, but this is beyond the scope of our paper and the truth is that all conflicting nodes receive low nodal support and/or stability across analyses.

Nemerteans continue to be neglected by many researchers due to a difficult taxonomy and hidden modes of life even though they constitute an important group of predatory invertebrates inhabiting many ecosystems. A well-resolved phylogeny of the group allows for detailed study of character evolution and evolutionary trends, e.g. transitions from marine to freshwater and terrestrial environments, from benthic to pelagic, and changes in feeding patterns. Recent efforts in documenting local biotas (e.g. Gibson, 1999; Collin et al., 2005; Sundberg et al., 2007; Thiel and Norenburg, 2009) are also important for discovering new lineages that are now often analysed with pre-existing molecular data sets (e.g. Sundberg et al., 2003; Junoy et al., 2010; Puerta et al., 2010; Strand and Sundberg, 2011), a

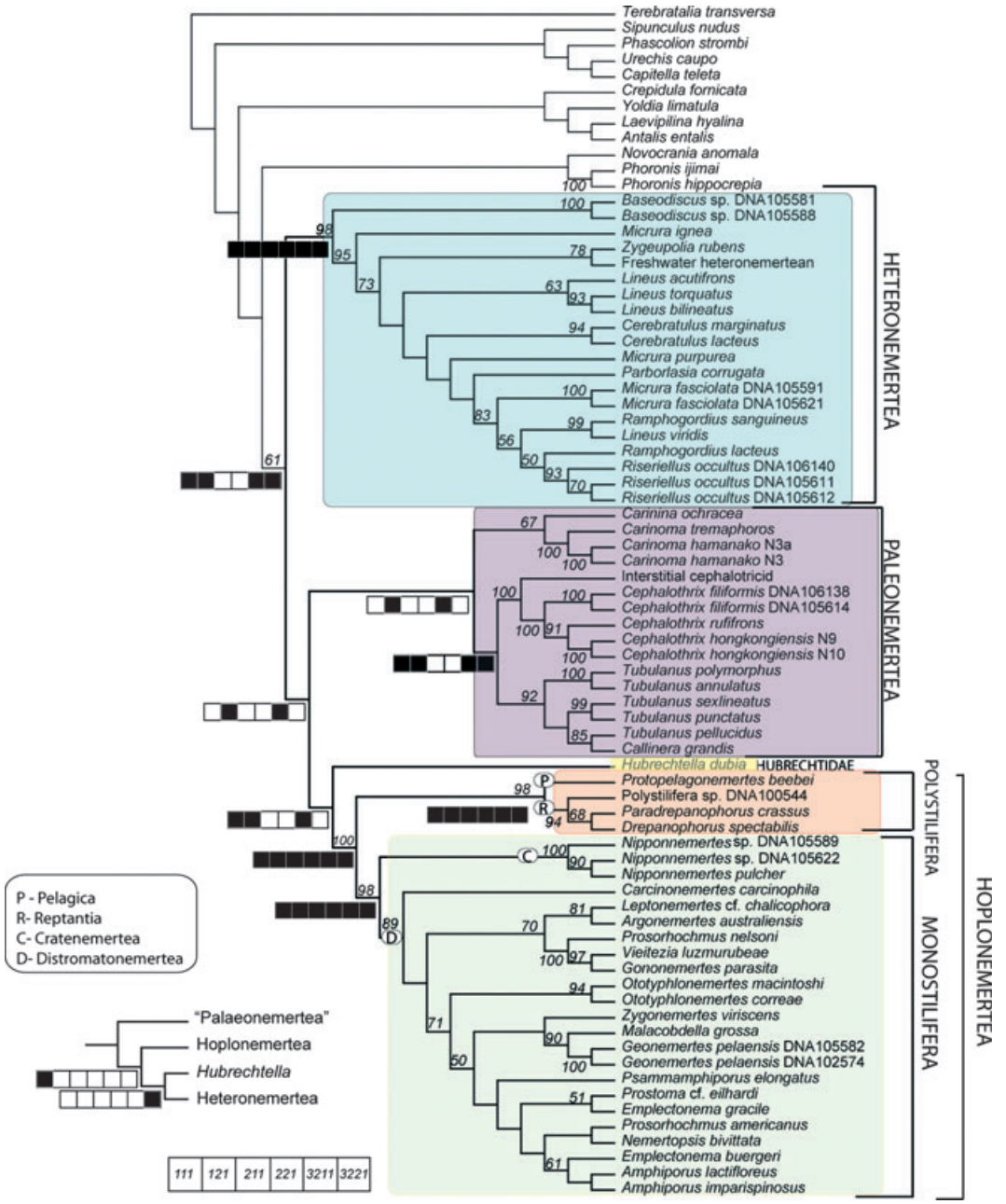

Fig. 4. Phylogenetic hypothesis based on the direct optimization analysis of all combined data under parameter set 3211 (42 030 weighted steps). Values on branches indicate jackknife resampling frequencies. Selected nodes show the sensitivity analysis under six parameter sets, with black squares indicating monophyly and white squares indicating non-monophyly. An alternative tree compatible with the Pilidiophora hypothesis is presented in the lower left corner.

practice that is becoming common among nemertean workers. Again, the present data set, with the addition of new markers, should serve this purpose well.

Relationships among the main groups

The relationships among the three major clades, Hoplonemertea, Heteronemertea, and Palaeonemertea, vary among the different methods of analysis. All analyses support the monophyly of Hoplonemertea [100% JF and BF; posterior probability (PP) = 1] and Heteronemertea (98% JF for direct optimization, 100% JF for TNT, PP = 1, and 100% BF), confirming previous results by Thollesson and Norenburg (2003). These results also agree with recent morphological approaches using sperm and nephridial structure

(Bartolomaeus and von Döhren, 2010; von Döhren et al., 2010). Based on these characters, both studies suggest that Heteronemertea and Hoplonemertea are monophyletic and palaeonemertean taxa retain the ancestral design in some of the structures.

ML and Bayesian analyses suggest that these clades are sister groups (87% BF, PP = 1), but that they include the palaeonemertean *Hubrechtella dubia*, as in the Pilidiophora hypothesis of Thollesson and Norenburg (2003). This recovered clade composed by Hoplonemertea and Pilidiophora was named as Neonemertea by the cited authors, as it appeared nested within a grade of palaeonemertean groups. By contrast, several parameter sets of the direct optimization parsimony analysis recovered *Hubrechtella* as sister to Hoplonemertea, both forming a clade with Palaeonemertea, although without jackknife support. This analysis also supports Heteronemertea as the sister group to the other clades, which partially agrees with results from Sundberg and Hylbom (1994), based on a parsimony analysis of morphological characters. Finally, the parsimony analysis of static homology finds Pilidiophora, but not a sister group to Hoplonemertea. All methods of analysis seem to firmly reject strict monophyly of Palaeonemertea, which has been in discussion in several previous studies (Sundberg et al., 2001, 2009; Thollesson and Norenburg, 2003; Sundberg and Strand, 2007), indicating that the group is in need of being thoroughly revised with emphasis on the position of *Hubrechtella* and its relatives, by including additional species.

The clade Hoplonemertea is split into two main groups, Monostilifera and Polystilifera, both of which are monophyletic and well supported. Within Monostilifera, *Nipponnemertes* is sister group to the remaining monostiliferans (Distromatonemertea) with high support by all phylogenetic approaches. Polystiliferans comprise reptant and pelagic species (Brinkmann, 1917), where the reptants are monophyletic (Figs 3 and 4). Approximately 100 species have been described as holopelagic (Maslakova and Norenburg, 2001), of which four are monostiliferans (Crandall and Gibson, 1998; Chernyshev, 2005; Crandall, 2006). The rest of the pelagic species comprise the polystiliferous Pelagica, suggesting that the pelagic lifestyle has evolved more than once among nemerteans, as suggested by the results of Thollesson and Norenburg (2003), but this remains untested here as the pelagic forms in the present study are only represented by *Protopelagonemertes beebei*. This is a hard group to study, as it is difficult to sample and the morphology is greatly simplified, but also because, as noted by Maslakova and Norenburg (2001), 51 out of 98 species were described based on a single specimen. Polystiliferans, in contrast to a previous study (Sundberg, 1990), are monophyletic with high support (98% JF, 100% BF, and PP = 1) and sister to other enoplan taxa.

The suggested clade Pilidiophora, here recovered by the ML, Bayesian, TNT analysis and parameter set 3221 in the direct optimization analysis, comprises Heteronemertea and the palaeonemertean genus *Hubrechtella*. The clade includes a total of approximately 450 species (Kajihara et al., 2008) and it is characterized by a long-lived pilidium larvae, while hoplonemerteans and palaeonemerteans develop into an adult form via a relatively non-specialized ciliated planktonic larva (e.g. Norenburg and Stricker, 2002; Maslakova et al., 2004a,b; but see Maslakova, 2010b). Based on the larval type, it was proposed by some that *Hubrechtella* is a heteronemertean (Cantell, 1969; Norenburg, 1985, 1993; Maslakova, 2010a). Therefore, a pilidium larva would be an autapomorphy of this clade and not plesiomorphic for nemerteans (Turbeville, 2002; Maslakova et al., 2004b; Maslakova, 2010a,b). This hypothesis also finds support in the study of Bürger (1895), where *Hubrechtia desiderata* is reported to have a protonephridial structure similar to that of heteronemerteans. However, this description is incomplete and requires verification (Bartolomaeus and von Döhren, 2010).

Resolution at family and genus level

As observed in previous studies (e.g. Sundberg et al., 2001; Thollesson and Norenburg, 2003; Strand and Sundberg, 2005b), the relationships among species within some of the main groups are not well resolved, even with addition of new markers and with the high number of different taxa analysed. Despite poor support for palaeonemertean relationships, the only traditional families recovered were those of the palaeonemerteans. All our results refute again the Archinemertea hypothesis, which placed Cephalothricidae apart from the remaining palaeonemerteans (Iwata, 1960). *Tubulanus sensu stricto* is paraphyletic, as *Callinera grandis* is nested within the genus with high support, supporting the results from a previous study using the 18S rRNA gene as marker (Sundberg et al., 2009). Additional sampling and a revision of the genera *Callinera*, *Carinina* and *Tubulanus* emerges as a priority to solve the relationships among these genera.

Although within the Monostilifera clade a few of the traditional families were supported, species representation for them is too sparse to discuss their validity. The genus *Ototyphlonemertes* is a specialized interstitial taxon with a large set of unambiguous synapomorphies, such as the absence of eyes in adults and the presence of statocysts in all species, which makes them easily distinguishable from the remaining monostiliferans. *Nipponnemertes*, as already discussed, is sister to the other monostiliferans. Although not fully understood phylogenetically, some morphological characters, such as the rhyncochoel musculature in *Nipponnemertes*, are most similar to those of the polystiliferan species

(Gibson, 1988), making *Nipponnemertes* a basal monostiliferan taxon, and therefore explaining its position in this phylogenetic hypothesis.

Diagnoses for several of the monostiliferan families come in so many versions that discussing their lack of monophyly verges on being self-evident. Nevertheless, it is worth noting that the important traditionally proposed families Amphiporidae, Prosorhochmidae, and Emplectonematidae are all without support, as expected, corroborating earlier analyses (e.g. Sundberg et al., 2001; Thollesson and Norenburg, 2003; Strand and Sundberg, 2005b). Some clades disrupting these families show high support, such as the prosorhochmids *Gononemertes parasita* + *Prosorhochmus nelsoni* as sister group of species of the family Acteonemertidae, while other prosorhochmids are found in other clades. In this clade, *Vieitezia luzmurubeae* is placed as sister taxon to *G. parasita* with high support, these two species being sister to *P. nelsoni*. Due to the lack of a robust phylogenetic hypothesis for *Tetrastemma*-related genera as well as for other monostiliferans, Junoy et al. (2010) chose not to place this species in a family. These results, which support previous studies, suggest that Distromatonemertea is in need of a thorough revision at the genus and family level. Of particular note, *Malacobdella* is again solidly nested within the Distromatonemertea, and the present results echo the finding of Thollesson and Norenburg (2003) for a strong relationship with *Pantinonemertes*, a supralittoral genus with morphological similarities and historical taxonomic ties to *Geonemertes*.

The order Heteronemertea shows a similar pattern as in Monostilifera. The traditional lineid genera are polyphyletic, as shown in earlier studies (Thollesson and Norenburg, 2003; Strand et al., 2005; Sundberg and Strand, 2007; Puerta et al., 2010). For example, there is one clade comprising *Lineus bilineatus*, *L. torquatus* and *L. acutifrons*, while *L. viridis* is sister to *Ramphogordius sanquineus* in a clade that includes members of the genera *Riseriellus* and *Micrura*.

The undescribed freshwater heteronemertean investigated here is consistently placed as the sister taxon of *Zygeupolia rubens* with high support (78% JF, 96%BF, and PP = 1). However, caution is necessary when placing this species in any group, due to non-monophyly of lineids and a lack of thorough descriptions as well as of good diagnostic morphological features for the genus. The genera *Lineus*, *Cerebratulus* and *Micrura* contain about 251 of the approximately 500 described species of heteronemerteans (Schwartz, 2009). The latter two genera are diagnosed traditionally as having a caudal cirrus and *Cerebratulus* by the presence of neurochord cells (Gibson, 1985; but see Schwartz, 2009). However, one or both character states are unknown for many of the species attributed to these genera (Schwartz and Norenburg, 2001; Schwartz, 2009). Riser (1998) suggested that the caudal cirrus appears to be a plesiomorphic character retained by burrowing species. Schwartz (2009, p. 28) suggested, based on molecular analyses, that the presence/absence of a caudal cirrus is "not informative for generic placement as it has been historically used". This also is seen in Puerta et al. (2010). *Baseodiscus* includes most (about 36 species) of the heteronemerteans that lack lateral horizontal cephalic slits. Both direct optimization parsimony and ML analyses agree that *Baseodiscus* is the sister group of other heteronemerteans, confirming prior results based on 16S rRNA data (Strand et al., 2005). This also confirms the prevalent views on division of the heteronemerteans based on morphology (McIntosh, 1873–1874; Bürger, 1895, 1904; Friedrich, 1935; Coe, 1940; Norenburg, 1993).

Further considerations

Nemerteans have fascinating lifestyles and have achieved many forms of parasitism/commensalism and multiple colonizations of freshwater and terrestrial environments. In these analyses, an unidentified freshwater heteronemertean and a species of the freshwater genus *Prostoma* corroborates the well-known recurrence of freshwater colonization. Terrestriality in nemerteans has fascinated probably more authors than existing species (e.g. Coe, 1929; Moore and Gibson, 1981, 1985; Sundberg, 1989; Moore et al., 2001; Mateos and Giribet, 2008), but in this case all species are restricted to the monostiliferan hoplonemerteans. Our analyses, despite not finding strong support for the hoplonemertean interrelationships, do suggest the polyphyly of terrestrial nemerteans, as shown in previous studies (e.g. Mateos and Giribet, 2008), but perhaps more surprising is the association of the terrestrial species to clades of marine nemerteans that contain parasites and commensals, such as *Malacobdella*, *Gononemertes*, and *Vieitezia*.

The present analyses reinforce several previous hypotheses in nemertean phylogenetics, character evolution, and ecology, and point to the most important issues in nemertean systematics. These include the further testing of the position of *Hubrechtella* and the Pilidiophora and Neonemertea hypotheses, which are sensitive to the analytical method, but adds support to several previously suggested clades, including Heteronemertea and its split into two main clades, as well as Hoplonemertea, Polystilifera, Pelagica, Reptantia, Monostilifera, Cratenemertea and Distromatonemertea. This study also shows that we are reaching the limits of a target-gene approach, even when using a thorough taxon sampling. Hence, we are testing remaining uncertainty at the deepest levels with high-throughput ("next-generation sequencing") approaches that have proven to be reliable for resolving pervasive phylogenetic problems within protostome animals (e.g. Hausdorf et al.,

2007; Dunn et al., 2008; Struck and Fisse, 2008; Hejnol et al., 2009; Witek et al., 2009; Struck et al., 2011).

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Appendix 1: Collection data

Amphiporus imparispinosus Griffin, 1898

NemPhyl 64 (MCZDNA106137): Cattle Point, San Juan Island (48°27′N, 122°58′W) Washington (USA); Leg. J. von Döhren, March 2007; upper intertidal between some rocks.

Amphiporus lactifloreus (Johnston, 1828)

NemPhyl 26 (MCZ DNA103901): Penmon, Isle of Anglesey (53°17′86″N, 004°03′46″W), Wales (UK); Leg. P. Sundberg, 14 December 2008; upper tidal, under stones.

Argonemertes australiensis (Dendy, 1892)

NemPhyl 40 (MCZ DNA105574): NW Tasmania (41°02′58″S, 145°35′19″E) (Australia); Leg. R. Mesibov, 6 August 2009.

Baseodiscus sp. DNA105581

NemPhyl 41 (MCZ DNA105581): DIVA-ALTABRIA II Expedition, station 27-AT (42°42.723634′N, 011°49.890279′W), off the coast of Vigo, Pontevedra, Galicia (Spain); Leg. G. Giribet et al., 5 October 2009.

Baseodiscus sp. DNA105588

NemPhyl 19 (MCZ DNA105588): Isla Solarte (09°18′36.6″N, 082°13′01″W), Archipiélago de Bocas del Toro, Bocas del Toro (Panama); Leg. G. Giribet, 30 March 2006.

Callinera grandis Bergendal, 1903

NemPhyl 16 & 16b (MCZ DNA105600): Tjärnö, Koster area, Skagerak (Sweden); Leg. M. Strand, 14 November 2008.

Carcinonemertes carcinophila (Kölliker, 1845)

NemPhyl 28 (MCZ DNA105576): Beaufort (34°43′7″N, 76°40′35″W), North Carolina (USA); Leg. J. Norenburg, October, 2005.

Carinina ochracea Sundberg, Chernyshev, Kajihara, Kaneby & Strand, 2009

MCZ DNA105601: Tjärnö (58°53′124″N, 11°07′275″E), Koster area, Skagerak (Sweden); Leg. M. Strand, August 2006.

Carinoma hamanako sp. nov. Kajihara, Yamasaki & Andrade, in press.

NemPhyl 3 & 3a (MCZ DNA105597): Ikarise Island (34°41′04″N, 137°35′59″E), Lake Hamanako, Shizuoka, Honshu (Japan); Leg. H. Kajihara, 30 March 2009.

Carinoma tremaphoros Thompson, 1900

NemPhyl 31 (MCZ DNA105579): Fort Pierce, Florida (USA); Leg. J. Norenburg, March 2009.

Cephalothrix filiformis (Johnston, 1828)

NemPhyl 46 (MCZ DNA105614): Rhos-on-Sea (53°18′46″N, 3°44′16″W), Wales (UK); Leg. P. Sundberg, 18 February 2010.

NemPhyl 65 (MCZ DNA106138): Sylt Island (55°2′N, 8°26′E), Nordfriesland, Schleswig-Holstein (Germany); Leg. J. von Döhren, March 2009; under stones on a sandflat close to the marine biological station.

Cephalothrix hongkongiensis Sundberg, Gibson & Olsson, 2003

NemPhyl 9 & 10 (MCZ DNA106145): Qingdao, Qingdao (China); Leg. S. Sun, 16 November 2008; intertidal coarse sand.

Cephalothrix rufifrons (Johnston, 1837)

NemPhyl 45 (MCZ DNA105613): Rhos-on-Sea (53°18′46″N, 3°44′16″W), Wales (UK); Leg. P. Sundberg, 18 February 2010.

Cerebratulus lacteus (Leidy, 1851)

NemPhyl 49 (MCZ DNA100912): Little Jim Sand Flat, Fort Pierce, Florida (USA); Leg. M. Schwartz, 2 April 2003.

Cerebratulus marginatus Renier, 1804

NemPhyl 2 (MCZ DNA105590): False Bay, San Juan Island, Washington (USA); Leg. M. Schwartz, 15 November 2008.

Drepanophorus spectabilis (Quatrefages, 1846)

NemPhyl 13 (MCZ DNA105587): Punta Santa Anna, Blanes, Girona (Spain); Leg. G. Giribet & G. Rouse, 20 June 2008; under rocks at 16 m depth.

Emplectonema buergeri Coe, 1901

NemPhyl 58 (MCZ DNA10567): Elliott Bay Marina, Dock N, Seattle, Washington (USA); Leg. M. Schwartz, 21 March 2010.

Emplectonema gracile (Johnston, 1837)

NemPhyl 53 (MCZ DNA10615): Crosby (53°30′17″N, 3°03′53″W), Liverpool (UK); Leg. P. Sundberg, 17 February 2010.

Geonemertes pelaensis Semper, 1863

NemPhyl 1 (MCZ DNA102574): St. Davis (Bermuda); Leg. W. Sterrer, 2006; in garden underside loose brick DD01, 6 Narrows Lane.

NemPhyl 1a (MCZ DNA105582): St. Davis (Bermuda); Leg. W. Sterrer, 20 November 2008.

Gononemertes parasita Bergendal, 1900

NemPhyl 14 (MCZ DNA105583): Koster area, Skagerak (Sweden); Leg. M. Strand, 14 November 2008; inside tunicate *Ascidia obliqua*, among mussels and cobble at 15 m depth.

Hubrechtella dubia Bergendal, 1902

NemPhyl 4 (MCZ DNA105599): Tjärnö (58°55.167′N, 11°06.048′W), Koster area, Skagerak (Sweden); Leg. M. Strand, 6 November 2008; organic mud 27 m deep.

Interstitial cephalotricid

MCZ DNA106139: Bogue Sound (34°38′49″N, 077°05′52″W), near Beaufort, North Carolina (USA); Leg. G. Giribet, K. Worsaae, G. Rouse, et al., 25 October 2007; in humid sand, above water level.

Leptonemertes cf. *chalicophora* (Graff, 1879)

NemPhyl 30 (MCZ DNA106131): Link Port, Fort Pierce, Florida (USA); Leg. J. Norenburg, March 2009.

Lineus acutifrons Southern, 1903

MCZ DNA104799: Praia de A Ladeira (42°34′N, 009°03′W), Corrubedo, Ribeira, A Coruña, Galicia (Spain); Leg. J. Junoy, 5 August 2009; intertidal beach.

Lineus bilineatus (Renier, 1804)

NemPhyl 50 (AToL 000087; MCZ DNA105620): Kristineberg (Station K12, between 58°19′36″N, 011°32′59″E and 58°19′11″N, 011°32′31″E), Skagerak (Sweden); Leg. G. Giribet et al. (AToL expedition 2004); Warén sledge in mud, 116–118 m deep.

Lineus torquatus Coe, 1901

NemPhyl 20 (MCZ DNA105828): Akkeshi, Hokkaido (Japan); Leg. H. Kajihara, 14 May 2010.

Lineus viridis (Müller, 1774)

NemPhyl 61 (MCZ DNA106128): Sylt Island (55°2′N, 8°26′E), Nordfriesland, Schleswig-Holstein (Germany); Leg. J. von Döhren, March 2009; crawling on stones on a sandflat during night-time low tide.

Malacobdella grossa (Müller, 1776)

NemPhyl 11 (MCZ DNA105592): Tjärnö, Koster area, Skagerak (Sweden); Leg. M. Strand, February 2009 and August 2009; dredged in shell/gravel bottom, inside *Arctica islandica*, 20 m deep.

Micrura fasciolata Ehrenberg, 1828

MCZ DNA105591: Tjärnö (Station T6, between 58°52′30″N, 011°06′02″E and 58°52′30″N, 011°06′13″E), Skagerak (Sweden); Leg. G. Giribet et al. (AToL expedition 2004), 30 July 2004; Trawling, 40 m deep.

NemPhyl 5 (MCZ DNA105621): Tjärnö (58°53.505′N, 11°0 6.239′W), Koster area, Skagerak (Sweden), Leg. M. Strand, 30 September 2008; dredged, bottom with mussels and cobble, 10 m deep.

Micrura ignea Schwartz & Norenburg, 2005

NemPhyl 78 (MCZ DNA103892): Isla Cristóbal (09°17.411′N, 82°15.292′W), Archipiélago de Bocas del Toro, Bocas del Toro (Panama); Leg. G. Giribet, 23 March 2009.

Micrura purpurea (Dalyell, 1853)

NemPhyl 51 (MCZ DNA105619): Tjärnö (Station T6, between 58°52′30″N, 011°06′02″E and 58°52′30″N, 011°06′13″E), Skagerak (Sweden); Leg. G. Giribet et al. (AToL expedition 2004), 30 July 2004; trawling, 40 m deep.

Nemertopsis bivittata (Delle Chiaje, 1841)

NemPhyl 60 (MCZ DNA106135): Pawleys Island (33°24′38.06″N, 79°07′53.54″W), South Carolina (USA); Leg. J.M. Turbeville, March 2010, under *Brachidontes exustus* mats on exposed granite boulders of groins.

Nipponnemertes pulchra (Johnston, 1837)

NemPhyl 15 (MCZ DNA105577): Tjärnö, Koster area, Skagerak (Sweden); Leg. M. Strand, 12 November 2008; mud, 50 m deep.

Nipponnemertes sp. 1

NemPhyl 55 (MCZ DNA105622): vessel R/V *Robert Gordon Sproul*, Santa Rosa-Cortes Ridge (32°59.0′N, 119°32.8′W), California (USA); Leg. G. Giribet, G.W. Rouse, N. Wilson, 28 November 2007; dredged with large Van Veen grab sampler (Kahl Scientific, volume 0.2 m^3) at depths of 367–389 m.

Nipponnemertes sp. 2

NemPhyl 25 (MCZ DNA105589): Talcahuano, west side of Península de Tumbes (36°42.80′S, 073°09.42′W), Región VIII: Biobío (Chile); Leg. P. Sundberg, 17 February 2009; exposed rocky shore, upper tidal.

Ototyphlonemertes correae Envall, 1996

NemPhyl 67 (MCZ DNA106134): Saltö (58°52′44.18″N, 011°7′25.35″E), Skagerrak (Sweden); Leg. J. Norenburg, September 2008.

Ototyphlonemertes macintoshi Bürger, 1895

NemPhyl 68 (MCZ DNA106133): Praia do Mindelo (41°18′36.72″N, 008°44′30.50″W), Vila do Conde (Portugal); Leg. J. Norenburg, September 2008.

Paradrepanophorus crassus (Quatrefages, 1846)

MCZ DNA1048000: Ribeira, A Coruña, Galicia (Spain); Leg. J. Pérez [Club de Buceo Hydronauta, Ribeira], 28 October 2009; under stones, 2 m deep.

Parborlasia corrugata (McIntosh, 1876)

NemPhyl 24 (MCZ DNA105584): West of Dream Island (64°48.00′S, 065°21.30′W), Palmer Archipelago, Antarctic Peninsula; Leg. T. Dahlgren (collected during FOODSBANCS project cruise), April 2009; 500–600 m deep.

Polystilifera sp. MCZ DNA100544

NemPhyl 48 (MCZ DNA100544): North Normand's Pond buoy and Iguana Cay (23°47′23″N, 076°08′16″W), Exuma Cays (Bahamas); Leg. G. Giribet, 24 March 2002.

Prosorhochmus americanus Gibson, Moore, Ruppert & Turbeville, 1986

NemPhyl 59 (MCZ DNA105665): Rudee Inlet (36°49′48.80″N, 75°58′05.5″W), Virginia (USA); Leg. J.M. Turbeville, 7 October 2007, under oysters and mussels on exposed granite boulders of jetty.

Prosorhochmus nelsoni (Sánchez, 1973)

NemPhyl 27 (MCZ DNA105586): Coquimbo (29°57.96′S, 71°21.14′W), Coquimbo Region, (Chile); Leg. P. Sundberg, 2 February 2009; upper tidal.

Prostoma cf. *eilhardi* (Montgomery, 1894)

MCZ DNA103928: Concord, Eastbrook Woods, Massachusetts (USA); Leg. C. Laumer, 19 April 2009; on moss and associated sediment.

Protopelagonemertes beebei Coe, 1936

NemPhyl 56 (MCZ DNA10632): Sample ORI33 (35°08.94′N, 139°17.19′E), Sagami Bay (Japan); Research cruise KT10-2 (Chief Scientist: S. Nishida, University of Tokyo) of the R/V *Tanseimaru*, operated by the Japan Agency for Marine-Earth Science and Technology (JAMSTEC sample numbers 1100021510 and 1100021511), 13 March 2010; net towed to a maximum depth of 1300 m and 200 m above the seafloor.

Psammamphiporus elongatus (Stephenson, 1911)

NemPhyl 77 (MCZ DNA106136): Praia de Vilar, (42°32′N, 009°01′W) Corrubedo, Ribeira, A Coruña, Galicia (Spain); Leg. J. Junoy, 8 May 2008; intertidal, fine sand.

Ramphogordius lacteus Rathke, 1843

NemPhyl 62 (MCZ DNA106129): Le Cabellou, Concarneaeu (47°51′N, 3°55′W), Finistère department, Brittany (France); Leg. J. von Döhren, March 2009; under stones in coarse sand in the upper mid-intertidal collected at low tide.

Ramphogordius sanguineus (Rathke, 1799)

NemPhyl 18 (MCZ DNA103903): Kresg Point, Maine, (USA); Leg. G.Y. Kawauchi, 19 April, 2009.

Riseriellus occultus Rogers, Junoy, Gibson & Thorpe, 1993

NemPhyl 42 (MCZ DNA105612): Crosby (53°30′17″N, 3°03′53″W), Liverpool (UK); Leg. P. Sundberg, 17 February 2010.

NemPhyl 43 (MCZ DNA105611): Rhos-on-Sea (53°18′46″N, 3°44′16″W), Wales (UK); Leg. P. Sundberg, 18 February 2010.

NemPhyl 63 (MCZ DNA106140): Le Cabellou, Concarneaeu (47°51′N, 3°55′W), Finistère department, Brittany (France); Leg. J. von Döhren, September 2009; in rock crevices in the upper intertidal during low tide.

Tubulanus annulatus (Montagu, 1804)

NemPhyl 6 (MCZ DNA105593): Tjärnö, Koster area (59°01.006′N, 011°07.393′W), Skagerak (Sweden); Leg. M. Strand, 2 October 2008; mud at 70 m.

Tubulanus pellucidus (Coe, 1895)

NemPhyl 17 (MCZ DNA105594): Pea Island (34°46′01″N, 75°31′35″W), North Carolina (USA); Leg. C. Runnels & C. Turbeville, 28 October 2007.

Tubulanus polymorphus Renier, 1804

NemPhyl 8 (MCZ DNA105595): Cattle Point, San Juan Island, Washington (USA); Leg. M. Schwartz, 14 November 2008.

Tubulanus punctatus (Takakura, 1898)

NemPhyl 38 (MCZ DNA105596): Akkeshi, Hokkaido (Japan); Leg. H. Kajihara, 14 May 2010.

Tubulanus sexlineatus (Griffin, 1898)

NemPhyl 57b (MCZ DNA105628): Elliott Bay Marina, Dock N, Seattle, Washington (USA); Leg. M. Schwartz, 21 March 2010.

Vieitezia luzmurubeae Junoy, Andrade & Giribet, submitted

MCZ DNA104801: Ría de Arousa (approx. 42°31′N, 8°59′W), Pontevedra, Galicia (Spain); Leg. J. Junoy, 6 August 2009; inside tunicate *Ciona intestinalis* from a mussel raft.

Undescribed freshwater heteronemertean

NemPhyl 44 (MCZ DNA106130): freshwater fish tank in Madrid (Spain); Leg. Y. Lucas Rodríguez, 30 November 2009.

Zygeupolia rubens (Coe, 1895)

NemPhyl 33 (MCZ DNA105580): Link Port, Fort Pierce, Florida (USA); Leg. J. Norenburg, March 2009.

Zygonemertes virescens (Verrill, 1879)

NemPhyl 34 (MCZ DNA105575): Link Port, Fort Pierce, Florida (USA); Leg. J. Norenburg, March 2009.

Appendix 2

Primers used in the study

Primer	Sequence	Reference
18S rRNA		
1F	5′-TACCTGGTTGATCCTGCCAGTAG-3′	Giribet et al. (1996)
5R	5′-CTTGGCAAATGCTTTCGC-3′	Giribet et al. (1996)
3F	5′-GTTCGATTCCGGAGAGGGA-3′	Giribet et al. (1996)
18Sbi	5′-GAGTCTCGTTCGTTATCGGA-3′	Whiting et al. (1997)
S2.0	5′-ATGGTTGCAAAGCTGAAAC-3′	Whiting et al. (1997)
9R	5′-GATCCTTCCGCAGGTTCACCTAC-3′	Giribet et al. (1996)
28S rRNA		
rd1a	5′-CCCSCGTAAYTTAGGCATAT-3′	Edgecombe and Giribet (2006)
rd4b	5′-CCTTGGTCCGTGTTTCAAGAC-3′	Edgecombe and Giribet (2006)
LSU5	5′-ACCCGCTGAAYTTAAGCA-3′	Littlewood (1994)
LSU3	5′-TCCTGAGGGAAACTTCGG-3′	Littlewood (1994)
D2f	5′-CTTTGAAGAGAGAGTTC-3′	Littlewood (1994)
28Z	5′-CTTGGTCCGTGTTTCAAGAC-3′	Hillis and Dixon (1991)
Sa	5′-GACCCGTCTTGAAACACGGA-3′	Whiting et al. (1997)
rd5b	5′-CCACAGCGCCAGTTCTGCTTAC-3′	Schwendinger and Giribet (2005)

Appendix 2

(Continued)

Primer	Sequence	Reference
rd4.8a	5′-ACCTATTCTCAAACTTTAAATGG-3′	Schwendinger and Giribet (2005)
rd7b1	5′-GACTTCCCTTACCTACAT-3′	Schwendinger and Giribet (2005)
F2012	5′-CCAAGGTKARYAGCCTCTRG-3′	Giribet et al. (2010)
R2762	5′-CCGCCCCAGCCAAACTCCCC-3′	Giribet et al. (2010)
16S rRNA		
ar-L	5′-CGCCTGTTTATCAAAAACAT-3′	Palumbi et al. (1991)
br-H	5′-CCGGTCTGAACTCAGATCACGT-3′	Palumbi et al. (1991)
COI		
LCO1490	5′-GGTCAACAAATCATAAAGATATTGG-3′	Folmer et al. (1994)
HCO2198	5′-TAAACTTCAGGGTGACCAAAAAATCA-3′	Folmer et al. (1994)
H3		
aF	5′-ATGGCTCGTACCAAGCAGAC-3′	Colgan et al. (1998)
aR	5′-ATATCCTTRGGCATRATRGTGAC-3′	Colgan et al. (1998)
H4		
2S	5′-TSCGIGAYAACATYCAGGGIATCAC-3′	Pineau et al. (2005)
ER	5′-CKYTTIAGIGCRTAIACCACRTCCAT-3′	Pineau et al. (2005)

When the first fragment of 28S rRNA was not easily amplified, the primers LSU5 and LSU3 were used instead of rd1a and rd4b. Primers 28Z and D2f are internal primers only used when sequencing fragments produced by the pair LSU3/LSU5.