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New insights into the phylogeny, systematics and DNA barcoding of Nemertea

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Abstract. Although some clades of ribbon worms (phylum Nemertea) are consistently recovered with high support in molecular phylogenies, the placement and inter-relationships of some taxa have proven problematic. Herein, we performed molecular phylogenetic analyses aimed at resolving these recalcitrant splits, using six loci (nuclear 18S rRNA, 28S rRNA, histones H3 and H4, and mitochondrial 16S rRNA and COI) for 133 terminals, with particular emphasis on the problematic families Hubrechtidae and Plectonemertidae. Three different datasets were used for phylogenetic analyses and both maximum likelihood and maximum parsimony methodologies were applied. All but one of the resulting tree topologies agree on the paraphyly of the class Palaeonemertea, whereas Heteronemertea, Hoplonemertea, Polystilifera, Monostilifera and Hubrechtidae are always recovered as reciprocally monophyletic. Hubrechtidae is sister group to Heteronemertea (the Pilidiophora hypothesis) only when length variable regions of 18S rRNA and 28S rRNA are excluded. Moreover, the terrestrial and freshwater family Plectonemertidae is recovered with high support and the implications of this finding are further discussed. Finally, we evaluate the utility of DNA barcoding for specimen identification within Nemertea using an extended dataset containing 394 COI sequences. Results suggest that DNA barcoding may work for Nemertea, insofar as a distinct barcoding gap (the gap between the maximum intraspecific variation and the minimum interspecific divergence) may exist, but its recognition is regularly hampered by low accuracy in species level identifications.

Additional keywords: cytochrome *c* oxidase subunit I, Hubrechtidae, Plectonemertidae, Pilidiophora.

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Introduction

Even though no universal agreement exists on the number of recognised species of ribbon worms (phylum Nemertea), current estimates suggest that the phylum accommodates over 1200 named species (Gibson 1995; Kajihara *et al*. 2008; Sundberg and Gibson 2008; Zhang 2011; Appeltans *et al*. 2012), placing it in an intermediate range among invertebrate phyla. Representatives are predominantly known from marine benthic intertidal communities, although some lineages have adapted to marine pelagic environments as well as terrestrial or freshwater habitats (Moore and Gibson 1981, 1985, 1988). The majority of nemerteans are active carnivores (e.g. Caplins *et al*. 2012) and use an eversible proboscis, enclosed by a rhyncocoel, to capture prey – a structure that is unique to the phylum (Schultze 1851; Gibson 1985). Others are scavengers or commensals, and some symbiotic bdellonemerteans have transitioned to become specialised suspension-feeders (Gibson 1967; Bell and Hickman 1985; McDermott and Roe 1985). Nemerteans are unsegmented or pseudosegmented (e.g. species of the genus *Annulonemertes*; Berg 1985; Kajihara *et al*. 2000; Sundberg and Strand 2007) bilaterally symmetrical worms with almost unrivalled variation in body length, ranging from only a few millimetres to over 30 m (McIntosh 1873). Despite this variation, there is a notable paucity of morphological characters useful for diagnosing nemertean species, genera or even families (e.g. Rogers *et al*. 1995; Chen *et al*. 2010; Strand *et al*. 2013). However, at the class level the characteristic stylet found at the end of the proboscis is a clear synapomorphy for Enopla (including Hoplonemertea and Bdellonemertea); this structure is lacking from other clades formerly grouped together in the paraphyletic class Anopla (including Heteronemertea and Palaeonemertea). Separation of Enopla from other clades is further supported by the relative placement of the mouth and proboscis pore: in Heteronemertea and Palaeonemertea the mouth and the proboscis pore are separate, whereas the pore is fused with the mouth in most monostiliferan hoplonemerteans (Härlin and Sundberg 1995; Chernyshev 2003). Considering the simplicity of the nemertean body plan (see Figs 1 and 2 for some representative taxa), especially after fixation, and the high degree of homoplasy in the commonly used morphological

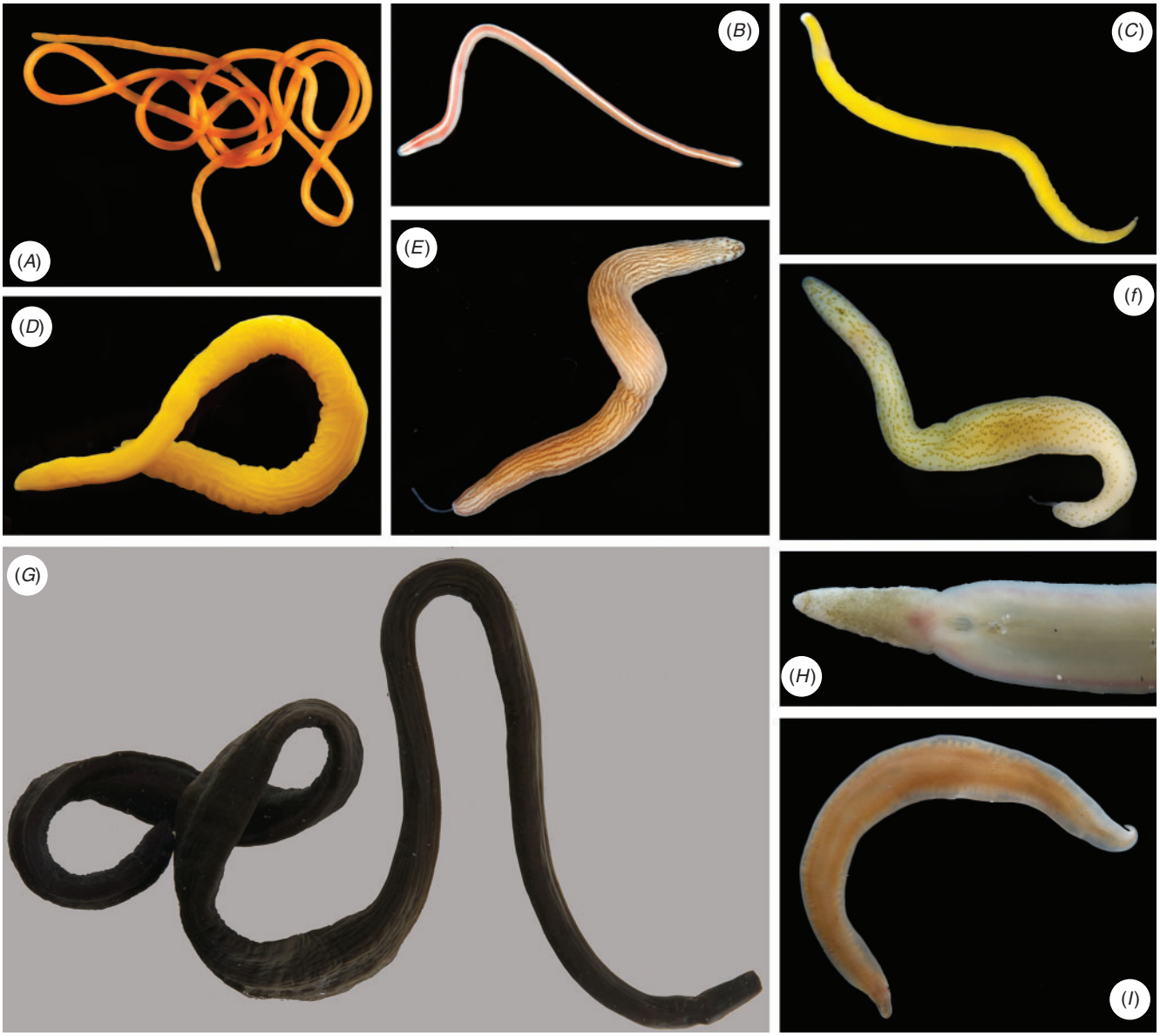

Fig. 1. Live habitus of selected specimens. Photos by G. Giribet, except where specified. (*A*) *Cephalotrix bipunctata* Bürger, 1892 IZ-133009 (Los Escullos, Cabo de Gata, Almería, Spain, 7.xii.2010; photo J. Junoy). (*B*) *Micrura* sp. IZ-132529 (Bocas del Toro, Panama, 21.iii.2013). (*C*) *Micrura* sp. IZ-132532 (Bocas del Toro, Panama, 22.iii.2013). (*D*) *Micrura* sp. IZ-133724 (Bocas del Toro, Panama, 17.iii.2010). (*E*) *Micrura rubramaculosa* (Bocas del Toro, Panama, 19.iii.2011), specimen conspecific with IZ-132531. (*F*) *Micrura chlorapardalis* IZ-132530 (Bocas del Toro, Panama, 20.iii.2013). (*G*) *Notospermus* sp. (Bocas del Toro, Panama, 15.iii.2010), conspecific with specimen IZ-132528. (*H*) '*Notospermus*' sp. IZ-134234 (Stradbroke Island, Queensland, Australia, 16.x.2008). (*I*) Reptantia sp. IZ-133024 (Bocas del Toro, Panama, 12.iii.2012). Additional specimen details can be found in MCZbase (http://mczbase.mcz.harvard.edu/).

characters (Sundberg and Svensson 1994; Schwartz and Norenburg 2001), it is probable that the phylum harbours a significant proportion of overlooked species diversity (Appeltans *et al*. 2012). One way of circumventing the inadequacy of morphological characters in identifying and diagnosing specimens is to employ molecular tools, such as DNA taxonomy or DNA barcoding, to alleviate this situation (e.g. Mahon *et al*. 2010; Sundberg *et al*. 2010; Strand and Sundberg 2011). However, such an endeavour presupposes both an *a priori* knowledge of the disposition of interspecific and intraspecific genetic variation within the target group, and a well-sampled genetic database for comparative purposes; these elements are largely lacking for nemerteans (Kvist 2013).

While Nemertea is often recovered as the sister group to Brachiopoda in metazoan phylogenies (e.g. Dunn *et al*. 2008; Hejnol *et al*. 2009; but see also supplementary material in Kocot *et al*. 2011 as a contrary example), together forming the clade Kryptrochozoa (Giribet *et al*. 2009), some internal relationships of the phylum remain unsupported. At the ordinal level, Sundberg *et al*. (2001) recovered a polyphyletic

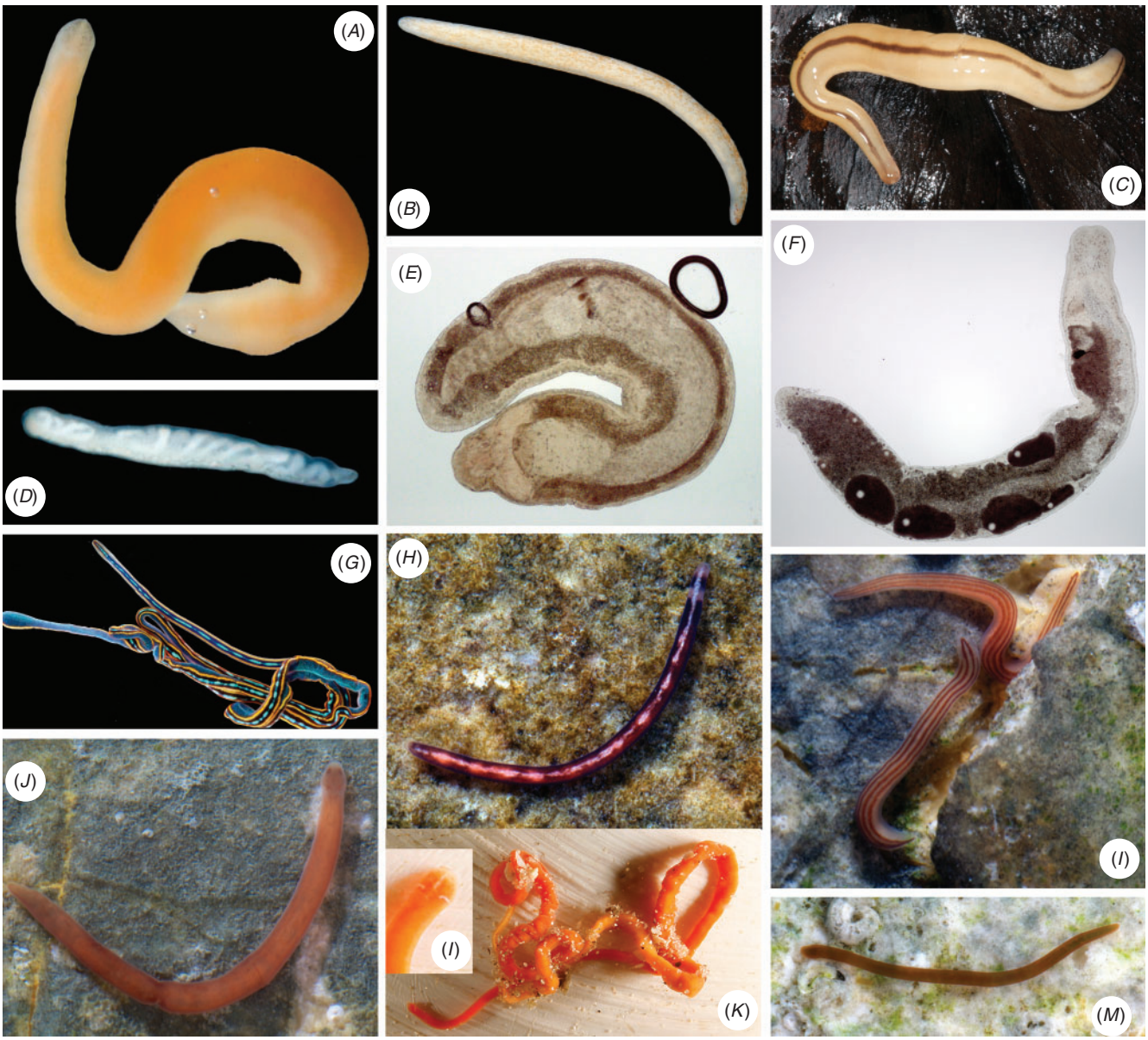

Fig. 2. Live habitus of selected specimens. Photos by G. Giribet, except where specified. (*A*) Amphiporidae sp. IZ-132476 (Isla Alborán, 22.ix.2011; photo J. Junoy). (*B*) Nemertea sp. IZ-132533 (Bocas del Toro, Panama, 22.iii.2013). (*C*) *Geonemertes* sp. IZ-132534 (Bocas del Toro, Panama, 20.iii.2013). (*D*) cf. *Potamonemertes percivali* IZ-25172 (Rangitata River, W of Ashburton, New Zealand; photo N. Boustead). (*E*) Plectonemertidae sp. A IZ-25174 (Kinglake Road, Melbourne, Australia; photo C. Laumer). (*F*) Plectonemertidae sp. B IZ-25175 (Yea River, Melbourne, Australia; photo C. Laumer). (*G*) Monostilifera sp. IZ-133023 (Bocas del Toro, Panama, 12.iii.2012). (*H*) *Oerstedia* sp. IZ-132743 (Cabrera, Balearic Islands, Spain, 29.vii.2012). (*I*) *Tetrastemma vittigera* IZ-25171 (Cabrera, Balearic Islands, Spain, 28.vii.2012). (*J*) Tetrastemmatidae sp. IZ-132537 (Cabrera, Balearic Islands, Spain, 28.vii.2012). (*K*, *L*) Hoplonemertea sp. IZ-135340 (Ribeira, A Coruña, Galicia, Spain, 14.xii.2010; photo J. Junoy). (*M*) *Tetrastemma* sp. IZ-132742 (Cabrera, Balearic Islands, Spain, 29.vii.2012). Additional specimen details can be found in MCZbase (http://mczbase.mcz.harvard.edu/).

Palaeonemertea when analysing 18S rRNA data, with some species nesting as the sister group to Heteronemertea while others were the sister species to Hoplonemertea. In line with this finding, Thollesson and Norenburg (2003), on the basis of two nuclear (28S rRNA and histone H3) and two mitochondrial (16S rRNA and cytochrome *c* oxidase subunit I, COI) genes, recovered a paraphyletic Palaeonemertea as sister group to a clade comprising Heteronemertea and Hoplonemertea (Neonemertea), with the monogeneric Bdellonemertea nested within monostiliferan hoplonemerteans (the latter placement was also recovered by Sundberg *et al*. 2001). In contrast to the common finding of a paraphyletic Palaeonemertea, Andrade *et al*. (2012) employed six markers (28S rRNA, 18S rRNA, histones H3 and H4, 16S rRNA and COI) and recovered the class (excluding *Hubrechtella dubia* Bergendal, 1902) as monophyletic, but its placement relative to other classes was

sensitive to the optimality criterion – under parsimony, the class is the sister group to Hoplonemertea + *Hubrechtella dubia*, whereas it is the sister group to Heteronemertea under a maximum likelihood approach.

From the limitations of morphological characters has arisen one of the most difficult challenges to deep nemertean phylogenetics: determining the placement of hubrechtiid palaeonemerteans. Based on morphology, Norenburg (1993) suggested an affinity between hubrechtiids and heteronemerteans, and underscored the finding of Cantell (1969) in that *Hubrechtella dubia* possesses a pilidium larva, a feature that is otherwise exclusive to the class Heteronemertea. However, in a phylogenetic study of Palaeonemertea based solely on morphology, Sundberg and Hylbom (1994) recovered a clade of hubrechtiid taxa nested well within Palaeonemertea – this was later corroborated by phylogenetic analysis of 18S rRNA sequences (Sundberg *et al*. 2001). In contrast, Thollesson and Norenburg (2003) recovered *Hubrechtella dubia* as the sister group to Heteronemertea, which impelled the authors to erect Pilidiophora, a clade comprising the taxa with a planktotrophic pilidium larva, a pelagic larval type found only within Nemertea, inside which the juvenile forms, eventually undergoing a catastrophic metamorphosis to the adult form (e.g. Maslakova 2010). Employing extensive taxon and data sampling, as well as both maximum likelihood and parsimony approaches, Andrade *et al*. (2012) found conflicting placements of *Hubrechtella dubia* depending on the optimality criterion used. Under the maximum likelihood approach, the Pilidiophora hypothesis was recovered (albeit with middling support) whereas parsimony analysis suggested the sister group relationship of *Hubrechtella dubia* and Hoplonemertea. In a recent investigation into the morphological features of the proboscis of *Hubrechtella juliae* Chernyshev, 2003, Chernyshev *et al*. (2013) found similarities between this species and species of *Baseodiscus* Diesing, 1850 (Heteronemertea), thereby adding to the list of potential synapomorphies for Pilidiophora. Given the above, the Pilidiophora hypothesis remains compelling, but deserves further testing.

The lack of a well-supported and taxon-rich phylogeny of Nemertea has not only limited our knowledge of the interrelationships of the taxa, but also constrained our understanding of their ecological adaptations, such as the mode and timing of nemertean transitions between sea, land and freshwater. Freshwater nemerteans occur in both Hoplonemertea and Heteronemertea, and terrestrial forms are almost unique to Hoplonemertea (Moore and Gibson 1985), and the current discussion on the evolutionary routes taken by nemerteans in their colonisation of freshwater and terrestrial habitats is divided between two main hypotheses (see Moore and Gibson 1985, 1988). The first suggests that marine nemerteans colonised land before a transition to freshwater habitats. This hypothesis is supported by the presence of terrestrial nemerteans on widespread island systems on which freshwater is missing (Moore and Gibson 1985). The second hypothesis is the psammolittoral-phreatic route, in which marine nemerteans, before invading land, have transitioned between fully marine interstitial compartments to the supralittoral zone where saturation of freshwater occurs (Pennak 1963). Moore and Gibson (1988) suggest that these hypotheses are not mutually exclusive and that it is likely that nemerteans have invaded freshwater and terrestrial habitats on several independent occasions and possibly through different modes of transition, and this is supported also by recent molecular analyses (Andrade *et al*. 2012).

The present account aims to elucidate four main topics: (i) the general phylogenetic relationships of the phylum in light of a largely increased taxon sampling; (ii) the precise placement of hubrechtiid nemerteans within the largest taxon set assembled for Nemertea by increasing sampling for these elusive nemerteans; (iii) the status and phylogenetic placement of terrestrial and freshwater taxa, with special reference to the family Plectonemertidae; and (iv) the utility of DNA barcoding as a potential tool for rapid and accurate specimen identification across this morphologically challenging phylum.

Materials and methods

Specimen collection

Specimens were collected in the field, or obtained from colleagues, and photographed, preserved in ~96% EtOH and stored in a −20°C freezer at the Museum of Comparative Zoology (MCZ) upon arrival. Collecting details and photographs of selected specimens are available in the MCZ online database (http://mczbase.mcz.harvard.edu), and can be accessed through their catalogue number (Table 1).

DNA extraction, amplification and purification

Tissue was cut from the most posterior part of each specimen; some specimens were already fragmented and, in such cases, tissue was taken from the posterior part of the fragment (for cf. *Ototyphlonemertes pallida* IZ-133745, the entire piece of the specimen was used owing to its minute nature). Total genomic DNA was subsequently extracted using the DNeasy Tissue Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. The remaining parts of the worms are stored as vouchers (except Nemertea sp. SK80, which was sent back to the collectors) at the Museum of Comparative Zoology at Harvard University (Table 1).

Six loci were amplified from the isolates: nuclear 18S rRNA, 28S rRNA, histones H3 and H4, and mitochondrial 16S rRNA and cytochrome *c* oxidase subunit I (COI). All primers (10 μM concentration) used for the amplification and sequencing reactions are listed in Table 2. These loci were chosen because they complement the available data for Nemertea, such that these can be analysed in conjunction to the highest extent possible. Amplification used one of two protocols. We first attempted to amplify the DNA using the GoTaq (Promega, Madison, WI, USA) DNA polymerase, with 0.5–1 μL DNA template, 0.25 μL of forward and reverse primers, 18 μL water, 5 μL 5X Green GoTaq Flexi Buffer and 0.13–0.20 μL taq. If unsuccessful, amplifications were carried out using Amplitaq DNA polymerase (Life Technologies, Waltham, MA) with 1 μL DNA template, 0.25 μL of forward and reverse primers, 20 μL water, 2.5 μL Buffer I and 0.13–0.20 μL taq. The following

Table 1. Newly sequenced specimens used in the present study with Museum of Comparative Zoology (Harvard University) voucher numbers and their corresponding GenBank accession numbers

Asterisks denote sequences downloaded from GenBank for which the same specimens were used to generate sequences for currently unavailable loci. Note that the below sequences were joined with those of Andrade *et al*. (2012) to form the final data matrix. Unfortunately, the short nature of the histone H4 sequences prohibits their deposition in GenBank. Therefore, these sequences can be attained from the first author upon request or from TreeBASE submission 14868

ID	Voucher number	18S	28S	H3	H4	16S	COI
Carinoma hamanako	IZ-135341	KF935278	KF935334	KF935390	N/A	KF935446	KF935500
Cephalothrix bipunctata	IZ-133009	KF935279	KF935335	KF935391	N/A	KF935447	KF935501
Baseodiscus cf. *delineatus*	IZ-133729	KF935280	KF935336	KF935392	N/A	KF935448	KF935502
Baseodiscus mexicanus	IZ-135321	KF935281	KF935337	KF935393	N/A	KF935449	KF935503
Nemertea sp.	SK80	KF935282	KF935338	KF935394	N/A		KF935504
Baseodiscus unicolor	IZ-132527	KF935283	KF935339	KF935395	N/A	KF935450	
Baseodiscus unicolor	IZ-135323	KF935284	KF935340	KF935396	N/A	KF935451	KF935505
Baseodiscus unicolor	IZ-135324	KF935285	KF935341	KF935397	N/A	KF935452	
Micrura ignea	IZ-133720	KF935286	KF935342	KF935398	N/A	KF935453	KF935506
Micrura ignea	IZ-135349	KF935287	KF935343	KF935399	N/A	KF935454	KF935507
Micrura verrilli	IZ-134451	KF935288	KF935344	KF935400	N/A	KF935455	KF935508
Micrura sp.	IZ-133724	KF935289	KF935345	KF935401	N/A	KF935456	KF935509
Micrura sp.	IZ-132532	KF935290	KF935346	KF935402	N/A	KF935457	KF935510
Micrura sp.	IZ-132529	KF935291	KF935347	KF935403	N/A	KF935458	KF935511
Micrura chlorapardalis	IZ-132530	KF935292	KF935348	KF935404	N/A	KF935459	KF935512
Micrura rubramaculosa	IZ-132531	KF935293	KF935349	KF935405	N/A	KF935460	KF935513
Micrura dellechiajei	IZ-132745	KF935294	KF935350	KF935406	N/A	KF935461	KF935514
Notospermus geniculatus	IZ-132741	KF935295	KF935351	KF935407	N/A	KF935462	
Notospermus sp.	IZ-132528	KF935296	KF935352	KF935408	N/A	KF935463	KF935515
Notospermus sp.	IZ-133726	KF935297	KF935353	KF935409	N/A	KF935464	
Notospermus sp.	IZ-134234	KF935298	KF935354	KF935410	N/A	KF935465	KF935516
Notospermus sp.	IZ-135356	KF935299	KF935355	KF935411	N/A	KF935466	
Cerebratulus leucopsis	IZ-135331	KF935300	KF935356	KF935412	N/A	KF935467	KF935517
Lineus sp.	IZ-132744	KF935301	KF935357	KF935413	N/A	KF935468	KF935518
Ramphogordius lacteus	IZ-135373	KF935302	KF935358	KF935414	N/A	KF935469	KF935519
Hubrechtella ijimai	IZ-135342	KF935303	KF935359			KF935470	KF935520
Hubrechtidae sp.	IZ-25168	KF935304	KF935360	KF935415		KF935471	KF935521
Reptantia sp.	IZ-132526	KF935305	KF935361	KF935416	N/A	KF935472	KF935522
Reptantia sp.	IZ-133024	KF935306	KF935362	KF935417	N/A	KF935473	KF935523
Amphiporidae sp.	IZ-132746	KF935307	KF935363	KF935418	N/A	KF935474	
Nemertea sp.	IZ-132533	KF935308	KF935364	KF935419	N/A	KF935475	KF935524
Argonemertes sp.	IZ-135315	KF935309	KF935365	KF935420	N/A	KF935476	KF935525
Plectonemertidae sp.	IZ-25166	KF935310	KF935366	KF935421		KF935477	KF935526
Plectonemertidae sp.	IZ-25167	KF935311	KF935367			KF935478	KF935527
Plectonemertidae sp.	IZ-25169	KF935312	KF935368	KF935422		KF935479	KF935528
Plectonemertidae sp.	IZ-25173	KF935313	KF935369	KF935423	N/A	KF935480	KF935529
Plectonemertidae sp.	IZ-25174	KF935314	KF935370	KF935424	N/A	KF935481	KF935530
Plectonemertidae sp.	IZ-25175	KF935315	KF935371	KF935425	N/A	KF935482	KF935531
cf. *Potamonemertes percivali*	IZ-25172	KF935316	KF935372	KF935426	N/A	KF935483	KF935532
Monostilifera sp.	IZ-133023	KF935317	KF935373	KF935427	N/A	KF935484	KF935533
Plectonemertidae sp.	NT000046	EU255585*		KF935428			EU255614*
Plectonemertidae sp.	NT000059	EU255592*		KF935429		KF935485	EU255621*
Plectonemertidae sp.	NT000072	EU255596*				KF935486	EU255626*
Tetranemertes antonina	IZ-132747	KF935318	KF935374	KF935430	N/A		KF935534
Oerstedia sp.	IZ-132740	KF935319	KF935375	KF935431	N/A	KF935487	KF935535
Oerstedia sp.	IZ-132743	KF935320	KF935376	KF935432	N/A	KF935488	KF935536
Antarctonemertes valida	IZ-134228	KF935321	KF935377	KF935433	N/A	KF935489	KF935537
Antarctonemertes riesgoae	IZ-134229	KF935322	KF935378	KF935434	N/A	KF935490	KF935538
Tetrastemma vititgera	IZ-132742	KF935323	KF935379	KF935435	N/A	KF935491	KF935539
Tetrastemma vittigera	IZ-25171	KF935324	KF935380	KF935436	N/A		KF935540
Tetrastemma sp.	IZ-132742	KF935325	KF935381	KF935437	N/A	KF935492	KF935541
Tetrastemmatidae	IZ-132537	KF935326	KF935382	KF935438	N/A	KF935493	KF935542
Hoplonemertea sp.	IZ-135340	KF935327	KF935383	KF935439	N/A	KF935494	KF935543
Vietezia luzmurubeae	IZ-133740	KF935328	KF935384	KF935440	N/A	KF935495	KF935544
cf. *Ototyphlonemertes pallida*	IZ-133745	KF935329	KF935385	KF935441	N/A	KF935496	KF935545

(*continued next page*)

Table 1. (*continued*)

ID	Voucher number	18S	28S	H3	H4	16S	COI
Emplectonema sp.	IZ-135333	KF935330	KF935386	KF935442		KF935497	KF935546
Amphiporus formidabilis	IZ-134452	KF935331	KF935387	KF935443	N/A	KF935498	KF935547
Malacobdella cf. *grossa*	IZ-25170	KF935332	KF935388	KF935444	N/A		
Geonemertes sp.	IZ-132534	KF935333	KF935389	KF935445	N/A	KF935499	KF935548

Table 2. List of primers used in the present study

Forward primer sequences are denoted in bold font. For 28S, the reverse primer rd4b was used with rd1a only when the primer set rd1a/b failed to amplify

Target locus	Primer name	Primer sequence	Reference
18S rDNA	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 rDNA	rd1a	**5′-CCCSCGTAAYTTAGGCATAT-3′**	Edgecombe and Giribet (2006)
	rd4b	5′-CCTTGGTCCGTGTTTCAAGAC-3′	Edgecombe and Giribet (2006)
	b	5′-TCGGAAGGAACCAGCTAC-3′	Whiting *et al.* (1997)
	a	**5′-GACCCGTCTTGAAACACGGA-3′**	Whiting *et al.* (1997)
	rd5b	5′-CCACAGCGCCAGTTCTGCTTAC-3′	Schwendinger and Giribet (2005)
	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 rDNA	ar-L	**5′-CGCCTGTTTATCAAAAACAT-3′**	Palumbi *et al.* (1991)
	br-H	5′-CCGGTCTGAACTCAGATCACGT-3′	Palumbi *et al.* (1991)
COI mtDNA	LCO1490	**5′-GGTCAACAAATCATAAAGATATTGG-3′**	Folmer *et al.* (1994)
	HCO2198	5′-TAAACTTCAGGGTGACCAAAAAATCA-3′	Folmer *et al.* (1994)
Histone H3	aF	**5′-ATGGCTCGTACCAAGCAGAC-3′**	Colgan *et al.* (1998)
	aR	5′-ATATCCTTRGGCATRATRGTGAC-3′	Colgan *et al.* (1998)
Histone H4	2S	**5′-TSCGIGAYAACATYCAGGGIATCAC-3′**	Pineau *et al.* (2005)
	ER	5′-CKYTTIAGIGCRTAIACCACRTCCAT-3′	Pineau *et al.* (2005)

thermal profiles were used for the PCR amplifications: 2 min initial denaturation at 94°C for all samples followed by 30–35 cycles of 30 s denaturation at 94°C, 30–60 s annealing at 45−48°C, 1 min extension at 72°C; all reactions were completed with a final extension step for 10 min at 72°C. Polymerase chain reaction (PCR) products were then visualised on a 1% agarose gel and purified using ExoSAP-IT (USB Corp, Cleveland, OH) following manufacturer's protocols. Purified products were cycle-sequenced using the same primers as for the amplifications and the following reagents: 3.2 μL sequencing primer (0.5 μM), 4.8 μL water, 1 μL purified DNA template, 0.5 μL ABI BigDye 5× sequencing buffer, and 0.5 μL ABI BigDye Terminator ver. 3.1 (Applied Biosystems, Foster City, CA) for a total volume of 10 μL. Cycle-sequencing products were further cleaned using Sephadex G-50 (GE Healthcare, Piscataway, NJ) columns and later sequenced using an ABI Prism 3730 Genetic Analyzer (Applied Biosystems).

Alignment and phylogenetic analysis

Forward and reverse sequences were assembled, and the contigs manually edited using Sequencher ver. 5.1 (Gene Codes Corporation, Ann Arbor, MI). Prior to alignment, BLASTn searches against the NCBI nr database were carried out for each sequence to control for potential contaminations.

In order to increase the taxon sampling and thereby more robustly infer the phylogenetic relationships, the newly generated dataset was then combined with that used by Andrade *et al.* (2012), both datasets having targeted the same markers. Multiple sequence alignments were carried out separately for each locus using MAFFT ver. 7 (Katoh and Standley 2013), and employing the L-INS-i strategy (recommended for sequences with one conserved domain and flanking gaps) for COI, histones H3 and H4, and the E-INS-i strategy (recommended for sequences with multiple conserved domains and interspersed gaps) for the ribosomal 18S, 28S and 16S rRNAs. The alignments used the following settings: 1.53 gap opening penalty for 16S rRNA, COI, and histones H3 and H4; 3.00 gap opening penalty for 18S rRNA and 28S rRNA; the 200PAM/K = 2 scoring matrix and an offset value of 0.0. From this, three different datasets were constructed in Mesquite ver. 2.5 (Maddison and Maddison 2010): the first including all of the newly generated data, as well as those of Andrade *et al.* (2012); the second including the same data but masking the hypervariable regions of 18S rRNA and 28S rRNA by employing the web version of Gblocks ver. 0.91b (Castresana

2000) (for this purpose, we allowed smaller final blocks, gap positions within the final blocks, less strict flanking positions but we did not allow many contiguous non-conserved positions); and the third including COI, histone H3, 16S rRNA and 18S rRNA sequences exclusively for Hubrechtidae and Hoplonemertea with the addition of four terrestrial taxa for which both GenBank sequences and unpublished sequences were used. These were *Antiponemertes novaezealandiae* (Dendy, 1895) (18S rRNA: AY928345), Acteonemertidae NT000046 (COI: EU255614, 18S rRNA: EU255585, histone H3: unpublished, 16S rRNA: unpublished), Acteonemertidae NT000059 (COI: EU255621, 18S rRNA: EU255592, histone H3: unpublished, 16S rRNA: unpublished), and Acteonemertidae NT000072 (COI: EU255626, 18S rRNA: EU255596, histone H3: unpublished, 16S rRNA: unpublished). The final data matrices and trees are available from TreeBASE under submission 14868.

Maximum likelihood (ML) and maximum parsimony (MP) analyses were then used to reconstruct the phylogenetic relationships. Prior to the ML analysis, optimal partitioning schemes and best-fitting models of nucleotide evolution (restricting the search to GTR and GTR + Γ) were identified for each dataset using PartitionFinder ver. 1.1.1 (Lanfear *et al*. 2012). PartitionFinder uses an heuristic algorithm (here we used the 'greedy' algorithm), beginning with a user-provided fully partitioned dataset (i.e. partitioned by locus and codon position, when appropriate) and identifies the best-fitting partitioning scheme by likelihood tests using a predetermined selection criterion (in this case the Bayesian information criterion). Employing the best partitioning scheme, an heuristic search was performed using RAxML ver. 7.6.3 (Stamatakis 2006) on the CIPRES Science Gateway platform (Miller *et al*. 2010) with a GTR + Γ model of sequence evolution for all partitions, and consisting of 1000 iterations with 25 initial GAMMA rate categories and final optimisation with four GAMMA shape categories (RAxML was called as follows: raxmlHPC-HYBRID -T 6 -s infile -n result -q part -p 12345 -m GTRGAMMA -o Terebratalia_transversa -f d -N 1000). Standard bootstrap support values were calculated using 1000 pseudoreplicates with a different starting tree for each iteration.

For MP, a new technology search was performed using TNT (Goloboff *et al*. 2008). Trees were recovered by using 1000 initial addition sequences, five rounds of ratcheting and three rounds of tree fusing after the initial Wagner tree builds, and requiring that the minimum length tree be found a total of 10 times before terminating the search. Using the command 'bbreak', trees resulting from the new technology search were then returned to TNT for TBR branch swapping. All characters were equally weighted and non-additive, and gaps were treated as missing data to use the same information employed in the probabilistic analyses. Bootstrap support was calculated from 1000 pseudoreplicates with the same settings as mentioned above. All trees were rooted at *Terebratalia transversa* (Sowerby, 1846) (Brachiopoda) following Andrade *et al*. (2012).

Tree constraints

Likelihood-based Shimodaira-Hasegawa (SH; Shimodaira and Hasegawa 1999) and approximately unbiased tests (AU; Shimodaira 2002) were carried out to assess statistical differences between *a posteriori* topological hypotheses of the completely unconstrained (i.e. the maximum likelihood) tree and a tree in which Hubrechtidae + Heteronemertea were forced to form a monophyletic group (Pilidiophora). Both SH and AU tests use nonparametric bootstrap resampling of estimated sitewise log-likelihood scores to assess the statistical significance (in a frequentist framework) of differences in likelihood scores between user-constrained phylogenetic trees, usually including the ML tree. The SH test is more conservative than the AU test, but this behaviour is most problematic only when many tree topologies are compared (Shimodaira 2002). Per site log-likelihood values (which summed constitute the log-likelihood of a phylogram) were calculated in RAxML using the '-f g' option (RAxML was called as follows: raxmlHPC-HYBRID -T 6 -s input_file –n results -p 12345 -m GTRGAMMA -f g -z best_constrained_tree) and these were subsequently submitted to CONSEL (Shimodaira and Hasegawa 2001) for statistical analysis under default parameters. Statistical significance ($p \leq 0.05$) was calculated based on the best-scoring unconstrained and constrained RAxML trees; because CONSEL does not consider partitions when performing resampling of sitewise log-likelihoods, the site likelihood values were derived from an unpartitioned analysis. In addition, an analysis forcing the monophyly of Pilidiophora was also performed for parsimony, using the 'force +' command in TNT; the number of extra steps needed to enforce the constraint were then counted.

Barcoding gap detection

To investigate the presence of a phylum-wide barcoding gap for Nemertea, all COI sequences connected to a binomial taxonomic label in GenBank were downloaded. Imprecise taxon labels (e.g. *Cerebratulus* sp.) were discarded to increase the certainty of comparing the same species in the intraspecific analyses and different species in the interspecific analyses. That is, in order to solidly infer intraspecific variations and interspecific divergences, we needed to know the exact taxonomic affiliations of the sequences – although the rate of erroneous identifications in nemerteans and other soft-bodied worms tends to be high. In addition, all sequences less than 200 bp long were removed from the dataset. The COI sequences downloaded from GenBank were combined with the newly generated COI sequences for which species level identifications were available and these were jointly aligned using MAFFT L-INS-i applying default gap opening costs. In four cases, MAFFT detected reverse complementation of sequences (this is an automated feature of version 7 when performing the analyses online at http://mafft.cbrc.jp/alignment/server/) and these were therefore reversed using the sequence manipulation suite (Stothard 2000). MEGA ver. 5 (Tamura *et al*. 2011) was used to calculate intraspecific variations and interspecific divergences among the samples using the following settings: uncorrected *p*-distances, uniform rates among sites and pairwise deletion of gaps for first, second and third codon positions.

Complementary to this, automatic barcode gap discovery (ABGD; Puillandre *et al*. 2012) was employed to verify the

distribution and size of a potential barcoding gap. Automatic barcode gap discovery was applied using default settings (pmin = 0.001, pmax = 0.1, steps = 10, and Jukes-Cantor [JC69] distances). A neighbour-joining tree was also constructed by MEGA ver. 5 for the full COI dataset, using uncorrected *p*-distances and applying mid-point rooting.

Results

In total, 56 specimens were newly sequenced for 18S rRNA, 28S rRNA, histones H3 and H4, 16S rRNA and COI; three additional plectonemertid specimens were sequenced for histone H3 and 16S (18S rRNA and COI are already available for these taxa; see Table 1), and *Antarctonemertes riesgoae* Taboada, Junoy, Andrade, Giribet, Cristobo & Avila, 2013 and *A. valida* (Bürger, 1893) were newly sequenced for parts of 28S rRNA and histones H3 and H4 (partial 28S rRNA, 16S rRNA and COI was already available for these taxa; see Table 1). All molecular sequences that were newly generated for the present study have been deposited in GenBank under accession numbers KF935278–KF935548 (Table 1), with the exception of histone H4 whose short sequence length prohibits their deposition in GenBank. The histone H4 sequences are therefore available from the first author upon request or from TreeBASE submission number 14868. The total number of aligned sites for the full dataset after inclusion of the data used by Andrade *et al*. (2012) was 8706 (18S rRNA: 2238 bp; 28S rRNA: 4615 bp; histone H3: 341 bp; histone H4: 166 bp; 16S rRNA: 681 bp; COI: 665 bp). For the alignment treated with Gblocks, the corresponding number was 4946 aligned sites (18S rRNA: 1650 bp; 28S rRNA: 1479 bp; histone H3: 341 bp; histone H4: 166 bp; 16S rRNA: 645 bp; COI: 665 bp). The extended hoplonemertean alignment, including four taxa for which sequences were downloaded from GenBank, comprised 3599 aligned sites (18S rRNA: 2030 bp; histone H3: 335 bp; 16S rRNA: 576 bp; COI: 658 bp).

Phylogeny – full dataset

The heuristic ML search of the full six-marker partitioned dataset produced a tree with an ln*L* of –195608.806782 (Fig. 3). Bootstrap values are rather low across the topology, especially in the deeper nodes. In the tree, Nemertea is recovered as monophyletic but with low likelihood bootstrap support (LBS: 54). As in previous studies, Palaeonemertea is recovered as paraphyletic with *Carinoma* Oudemans, 1885 species + *Carinina ochracea* Sundberg, Chernyshev, Kajihara, Kånneby & Strand, 2009 (LBS: 89) and the remaining tubulanids + cephalothricids (LBS: 85) forming two consecutive clades with *Carinoma* spp. + *C. ochracea* as the earliest diverging lineage, sister group to the remaining nemerteans (LBS: 85). The monophyly of Heteronemertea received maximum support (LBS: 100) and the class nests as the sister group to Hubrechtidae + Hoplonemertea (LBS: 65), and both of the latter are also reciprocally monophyletic and maximally supported.

Within the paraphyletic Palaeonemertea, *Callinera grandis* Bergendal, 1903 nests within *Tubulanus* Renier, 1804 rendering the genus paraphyletic (LBS: 100), whereas Cephalothricidae is recovered as its monophyletic (LBS: 100) sister group.

Within Heteronemertea, the included specimens of *Baseodiscus* are recovered as a clade (LBS: 89), as the sister group to the remaining heteronemerteans. Much like in the tree produced by Andrade *et al*. (2012), numerous heteronemertean genera are rendered non-monophyletic. For example, the included species of *Micrura* Ehrenberg, 1871 are recovered in six separate places in the tree (see also Schwartz and Norenburg 2005): the three specimens of *Micrura ignea* Schwartz & Norenburg, 2005 (monophyletic with LBS: 100) are the sister species to the remaining non-*Baseodiscus* heteronemerteans (LBS: 55); a clade comprising *Micrura verrilli* Coe, 1901 and three undetermined *Micrura* species (LBS: 77) nest on a consecutive branch to *Micrura ignea*, as sister group to the remaining taxa (LBS: 95); *Micrura dellechiajei* (Hubrecht, 1879) appears as the sister group to three specimens of *Notospermus* Huschke, 1829 (LBS: 95); *Micrura chlorapardalis* Schwartz & Norenburg, 2005 groups with *Micrura rubramaculosa* Schwartz & Norenburg, 2005 (LBS: 100) as sister clade to *Cerebratulus marginatus* Renier, 1804 (LBS: 78); and *Micrura purpurea* (Dalyell, 1853) nests as sister species to a clade (LBS: 73) containing *Parborlasia corrugatus* (McIntosh, 1876), the monophyletic *Micrura fasciolata* Ehrenberg, 1828 (LBS: 100), the two paraphyletic genera *Lineus* Sowerby, 1806 and *Ramphogordius* Rathke, 1843, and the monophyletic *Riseriellus occultus* Rogers, Junoy, Gibson & Thorpe, 1993 specimens (LBS: 100).

The three hubrechtiid specimens form a clade (LBS: 100) sister to Hoplonemertea (LBS: 100), which, in turn, splits into Polystilifera (LBS: 100) and Monostilifera (LBS: 100). Interestingly, a clade of terrestrial and freshwater hoplonemerteans is recovered with high bootstrap support (LBS: 100) (see below and 'Discussion'). This clade includes *Argonemertes australiensis* (Dendy, 1892), *Leptonemertes* cf. *chalicophora* (Graff, 1879), cf. *Potamonemertes percivali* Moore & Gibson, 1973, and four undescribed plectonemertid species from Australia.

The strict consensus of nine equally parsimonious trees (length: 42 678 steps; consistency index (CI): 0.253; retention index (RI): 0.610) produced by TNT (Fig. 4) for the same dataset is highly congruent with that of the ML analysis, albeit with minor differences in the detailed placement of some taxa. Overall, however, support values associated with the parsimony tree are relatively lower than those of the ML tree. Palaeonemertea is again recovered as paraphyletic, but in this tree, the *Tubulanus* + *Callinera* and Cephalotricidae clade (parsimony bootstrap support (PBS): 69) is recovered as the earliest diverging lineage (PBS: 98). The remaining major lineages are recovered as monophyletic; Heteronemertea with PBS: 100; Hubrechtidae with PBS: 100; Hoplonemertea with PBS: 99; Polystilifera with PBS: 100; and Monostilifera with PBS: 99).

Both the SH test and AU test agree that the unconstrained tree and the tree forced to show a monophyletic Pilidiophora are statistically different ($p = 0.00$ and $p = 3E^{-64}$, respectively). However, the constrained parsimony analysis resulted in nine equally parsimonious trees (not shown) with 42 686 steps (CI: 0.253; RI: 0.610); only eight extra steps (~0.02% of total tree length) were needed to invoke a monophyletic Pilidiophora.

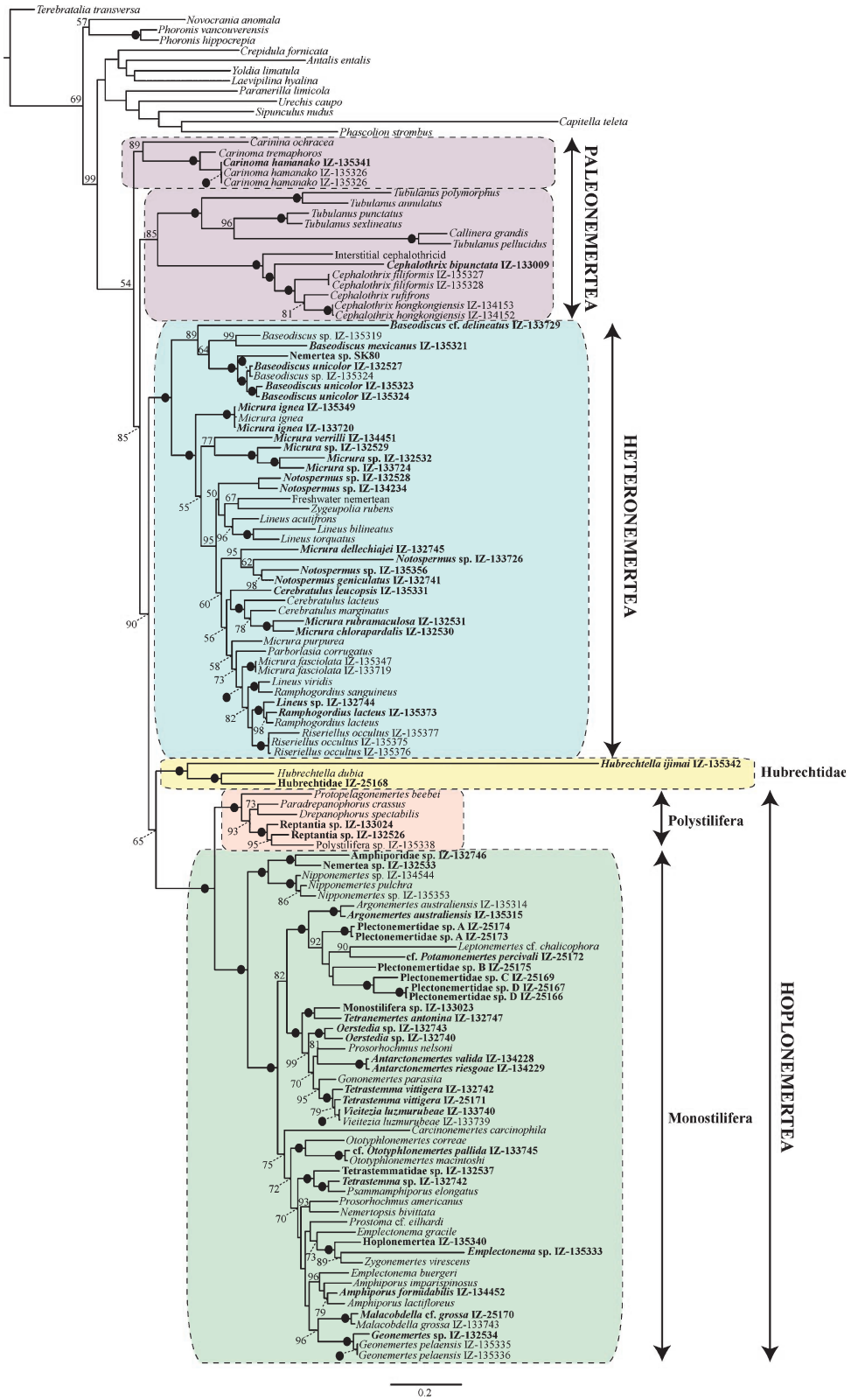

Fig. 3. Best scoring tree from the maximum likelihood analysis of the full six-marker dataset (ln*L* = –195608.806782). Likelihood bootstrap values >50% are shown above each node and solid circles indicate full bootstrap support. Specimens sequenced for the present study are denoted in bold font and IZ numbers refer to the morphological voucher ID deposited in the Department of Invertebrate Zoology collection of the MCZ.

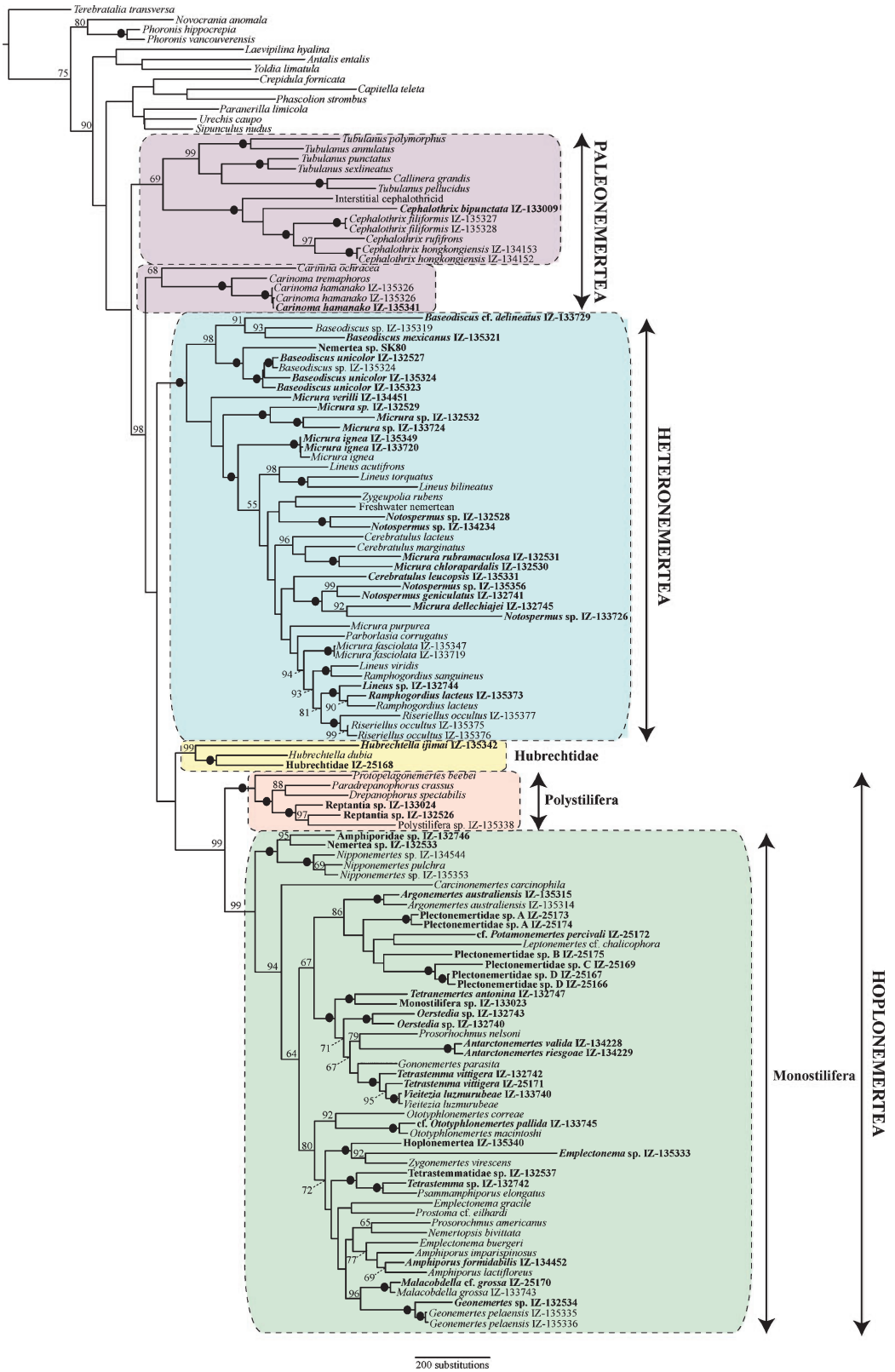

Fig. 4. Strict consensus of nine equally parsimonious trees (length: 42 678 steps; consistency index: 0.253; retention index: 0.610) produced by TNT for the full six-marker dataset. Legends as in Fig. 3.

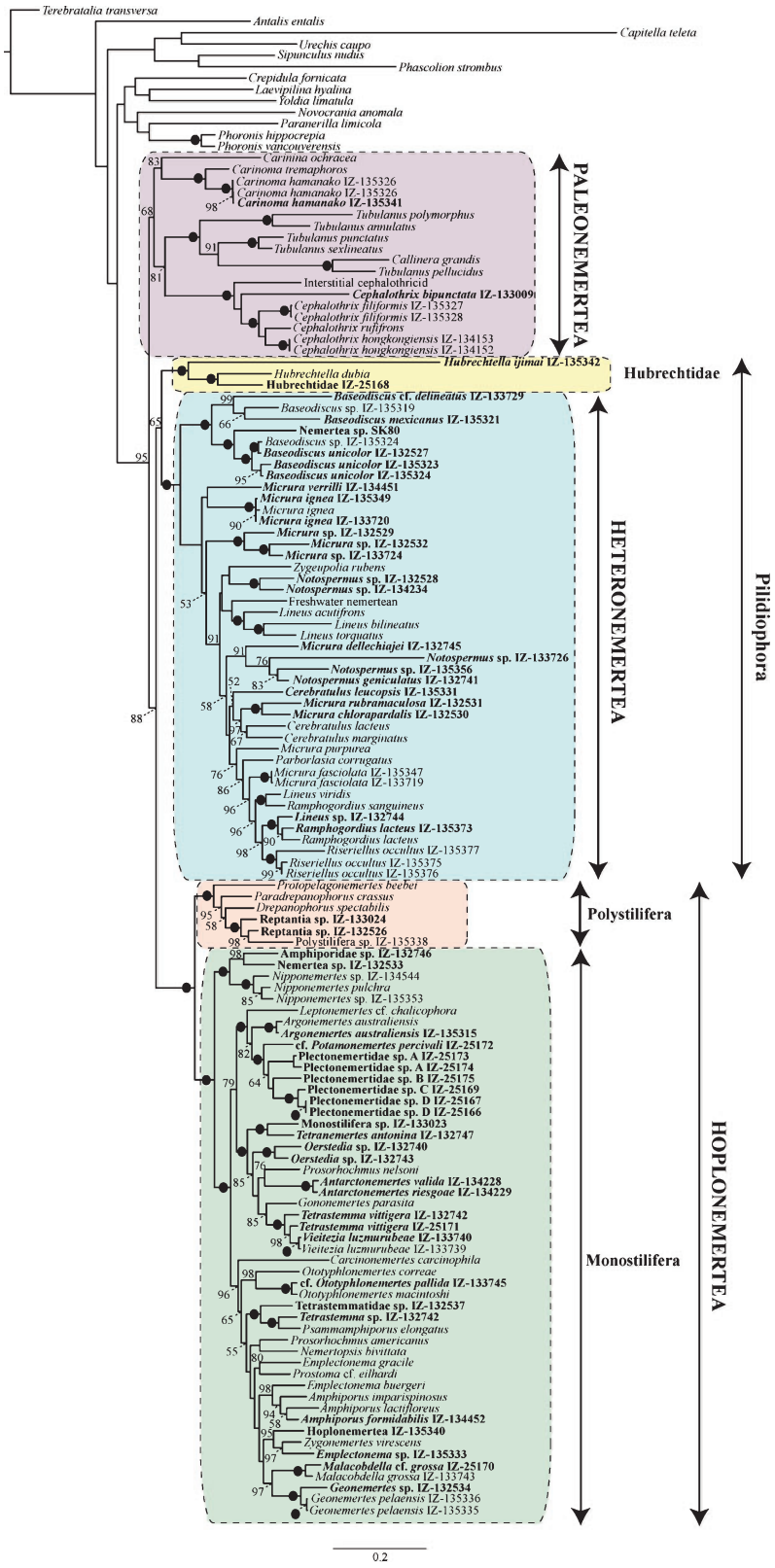

Fig. 5. Best scoring tree from the maximum likelihood analysis of the six-marker dataset with hypervariable regions removed by Gblocks (ln*L* = –122882.420713). Legends as in Fig. 3.

Phylogeny – Gblocks-trimmed dataset

The ML analysis of the dataset with variable regions removed by Gblocks returned a tree with a ln*L* of –122882.420713 (Fig. 5). The resulting tree is highly congruent with that of the full dataset concerning the intrafamilial relationships, but some discrepancies do exist between the trees. Most importantly, in the ML Gblocks tree Palaeonemertea is recovered as a monophyletic (LBS: 68) sister group to the remaining nemerteans (LBS: 88). In addition, the Pilidiophora hypothesis is supported (LBS: 65) as Hubrechtidae (monophyletic with LBS: 100) is recovered as sister group to Heteronemertea, as opposed to Hoplonemertea for the full dataset. Each class is recovered as monophyletic with high support, and the plectonemertid clade is again recovered as monophyletic (LBS: 100) but with a slightly different internal topology compared with the tree of the full dataset.

The single most parsimonious tree recovered by TNT (length: 26 238 steps; CI: 0.223; RI: 0.601) is again largely compatible with the ML tree, but displays an overall decrease in bootstrap values (Supplementary Fig. S1). As opposed to the ML tree, however, Palaeonemertea is recovered as paraphyletic in the MP tree, with the Tubulanidae + Cephalothricidae clade as sister group to the larger clade including Hubrechtidae, Heteronemertea and Hoplonemertea. Hubrechtidae is recovered as sister group to Heteronemertea but with low bootstrap support (PBS: <50).

Phylogeny – Hoplonemertea

Maximum likelihood analysis of the four-marker extended hoplonemertean dataset, including four additional terrestrial taxa for which sequences were downloaded from GenBank, resulted in a tree with a ln*L* of –41886.101242 (Fig. 6). Polystilifera and Monostilifera are both monophyletic with maximum support and the four additional terrestrial taxa nest within the plectonemertid clade (LBS: 97) that was also recovered in the analyses of the full dataset and in the dataset treated with Gblocks. Within this clade, an unidentified plectonemertid species from Spain (NT000059) (see Mateos and Giribet 2009) is weakly recovered as the sister species to the remaining taxa (LBS: <50), which are further divided into four main subclades: the first including the *Argonemertes australiensis* specimens (LBS: 99); the second including two unidentified plectonemertid specimens (NT000072 and NT000046; LBS: 100; see Mateos and Giribet 2009); the third including *Antiponemertes novazealandiae* and *Leptonemertes* cf. *chalicophora* (LBS: 99); and the fourth comprising the remaining undescribed Australian plectonemertid taxa and cf. *Potamonemertes percivali* (LBS: 100).

The MP analysis of the same dataset resulted in two equally parsimonious trees with 8918 steps (CI: 0.299; RI: 0.503). The strict consensus of these (Supplementary Fig. S2) is relatively unresolved but does not conflict with the ML tree. Regarding Plectonemertidae, the exact same topology as the ML tree was recovered by the MP analysis and with high support (PBS between 82 and 100) for the same subclades.

Barcoding gap detection

The full COI dataset, including all sequences from GenBank that were associated with a binomial taxonomic label, comprised 394 terminals with 137 unique taxonomic labels (i.e. putatively different species); the multiple sequence alignment comprised 674 sites. In total, 9452 interspecific and 4453 intraspecific variation values were compared (these are fully presented in Supplementary Tables 1 and 2); the average interspecific uncorrected *p*-distance within the entire dataset was 19.61% ± 3.58 (max = 40.13%; min = 0.10%) and the average intraspecific variation was 1.14% ± 2.87 (max = 20.87%; min = 0.00%). The uncommonly low minimum interspecific distance and uncommonly high maximum intraspecific distance are discussed further below. In total, 3827 out of the 4453 intraspecific comparisons (86%) showed distances below 1%, and 3937 (88%) showed distances below 2%. Moreover, fully 9444 out of the 9452 interspecific comparisons (99.9%) showed distances above 2%, with 9422 comparisons (99.6%) showing distances of 10% or higher. Fig. 7 shows the distribution of interspecific versus intraspecific values across the full dataset and the figure indicates that there is an absence of a fully discrete and sufficiently sized barcoding gap, although a tendency towards separation of maximum intraspecific variation and minimum interspecific divergence is present. Contrary to this, the results from the ABGD show a distinct disjunction between what is presumed by the software to be intraspecific variation (~2%) and interspecific divergence (~8%) (Fig. 8*A*). This is likely a result of ABGD's *a priori* assumption of the distribution of genetic variation within the dataset, seeing as several of the empirical intraspecific variation values from the dataset exceed the lower limit of the interspecific divergence recovered by ABGD. In other words, the results from ABGD could easily have been misinterpreted to suggest the presence of a relatively large barcoding gap, had the empirical values not been calculated. The 'gap' depicted by ABGD is, in fact, flanked by intraspecific distance values on both sides. Note that this does not necessarily diminish the value of ABGD but, rather, suggest the difficulty of inferring species level identifications for nemerteans (discussed further below).

As a corroborative element to this, we investigated the topology, with special attention to branch lengths, of the neighbour-joining tree derived from the same 394-terminal dataset. At first glance, the tree (Fig. 9) suggests a large length difference between branches that connect conspecifics and those that connect non-conspecifics. That is, branches linking sister groups of different species are much longer than those terminal branches that link the same species. After considering the detailed positions of the different species in the tree, however, it is evident that several specimens with the same taxonomic label (e.g. *Cerebratulus leucopsis* (Coe, 1901), *Lineus bilineatus* (Renier, 1804) and *Tetrastemma vermiculus* (Quatrefages, 1846); see Fig. 9) are present in several remote places in the tree. There seems to be little tendency towards clustering of taxonomic groups in the tree, although some clusters of specimens from the same group do exist. For example, a large cluster of specimens belonging to Cephalothricidae is present in the tree (a second cluster of three specimens of *Cephalothrix major* Coe, 1930 exists in a separate part of the tree; not shown), and specimens of *Malacobdella* Blainville, 1827 group together in the tree, and so do the specimens of *Tetrastemma* Ehrenberg, 1831.

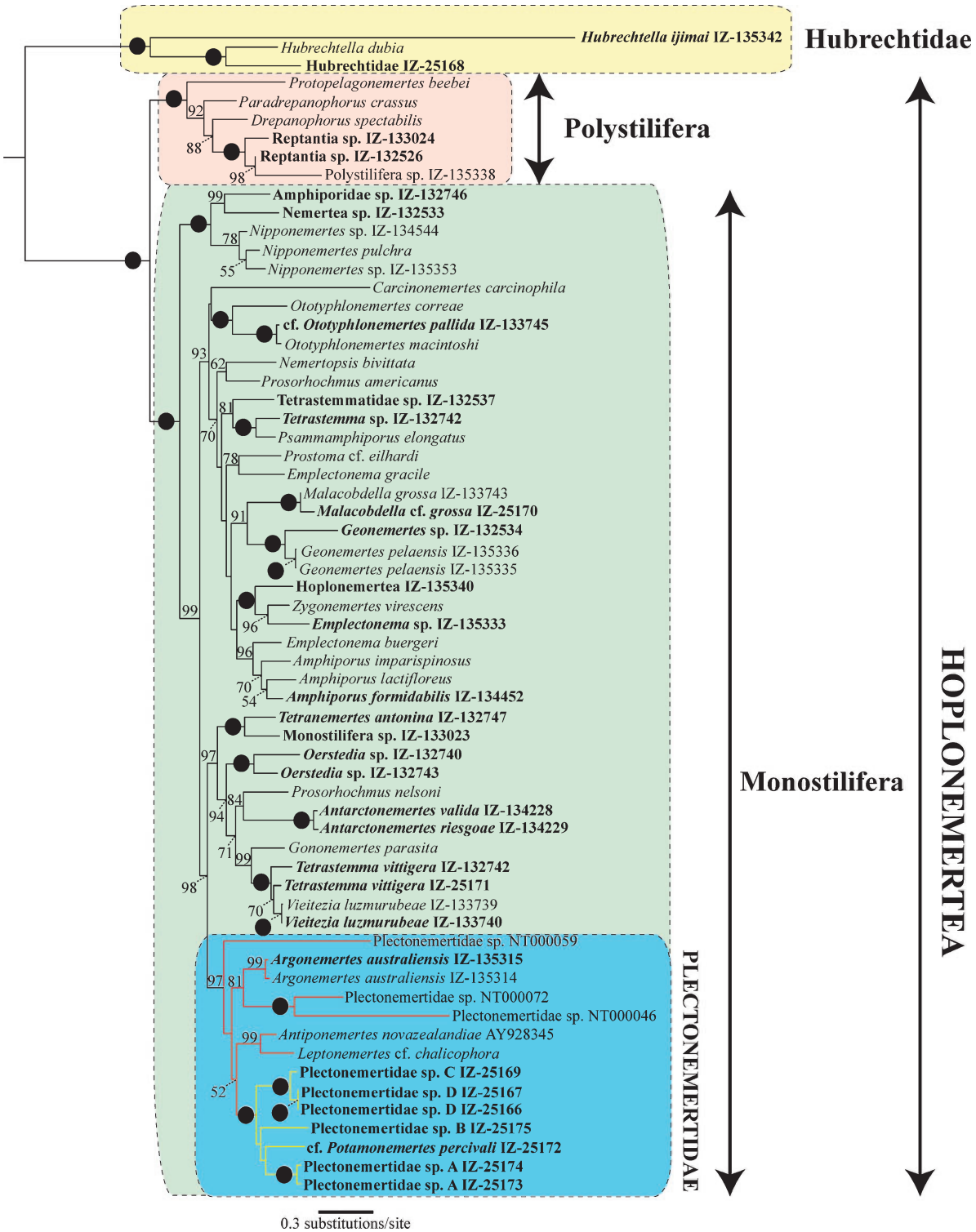

Fig. 6. Best scoring tree from the maximum likelihood analysis of the extended hoplonemertean dataset with sequence data for four taxa added from GenBank (ln*L* = –41886.101242). Legends as in Fig. 3. Within the plectonemertid clade, red and yellow branches lead to terrestrial and freshwater taxa, respectively.

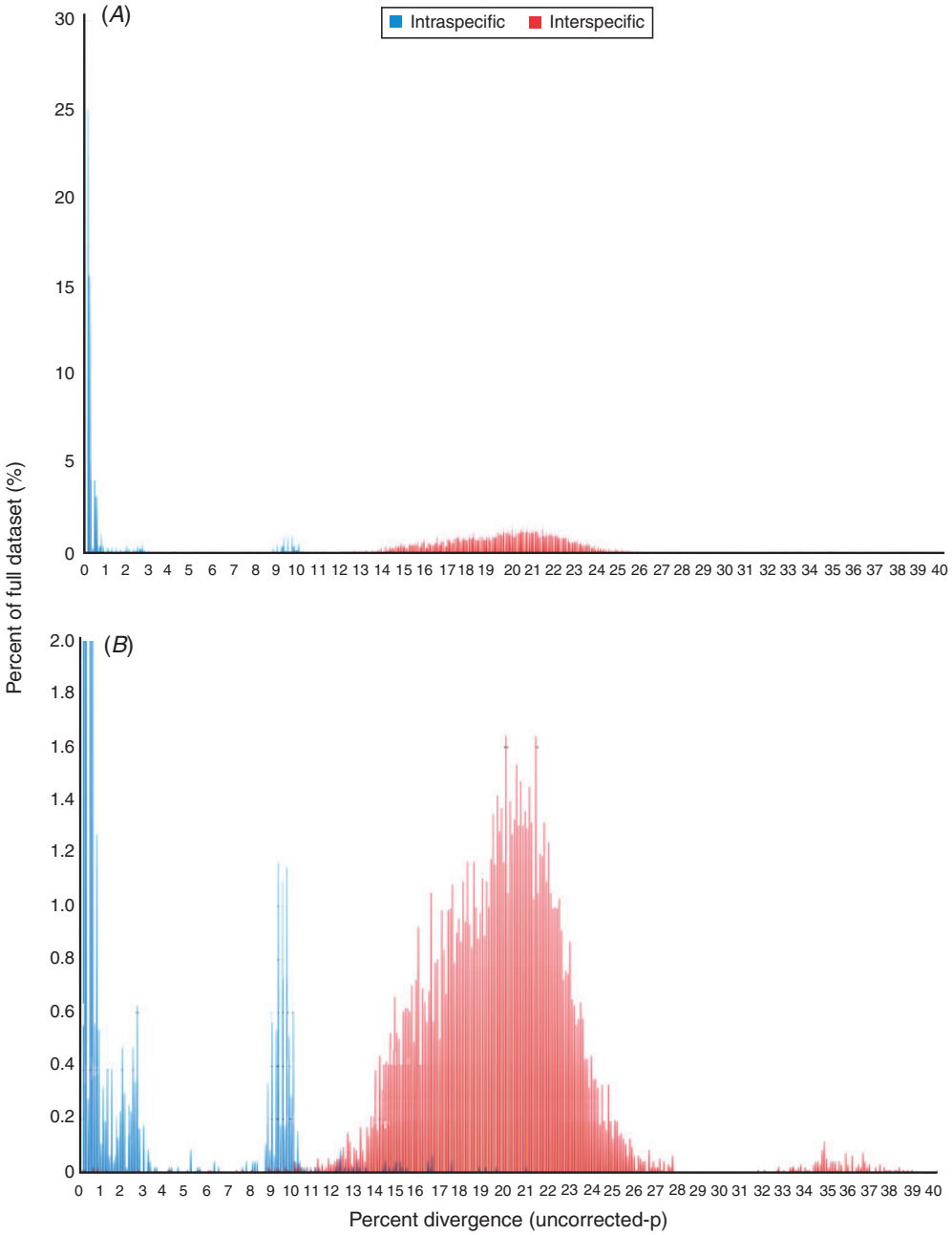

Fig. 7. The distribution of interspecific versus intraspecific distance values across the full COI dataset for Nemertea (n = 394). (*A*) Chart showing the entire range of the *y*-axis; (*B*) enlarged view of the full dataset with the *y*-axis set to a maximum of 2%. Blue bars indicate intraspecific variation and red bars indicate interspecific divergences.

Discussion

Employing the largest gene and taxon sampling to date for nemertean taxa, the phylogenetic hypotheses presented here agree well with previous hypotheses, especially concerning the phylogenetic status and inter-relationships of higher taxonomic ranks. In three out of the four analyses that employed the full taxon dataset, Palaeonemertea (excluding Hubrechtidae) is recovered as paraphyletic; only the ML analysis of the dataset with hypervariable regions removed recovered the class as monophyletic (again, excluding Hubrechtidae). This result mirrors that of Sundberg *et al*. (2001) and Thollesson and Norenburg (2003), but contradicts the topology recovered by Andrade *et al*. (2012) in which Palaeonemertea was recovered as monophyletic (but always with negligible support) regardless of optimality criterion and alignment masking. Beyond this, the trees recovered here support previous morphological and molecular hypotheses insofar as each of Heteronemertea, Hoplonemertea, Polystilifera, Monostilifera and Hubrechtidae are monophyletic. A basal split between

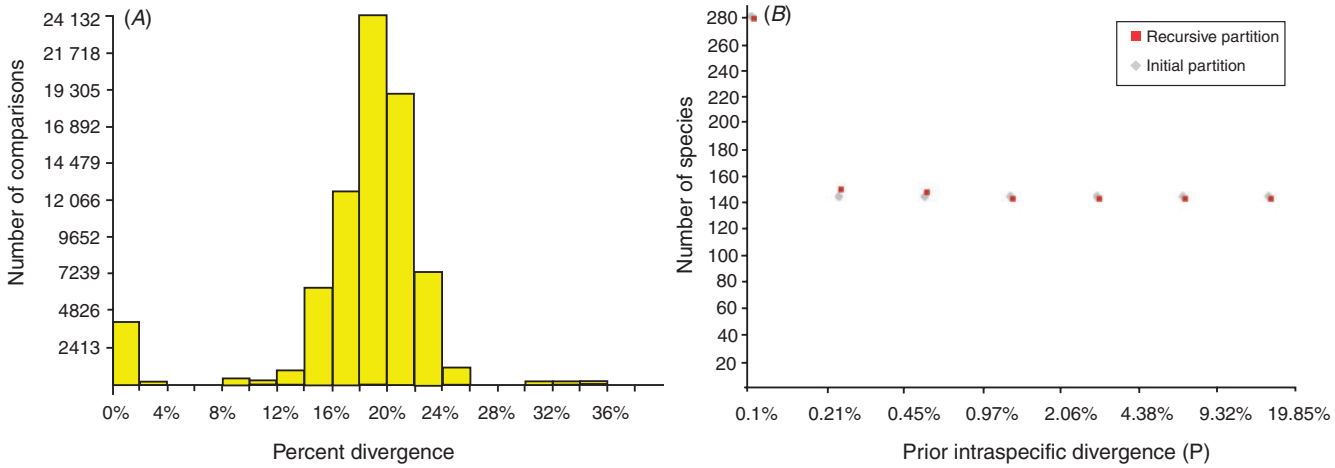

Fig. 8. Schematic illustrations of the results from the automatic barcode gap discovery software. (*A*) Histogram of distances with the distribution showing two modes. Without prior knowledge of the taxonomic labels associated with each comparison, the figure suggests the presence of a barcoding gap between ~2% and ~8% when, in reality, this gap is flanked on both sides by intraspecific distances. Note that the total number of specimen-to-specimen comparisons (*y*-axis) is not comparable with the number of interspecific or intraspecific comparisons conveyed in the text; (*B*) the number of groups (i.e. species) within the partitions (initial and recursive) as a function of the prior limit between intraspecific variation and interspecific divergence. Note that the dataset included 137 unique taxonomic labels and that, to maintain the same amount of groups in the data, the prior intraspecific variation needs to be allowed to vary between 0% and 9.32% (see text for further discussion).

Baseodiscus and the remaining Heteronemertean taxa (Figs 2–4; Supplementary Fig. S1) was also recovered by both Thollesson and Norenburg (2003) and Andrade *et al*. (2012) and this topology is further solidified by the present study, which increases the sampling within *Baseodiscus* considerably. Interestingly, an unidentified nemertean specimen (denoted Nemertea sp. SK80 in the trees) collected in freshwater (conductivity: 774 µS/cm), on Campbell Island, off the south coast of New Zealand (52°32′52″S, 169°4′11″E), nests well within *Baseodiscus*. To our knowledge, this is the first record of a freshwater putative *Baseodiscus* species. However, because of the proximity of the windswept collecting site to the ocean, it is possible that salt spray reaches the site (S. McMurtrie, pers. comm.). Further collection of nemertean specimens from this region needs to be carried out in order to secure the species level identity of the specimen.

With the exception of *Riseriellus*, all genera within Heteronemertea for which more than one specimen was sampled are non-monophyletic. In some cases, this may be a further testament to the difficulty of species level diagnosis within Nemertea but, in others, it is most likely a confirmation of a much needed large-scale genus level revision of the phylum in general, and of Heteronemertea in particular.

Phylogenetic placement of Hubrechtidae

The validity of the Pilidiophora clade is reinforced by several autapomorphic morphological and behavioural features, including the pilidium larva (Cantell 1969; Norenburg 1993; Maslakova 2010) and the sharing of similar protonephridial structures (Bürger 1895; but see also Bartolomaeus and von Döhren 2010). A recent study also suggests a relationship between *Hubrechtella* and *Baseodiscus* (Heteronemertea) based on their shared subendothelial diagonal muscles in the proboscis and the absence of outer diagonal musculature (Chernyshev *et al*. 2013), but this would optimise as a plesiomorphy for Heteronemertea under the current scheme, where Valenciniidae constitutes the sister group of all the remaining heteronemerteans. In terms of molecules, however, despite our increased taxon sampling for Hubrechtidae – including *Hubrechtella dubia*, *Hubrechtella ijimai* (Takakura, 1922) and a Panamanian hubrechtid specimen with unknown specific identity – the phylogenetic placement of the family differs depending on the dataset used. Whereas Hubrechtidae is the sister group of Hoplonemertea in both the ML and MP analyses when employing the full dataset (with negligible support, LBS: 65; PBS: <50), the family is sister group to Heteronemertea when length-variable regions of 18S rRNA and 28S rRNA are excluded, regardless of optimality criterion (LBS: 88; PBS: <50). As such, the Pilidiophora hypothesis (Heteronemertea + Hubrechtidae) is upheld only when excluding these hypervariable regions. This contradicts the finding by Andrade *et al*. (2012), in which the placement of *Hubrechtella dubia* was sensitive to optimality criterion but not to the amount and type of data included. It is likely that their hypervariable nature make these regions prone to misalignment, which could affect the placement of these taxa in the phylogeny, although the importance of using such regions has been highlighted in other empirical cases (e.g. Lindgren and Daly 2007; Giribet and Edgecombe 2013). We also employed alignments that used the L-INS-i strategy for all loci, and performed partitioned and unpartitioned ML analyses, as well as a MP analysis for these and the resulting topology (not shown) was always equivalent, indicating that putative misalignments may be difficult to overcome and this question may need to be re-examined with other datasets. Insofar as two recent large-scale phylogenetic analyses of Nemertea included either 28S rRNA (Thollesson and

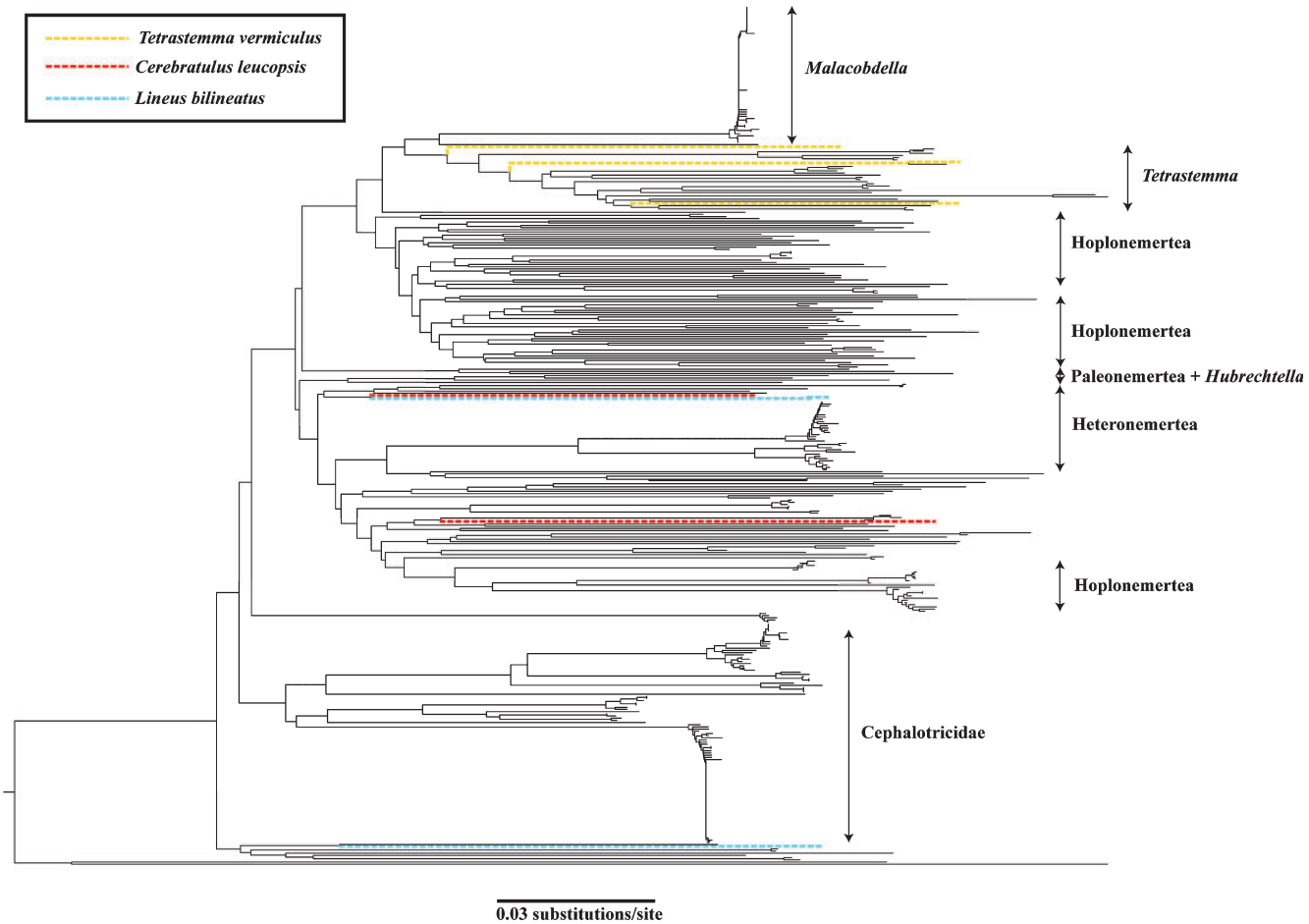

Fig. 9. Neighbour-joining tree derived from the full COI dataset (n = 394), with taxonomic affiliations denoted for some major clusters. Light blue terminal branches lead to specimens of *Lineus bilineatus*, red lines lead to specimens of *Cerebratulus leucopsis*, and yellow lines lead to specimens of *Tetrastemma vermiculus*, which are all further discussed in the text.

Norenburg 2003) or 18S rRNA (Sundberg *et al*. 2001) and only one of them (Thollesson and Norenburg, op. cit.) recovers Pilidiophora as monophyletic, we were impelled to evaluate the separate relative impact of the hypervariable regions of 28S and 18S rRNAs on the placement of Hubrechtidae. We therefore sequentially excluded either the hypervariable regions of 28S or 18S rRNA and re-ran the ML analysis with the best scoring partitioning scheme, as suggested by PartitionFinder, and using the same settings as mentioned in 'Materials and methods'. The resulting trees (not shown) indicate that the hypervariable regions of both 18S rRNA and 28S rRNA influence the placement of Hubrechtidae as sister group to Hoplonemertea, as analyses of both datasets recover this relationship.

Interestingly, both statistical tests (SH and AU), used here for evaluating ML tree differences, unequivocally agree (P = 0.00 for the SH test, $P = 3E^{-64}$ for the AU test) that the unconstrained tree is significantly different from the tree forced to show monophyly of Pilidiophora. These results indicate that the unconstrained ML analysis (Fig. 3) accurately recovered hubrechtid relationships supported by the six-marker dataset, and that forcing the monophyly of Pilidiophora produces a tree with largely suboptimal likelihood scores. By contrast, in the constrained parsimony analysis, only eight additional steps (~0.02% of full tree length) were needed to infer the monophyletic status of Pilidiophora. Although the parsimony scheme used here does not test for statistical significance of the result, it serves as an initial control that, under parsimony, the Pilidiophora hypothesis seems to be only slightly suboptimal compared with the hypothesis presented in Fig. 4.

Given the sensitivity of the placement of Hubrechtidae to different treatments of the available rRNA data, and the meagre support for their placement in all treatments, it is clear that new datasets will be needed to cement the position of this problematic taxon. To this end, large-scale phylogenomic analysis is a promising approach and may also prove useful in solidifying the phyletic status and placement of several other problematic groups, e.g. Palaeonemertea, *Micrura* and *Tetrastemma* (Sundberg and Hylbom 1994; Sundberg and Saur 1998; Strand and Sundberg 2005*a*, 2005*b*; Strand *et al*. 2013).

Plectonemertidae and the terrestrial nemerteans

There has been some contention regarding the natural groupings of terrestrial and freshwater nemerteans (for summaries see

Mateos and Giribet 2008; Sundberg and Gibson 2008). Presently, there is consensus that, although all higher nemertean taxa have a marine origin, different groups have transitioned from marine to freshwater environments through different modes and on several separate occasions, some via terrestrial habitats and others through purely aquatic habitats (Moore and Gibson 1985; Sundberg 1989*a*, 1989*b*; Moore *et al*. 2001). Moore and Gibson (1988), in revising the classification of terrestrial nemerteans on the basis of similarity in morphological features, split *Geonemertes* Semper, 1963 into the modern genera of terrestrial nemerteans, recognising that the characters used to diagnose this artificial genus were, in fact, convergent adaptations to terrestriality. Moore and Gibson (1988) placed the supralittoral genus *Acteonemertes* Pantin, 1969, and the terrestrial genera *Argonemertes* Moore & Gibson, 1981 and *Antiponemertes* Moore & Gibson, 1981, as well as the freshwater genera *Campbellonemertes* Moore & Gibson, 1972 and *Potamonemertes* Moore & Gibson, 1973 in the family Plectonemertidae. Plectonemertidae had, until then, been a monotypic family (the marine *Plectonemertes* Gibson, 1990 being the only genus; note that Gibson's (1990) contribution was in press when Moore and Gibson (1988) was published). Moore and Gibson (1988) also placed the terrestrial genera *Katechonemertes* Moore & Gibson, 1981 and *Leptonemertes* Girard, 1893 within Plectonemertidae but noted that the geographic isolation and non-specialisation of these genera made their inclusion in the family questionable. Later, the results of Crandall's (2001) phylogenetic analysis based on morphology suggested that (i) Plectonemertidae *sensu* Moore and Gibson 1988 formed a paraphyletic assemblage and indeed should be regarded as a monotypic family (i.e. *Plectonemertes* did not group with any of the suggested plectonemertid genera), (ii) *Acteonemertes*, *Argonemertes*, *Antiponemertes*, *Leptonemertes* and *Katechonemertes* formed a distinct family (later named Acteonemertidae; Chernyshev 2005) and (iii) *Campbellonemertes* and *Potamonemertes* either formed a distinct family or two monotypic families. Unfortunately no phylogenetic study attempted to re-evaluate these results, and the taxonomy of the terrestrial nemerteans has remained largely untouched, and consisting of several often monotypic families (see, for example, Mateos and Giribet 2008). To date, molecular sampling of these genera has remained insufficient to provide an independent test of these ideas.

The expanded hoplonemertean phylogenetic hypothesis presented here (Fig. 6 and Supplementary Fig. S2) included *Argonemertes australiensis* from Tasmania, several taxa labelled as 'Acteonemertidae sp.' by Mateos and Giribet (2008) from the Iberian Peninsula, as well as cf. *Potamonemertes percivali* from New Zealand, several undescribed plectonemertid species from Australia, *Leptonemertes* cf. *chalicophora* from the Iberian Peninsula and *Antiponemertes novazealandiae* (sequence obtained from Strand and Sundberg 2005*b*). Both likelihood and parsimony trees show these taxa to form a monophyletic group (LBS: 97; PBS: 56), suggesting the inclusion of (at least) *Argonemertes*, *Leptonemertes*, *Antiponemertes* and *Potamonemertes* within Plectonemertidae. Contraindicating the proposed exclusion of *Potamonemertes* from Acteonemertidae by Chernyshev (2005), and the exclusion of Acteonemertidae *sensu* Chernyshev (2005) from Plectonemertidae by Crandall (2001), in our analysis, these taxa form a monophyletic family (Plectonemertidae *sensu* Moore and Gibson 1988) with high nodal support. We also sampled four undescribed freshwater Australian species, which we show fall within Plectonemertidae in a strongly supported clade with a New Zealand specimen provisionally identified as *Potamonemertes percivali*, and whose collection sites provide further insight into the remarkable habitat diversity of this clade. IZ-25173 and IZ-25174 (sp. A; see Figs 3–6), as well as IZ-25175 (sp. B) are unpigmented, eyeless species found in groundwater-fed surface waters, in these respects similar to *Potamonemertes*, whereas IZ-25169 (sp. C), and IZ-25166 and IZ-25167 (sp. D) were recovered from boreholes used to sample subsurface calcrete aquifers in the arid Pilbara region of Western Australia; Nemertea thus joins the extremely diverse assemblage of stygofauna known from such habitats (Humphreys 2008; Guzik *et al*. 2011). Groundwater-associated fauna are well known for their poor dispersal ability and, given the wide geographic range (Western Australia, Victoria and New Zealand) of this clade within Australasia, the existence of this clade may be evidence for a large and possibly geologically old radiation of stygofaunal nemerteans, as has been demonstrated for several other taxa (e.g. phreatoicidean isopods; Wilson 2008). Further sampling of groundwater-associated nemerteans (a methodologically challenging prospect, given the need to observe living specimens and fix appropriately for histological study) within Australia, New Zealand, and possibly other former Gondwanan continental fragments, will be necessary to test this idea. In any case, the existence of this clade is consistent with the notion of a much larger diversity of continental Plectonemertidae than has perhaps been fully appreciated to date (Mateos and Giribet 2008). Judging from the partially fragmented dataset used here (e.g. only 18S rRNA was available for *Antiponemertes novazealandiae*), it would be premature to synonymise Acteonemertidae and *Potamonemertes* with Plectonemertidae, especially in the absence of sequence data from *Plectonemertes* and *Acteonemertes*, but the recovery of this clade by the present study represents yet another line of evidence in support of Plectonemertidae *sensu* Moore and Gibson (1988). Pending inclusion of marine taxa such as *Acteonemertes* and *Plectonemertes* it is difficult to comment on the specific macroevolutionary routes by which plectonemertid taxa have come to flourish in freshwater and terrestrial habitats (Moore and Gibson 1985, 1988). A fuller taxon sampling should illuminate relationships within this enigmatic nemertean family and hence the specific sequence of habitat colonisation.

The other clade of terrestrial nemerteans includes *Geonemertes pelaensis* Semper, 1863, which appears related to *Malacobdella* and not to *Prosorhochmus* Keferstein, 1862, with whom it was once grouped in the family Prosorhochmidae. *Geonemertes* thus appears nested in a clade that includes a diversity of monostiliferan genera with interesting reproductive or life-strategy behaviours, like the parasitic and commensal genera *Malacobdella*, *Gononemertes* Bergendal, 1900 and *Vieitezia* Junoy, Andrade & Giribet, 2011 (see discussion in Junoy *et al*. 2011), cocoon-forming species (Taboada *et al*. 2013), plus other free-living forms including *Tetranemertes* Chernyshev, 1992, *Oerstedia* Quatrefages, 1846,

Prosorhochmus and *Tetrastemma*. This clade is well supported (LBS: 97) and appears as sister group to Plectonemertidae (LBS: 98).

DNA barcoding gap

DNA barcoding is a promising tool for specimen identification (e.g. Fernández-Álvarez and Machordom 2013), especially for organisms that are relatively difficult to identify, such as nemerteans. Indeed, recent nemertean species descriptions are associated with COI barcodes (Strand and Sundberg 2011; Strand *et al*. 2013; see also Junoy *et al*. 2011; Kajihara *et al*. 2011; Taboada *et al*. 2013). However, among other factors, the functionality of DNA barcoding is contingent on two presumptions: the presence of a high-coverage barcode database, and the presence, within any given group of organisms, of a wide DNA barcoding gap – the difference between the highest intraspecific variation and the lowest interspecific divergence (for discussion, see Tautz *et al*. 2002, 2003; Lipscomb *et al*. 2003; Moritz and Cicero 2004; Will and Rubinoff 2004; Schander and Willassen 2005; Ebach and Holdredge 2005; DeSalle *et al*. 2005; Rubinoff *et al*. 2006; Kvist 2013). At first glance, our average intraspecific and interspecific variation values suggest the presence of a wide barcoding gap (intraspecific: 1.14% ± 2.87; interspecific: 19.59% ± 3.55; Fig. 7), but a closer investigation of the overall values presents a more dubious scenario. Inasmuch as the lowest interspecific divergence values (<1%) occur between either synonymous taxa (e.g. *Myoisophagus sanguineus* and *Ramphogordius sanguineus* (Rathke, 1799); Riser 1994; Kajihara *et al*. 2008) or between somewhat cryptic taxa (e.g. *Prosorhochmus claperedii* Keferstein, 1862, *Prosorhochmus americanus* Gibson, Moore, Ruppert & Turbeville, 1986 and *Prosorhochmus chafarinensis* Frutos, Montalvo & Junoy, 1998), the low interspecific values may merely illustrate the difficulty of species level identifications of some taxa. This difficulty is indeed reflected in the implausibly high intraspecific variation present between some specimens with identical taxonomic labels (e.g. 20.87% within *Cerebratulus leucopsis*, 19.01% within *Lineus bilineatus*, and 17.38% within *Tetrastemma vermiculus*). As mentioned above, most (88%) of the intraspecific comparisons resulted in distance values below 2%, a figure that has become somewhat standard in barcoding practice for conspecific variation (Smith *et al*. 2005; but see Boyer *et al*. 2007 for a counter example), and it is possible that the remaining 12% are represented by wrongly labelled taxa. As such, any user of GenBank COI sequences pertaining to the aforementioned taxa (and surely numerous other sequences) should indeed be cautious of the inferred taxonomic labels. In this regard, DNA barcoding holds particular promise, as it may allow for more unambiguous and objective identification, free of homoplastic characters or convergent evolution (although a rigorous database of authoritative sequences need first be created, as mentioned above). Regardless of this, however, it is difficult to set an arbitrary cut-off limit on interspecific versus intraspecific variation for the inclusion or exclusion of a specimen in a species complex, especially as the values recovered by the present study are present as a somewhat continuous range (see Supplementary Tables 1 and 2).

The unreasonably high intraspecific distance values are further underscored by ABGD. Species diversity estimation by ABGD (Fig. 8*B*) shows that, to maintain the species diversity indicated by the taxonomic labels (137 unique taxonomic labels, i.e. putative species), the prior intraspecific distance needs to be allowed to vary between 0.00% and 9.32%, an upper limit that is much higher than normal estimations of intraspecific variation (e.g. Hebert *et al*. 2003*a*, 2003*b*; Smith *et al*. 2005; Ratnasingham and Hebert 2007). Importantly, ABGD calculates all pairwise distances without considering species affinities and uses an *a priori* determined range of intraspecific distances to infer a model-based confidence limit for the empirical divergences. As a result, relatively high intraspecific variation values can easily be confused with low interspecific divergence values, making the resulting distribution of values difficult to interpret. That is, correct interpretation of the results from ABGD presupposes a reasonable range of intraspecific and interspecific divergences. This is clearly not the case within Nemertea, likely due to the aforementioned problems of specimen identification, such that Fig. 8*A* indicates the presence of a barcoding gap between ~2% and ~8% divergence. Unbeknownst to any investigator operating without knowledge of the taxonomic labels associated with the sequences, this gap is flanked on both sides by intraspecific distance values. Strictly speaking, the putative 'barcoding gap' of this dataset occurs in the centre of the distribution of intraspecific values, as opposed to its normal occurrence *between* intraspecific variation and interspecific divergence. The neighbour-joining tree, constructed from the uncorrected *p*-distances of the COI sequences (Fig. 9), corroborates this finding. The general topology of the tree implies that branches between conspecifics are much shorter than those between non-conspecifics, notwithstanding the several taxa with identical taxonomic labels, which are recovered in very disparate parts of the tree (see branch colours in Fig. 9 for three examples).

Taking into account the full body of results presented here, it seems reasonable to suggest that DNA barcoding may work for Nemertea, insofar as a distinctly wide barcoding gap may exist, pending higher accuracy in species level identifications (authoritative barcodes *sensu* Kvist *et al*. 2010; see Strand and Sundberg 2011) of the specimens that will provide the backbone of the barcode database.

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plectonemertids), J. T. Cannon and K. M. Kocot (*Malacobdella*), N. C. Boustead and D. P. Gray (cf. *Potamonemertes percivali*). The putative *Baseodiscus* specimen (SK80) and associated information was provided by EOS Ecology and was collected during the 2010–11 Campbell Island Biocentennial Expedition (CIBE). We also thank the Willi Hennig Society for making TNT freely available. Two anonymous reviewers and editor Nerida Wilson provided comments that helped improve this article.

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