

A Transcriptomic Approach to Ribbon Worm Systematics (Nemertea): Resolving the Pilidiophora Problem

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Abstract

Resolving the deep relationships of ancient animal lineages has proven difficult using standard Sanger-sequencing approaches with a handful of markers. We thus reassess the relatively well-studied phylogeny of the phylum Nemertea (ribbon worms)—for which the targeted gene approaches had resolved many clades but had left key phylogenetic gaps—by using a phylogenomic approach using Illumina-based de novo assembled transcriptomes and automatic orthology prediction methods. The analysis of a concatenated data set of 2,779 genes (411,138 amino acids) with about 78% gene occupancy and a reduced version with 95% gene occupancy, under evolutionary models accounting or not for site-specific amino acid replacement patterns results in a well-supported phylogeny that recovers all major accepted nemertean clades with the monophyly of Heteronemertea, Hoplonemertea, Monostilifera, being well supported. Significantly, all the ambiguous patterns inferred from Sanger-based approaches were resolved, namely the monophyly of Palaeonemertea and Pilidiophora. By testing for possible conflict in the analyzed supermatrix, we observed that concatenation was the best solution, and the results of the analyses should settle prior debates on nemertean phylogeny. The study highlights the importance, feasibility, and completeness of Illumina-based phylogenomic data matrices.

Key words: phylogeny, Palaeonemertea, Neonemertea, Pilidiophora, supermatrix, concatenation, Illumina.

Introduction

Of all the animal phyla, nemerteans are unique in presenting an excretory system similar to that of some acoelomates while possessing true coeloms, the rhynchocoel and the closed circulatory system (see Turbeville 2002). This taxon includes about 1,280 species (Gibson 1995; Kajihara et al. 2008), which is more than many other well-known animal phyla, but it is still considered a “minor” phylum by some, despite inhabiting marine, freshwater and some terrestrial environments. The phylum includes what is considered to be the longest metazoan ever recorded, *Lineus longissimus*, which can reach more than 30 m in length (McIntosh 1873–1874), but many species are small, and some even microscopic. Most nemertean species are carnivorous or scavengers, using their unique proboscis apparatus for capturing prey.

The classification of nemerteans has been in flux for decades but a consensus has arisen with the relatively recent use of molecular systematics (e.g., Sundberg et al. 2001; Thollesson and Norenburg 2003; Sundberg and Strand 2007; Andrade et al. 2012; Kvist et al. 2014). The traditional classification system of nemerteans for most of the last 100 years followed largely Stiasny-Wijnhoff (1936), which accepted as classes Schultze's (1851) division of nemerteans into Anopla and Enopla. Stiasny-Wijnhoff (1936) divided Anopla into Palaeonemertea and Heteronemertea, and Enopla into Hoplonemertea and Bdelonemertea. Hoplonemertea was further subdivided into Monostilifera and Polystilifera. Recent accounts of the systematics of Nemertea were provided by Andrade et al. (2012) and Kvist et al. (2014). In those studies, the authors produced a

comprehensive analysis of ribbon worm relationships based on six molecular markers obtained by Sanger sequencing for a large taxon sample, including all major lineages. Although many relationships were conclusive, and the major groupings (e.g., Heteronemertea and Hoplonemertea) were well supported, their relationship to Palaeonemertea and Hubrechtidae, and the monophyly of Palaeonemertea were not satisfactorily resolved. Andrade et al. (2012) therefore concluded their study with a plea for Next Generation Sequence data to resolve the deepest nodes in the tree, as phylogenomic matrices assembled this way have proven informative at resolving basal metazoan and protostome relationships (e.g., Dunn et al. 2008; Hejnal et al. 2009; Pick et al. 2010; Kocot et al. 2011; Smith et al. 2011; Struck et al. 2011; von Reumont et al. 2012; Fernández, Laumer, et al. 2014).

Nemertean transcriptomic data are scarce and based on Sanger data for the species *Cerebratulus lacteus* (Heteronemertea) and *Carinoma mutabile* (Palaeonemertea) (Dunn et al. 2008). More recently, two additional species have been sequenced using Illumina, *Cephalothrix hongkongiensis* (Palaeonemertea) and *C. marginatus* (Heteronemertea) (Riesgo, Andrade, et al. 2012), both included in this study. Here, we use an RNA-seq approach to generate new transcriptomes for 11 nemertean species with Illumina (including the ones from Riesgo, Andrade, et al. 2012) and Roche-454 to address four outstanding questions in nemertean phylogeny. We selected species from all the major ribbon-worm lineages, spanning their entire diversity, and obtained fresh RNA from live or RNAlater preserved specimens. Our aims were to 1) test the monophyly of Palaeonemertea by including a member of each of the three lineages obtained by Andrade et al. (2012); 2) test the monophyly of Anopla, which has been falsified by most phylogenetic analyses; 3) test the position of Heteronemertea, which has remained in flux in most studies; and 4) address the position of Hubrechtidae, a family of uncertain affinities, originally classified in Palaeonemertea, but more recently found to be the possible sister group of Heteronemertea, thus forming a clade named Pilidiophora for the presence of a pilidium larva which occurs in both taxa (Thollessen and Norenburg 2003).

Results

The number of sequence reads, used reads, contigs, and other values to assess the quality of the assembled transcriptomes can be found in table 1. Our smallest Illumina library used approximately 12 million reads (assembled into 29,292 contigs for *Protopelagonemertes beebei*), whereas our largest one used almost 80 million reads (assembled into 70,286 contigs for *Carinoma hamanako*). Interestingly, smaller libraries yielded more assembled contigs (see table 1), although some with a smaller $n50$, such as *Nipponnemertes* sp., which had 62 million used reads assembled into 34,065 contigs greater than 199 bp, $n50 = 816$, whereas *Cer. marginatus* had 28 million used reads assembled into 117,335 contigs greater than 150 bp, $n50 = 1,103$. In fact, the relatively small library of *C. marginatus* had some of the longest contigs, the largest number of contigs greater than 999 bp, and the longest total

number of assembled base pairs. This indicates that transcriptome quality may not be directly correlated to the number of raw reads or the number of used reads, but instead with library quality and diversity.

We obtained a total of 42,730 clusters from the ortholog clustering analyses, from which 7,581 had five taxa or more. Although previous studies have compared alternative matrices (e.g., Hejnal et al. 2009; Smith et al. 2011), this was done to compare results between large numbers of genes (which came with low matrix completeness) and more complete matrices. The most densely populated matrix from Hejnal et al. (2009), with only 53 genes, was only 50% complete. Likewise, the “small” matrix of Smith et al. (2011), consisting of 301 genes, has 50% gene occupancy and 27% character occupancy, whereas the “big” matrix consists of 1,185 genes with 40% gene occupancy and 21% character occupancy. Our original gene occupancy threshold is much higher than any of these matrices, and we obtained 89% and 80% of character occupancy for the reduced and large matrices, respectively. As support is optimal for almost all nodes, we did not find it necessary to evaluate more than two alternative matrices with more or less occupancy (fig. 1). The number of ortholog groups represented per taxon ranged from 704 to 2,613 for the large matrix and from 247 to 458 for the reduced matrix (table 2).

The maximum likelihood (ML) tests using the two alternative models, LG and LG4X, produced an identical topology on the best-scoring trees. The log-likelihood score for them was $-6,056,455.0098$ and $-5,973,224.5499$, respectively. For the reduced matrix, only the LG4X model was used, and the best-scoring tree had a log-likelihood value of $-1,236,158.9849$. The phylogenetic analyses of our two data matrices yielded identical topologies for the ML analysis using a partitioned model approach and for the Bayesian analyses (fig. 2), the only differences being in the internal resolution of Palaeonemertea and in one of the outgroup taxa. Each node in the nemertean tree received 100% bootstrap support or a posterior probability of 1.00, with the exception of the sister group relationship of *Carinoma* and *Cephalothrix*, not supported in the small data matrix. Monophyly of Nemertea, Palaeonemertea, Neoneemertea, Hoplonemertea, Monostilifera, Pilidiophora, and Heteronemertea are thus found in all analyses with maximum support. One result evident in our analyses, as well as in previously published work, is the rejection of the order Bdellemertea (with its only representative genus *Malacobdella*), as it nests deep within the Monostilifera. Monostilifera also shows the deep split between the clades Cratenemertea (*Nipponnemertes*) and Distonematonemertea (*Argonemertes*, *Paranemertes*, and *Malacobdella*). Within Heteronemertea our data also support a deep split between *Baseodiscus* and the other represented genera.

Outgroup relationships are outside the scope of this article, but our analyses support the monophyly of all the represented mollusks and annelids (including the represented sipunculan). Other well-established nodes in molluscan and annelid phylogeny, including a relationship of the three conchiferan mollusks or the two clitellate annelids, are also well

Table 1. Species Included in the Analysis, Including New and Publicly Available Data.

Species	MCZ Voucher	Sampling Location	Sequence Method/Source	N Raw Reads	N Reads after Filtering	Assembler	N Contigs (> 150 bp)	n50	Longest Contig	N Contigs > 999 bp	Average Contig Length	Total Length (bp)
<i>Tubulanus punctatus</i>	IZ-134221	Akkeshi, Hokkaido, Japan	454	69,472	63,219	Newbler	4,217	501	2,374	223	488.9	2,061,617
<i>Carinoma hamanako</i>	IZ-135326	Ikarise Island, Honshu, Japan	Illumina-PE	103,234,742	80,199,393	Velvet/Oases	70,286	1,674	13,084	22,664	825.4	72,037,381
<i>Cephalothrix hongkongiensis</i>	IZ-135329	Akkeshi, Hokkaido, Japan	Illumina-PE	51,634,374	41,432,780	Velvet/Oases	73,445	1,047	9,337	14,514	539.6	52,901,581
<i>Hubschreilla ijimai</i>	IZ-135342	Hamanako, Honshu, Japan	Illumina-PE	72,768,612	50,847,602	Velvet/Oases	110,394	391	5,458	4,110	343.2	49,058,203
<i>Baseodiscus unicolor</i>	IZ-135322	Bocas del Toro, Panama	Illumina-PE	175,593,324	78,906,444	Velvet/Oases	89,321	1,443	24,550	17,032	745.3	72,397,184
<i>Cerebratulus marginatus</i>	IZ-134484	San Juan Island, WA	Illumina-PE	52,515,657	28,486,396	Velvet/Oases	117,305	1,103	16,854	24,435	503	90,860,260
<i>Riseriellus occultus</i>	IZ-135375	Liverpool, UK	Illumina-PE	115,328,860	71,403,264	Velvet/Oases	91,728	800	14,082	3,359	368.5	53,276,504
<i>Argonemertes australiensis</i>	IZ-135314	Tasmania, Australia	Illumina-PE	128,371,852	39,211,148	Velvet/Oases	43,999	581	6,810	4,515	471.3	24,144,649
<i>Malacobdella grossa</i>	IZ-133743	Tjörnö, Skagerak, Sweden	Illumina-PE	47,277,586	35,241,626	Velvet/Oases	49,596	1,508	9,108	17,380	688.9	52,064,433
<i>Nipponnemertes</i> sp.	IZ-135354	Biobío, Chile	Illumina-PE	178,923,604	62,354,396	Velvet/Oases	28,772	816	12,963	5,178	584.4	20,995,957
<i>Paranemertes peregrina</i>	IZ-134480	San Juan Island, WA	Illumina-SE	24,392,996	16,521,215	Velvet/Oases	30,456	1,721	21,949	16,859	913.1	52,515,685
<i>Protoplegonemertes beebei</i>	IZ-135370	Sagammi Bay, Japan	Illumina-SE	22,321,639	12,011,532	Velvet/Oases	29,295	1,225	19,285	6,275	709.4	23,997,464
Outgroups												
<i>Octopus vulgaris</i>	DNA 106283	Bianes, Girona, Spain	Illumina-PE	94,283,86	16,501,336	Velvet/Oases	56,949	1,158	19,747	10,435	689.4	39,263,022
<i>Chiton olivaceus</i>	MAL-378064	Tossa de Mar, Girona, Spain	Illumina-PE	82,814,428	55,901,966	Velvet/Oases	109,413	784	7,118	15,108	557.2	60,964,316
<i>Gadilla tolmiei</i> ^a			Smith et al. (2011)			Velvet/Oases	78,360	764	17,170	9,932	542.2	42,641,982
<i>Emmucula tenuis</i> ^a		Greenland	Smith et al. (2011)			Velvet/Oases	64,402	1,258	18,317	13,928	747.5	48,144,794
<i>Sipunculus nudus</i>	IZ-130438	Fort Pierce, FL	Illumina-PE	195,601,190	34,173,928	Velvet/Oases	121,416	733	7,399	13,337	455.6	55,305,482
<i>Hormogaster samnitica</i>	GEL6 ^b	Gello, Toscana, Italy	Illumina-PE	53,956,780	31,623,984	Velvet/Oases	59,853	1,156	9,307	11,613	677.1	40,526,740
<i>Capitella teleta</i>			JGI database ^c									
<i>Helobdella robusta</i>			JGI database ^c									

NOTE.—The sequencing method for new data or the source of the data is indicated in the third column. The public archive used was JGI—<http://www.jgi.doe.gov/> (last accessed September 7, 2014). MCZ voucher accession numbers beginning with IZ and DNA are at the Harvard Museum of Comparative Zoology. See Materials and Methods for details on sample preparation protocols.

^aAssemblies were obtained directly from Casey W. Dunn referring to Smith et al. (2011).

^bMaterial deposited in the Department of Zoology and Physical Anthropology, Universidad Complutense de Madrid.

^cUsed the translated assemblies from the JGI database. N, number.

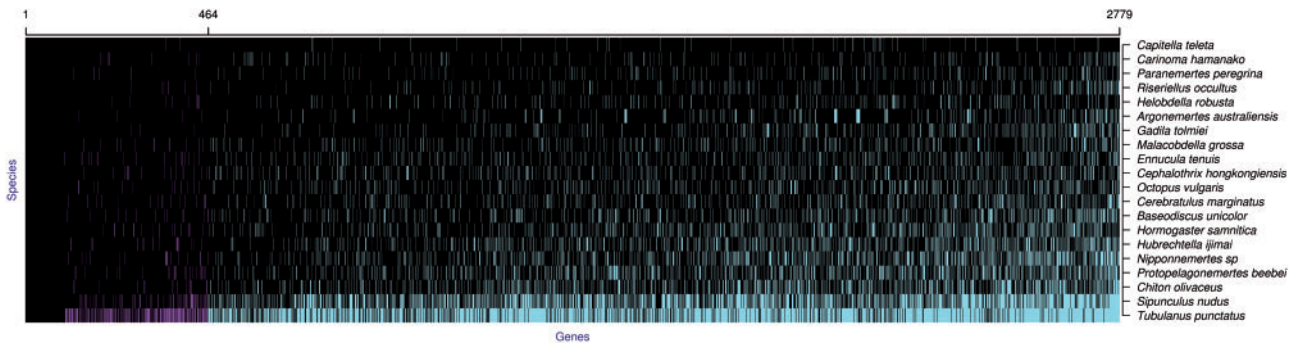


Fig. 1. Gene occupancy representation per species, with maximum occupancy toward the top left. *Capitella teleta* is the best represented species, whereas the 454 library of *Tubulanus punctatus* is the worst represented one. Large matrix (2,779 orthogroups) represented in blue, and the reduced subset (95% occupancy, 464 orthogroups) is represented in purple.

Table 2. Number of Sequences Used in the Ortholog Clustering Analysis and on the Final Matrices per Taxon.

Species	N Peptide Sequences Analyzed in OrthoMCL	N Orthologs Included in Large Matrix	Missing Data in Large Matrix (%)	N Orthologs Included in Reduced Matrix	Missing Data in Reduced Matrix (%)
<i>Tubulanus punctatus</i>	4,084	704	74.7	247	46.8
<i>Carinoma hamanako</i>	43,936	2,613	6.0	456	1.7
<i>Cephalothrix hongkongiensis</i>	67,577	2,400	13.6	444	4.3
<i>Hubrechtella ijimai</i>	109,839	2,157	22.4	442	4.7
<i>Baseodiscus unicolor</i>	70,859	2,280	18.0	450	3.0
<i>Cerebratulus marginatus</i>	105,512	2,360	15.1	446	3.9
<i>Riseriellus occultus</i>	91,160	2,565	7.7	449	3.2
<i>Argonemertes australiensis</i>	41,675	2,539	8.6	447	3.7
<i>Malacobdella grossa</i>	32,219	2,453	11.7	451	2.8
<i>Nipponnemertes sp.</i>	28,538	2,067	25.6	434	6.5
<i>Paranemertes peregrina</i>	29,918	2,583	7.1	458	1.3
<i>Protopelagonemertes beebei</i>	22,618	2,061	25.8	441	5.0
Outgroups					
<i>Octopus vulgaris</i>	55,002	2,371	14.7	457	1.5
<i>Chiton olivaceus</i>	105,806	2,039	26.6	427	8.0
<i>Gadila tolmiei</i>	77,870	2,463	11.4	454	2.2
<i>Ennucula tenuis</i>	63,517	2,422	12.8	443	4.5
<i>Sipunculus nudus</i>	119,303	1,333	52.0	398	14.2
<i>Hormogaster samnitica</i>	59,134	2,235	19.6	444	4.3
<i>Capitella teleta</i>	32,415	2,727	1.9	464	0.0
<i>Helobdella robusta</i>	23,432	2,552	8.2	462	0.4

supported in our data set, serving as a test for interpreting the nemertean support values.

The different incongruence inferences presented some different results, probably due to limitations of “internode certainty” when dealing with missing data (Salichos et al. 2014). The relative tree certainty (TC) (Salichos and Rokas 2013) for the large matrix (2,779 partitions) including all conflicting bipartitions (TC-All) was 0.996, whereas for the reduced matrix (464 partitions), retrieved a value of 0.924, clearly indicating no conflict. The split network from SuperQ v1.1 identifies some intergene conflict with respect to the specific positions of *Hubrechtella ijimai* and the hoplonemerteans (fig. 3) for both matrices. Conclustador resulted

in just one cluster for each matrix, and the small matrix supernetwork also showed conflict between *Cep. hongkongiensis* and *Ca. hamanako*, as evidenced on the tree presented on figure 2.

Discussion

Resolving the Tree of Life has been seen as one of the most important 125 unresolved scientific questions in 2005 by Science Magazine (July 1, 2005, vol. 309, p. 96), and the advent of phylogenomics has aided in resolving many contentious aspects in animal phylogeny (Delsuc et al. 2005; Dunn et al. 2008; Bleidorn et al. 2009; Hejnol et al. 2009; Meusemann et al. 2010; Kocot et al. 2011; Rehm et al. 2011;

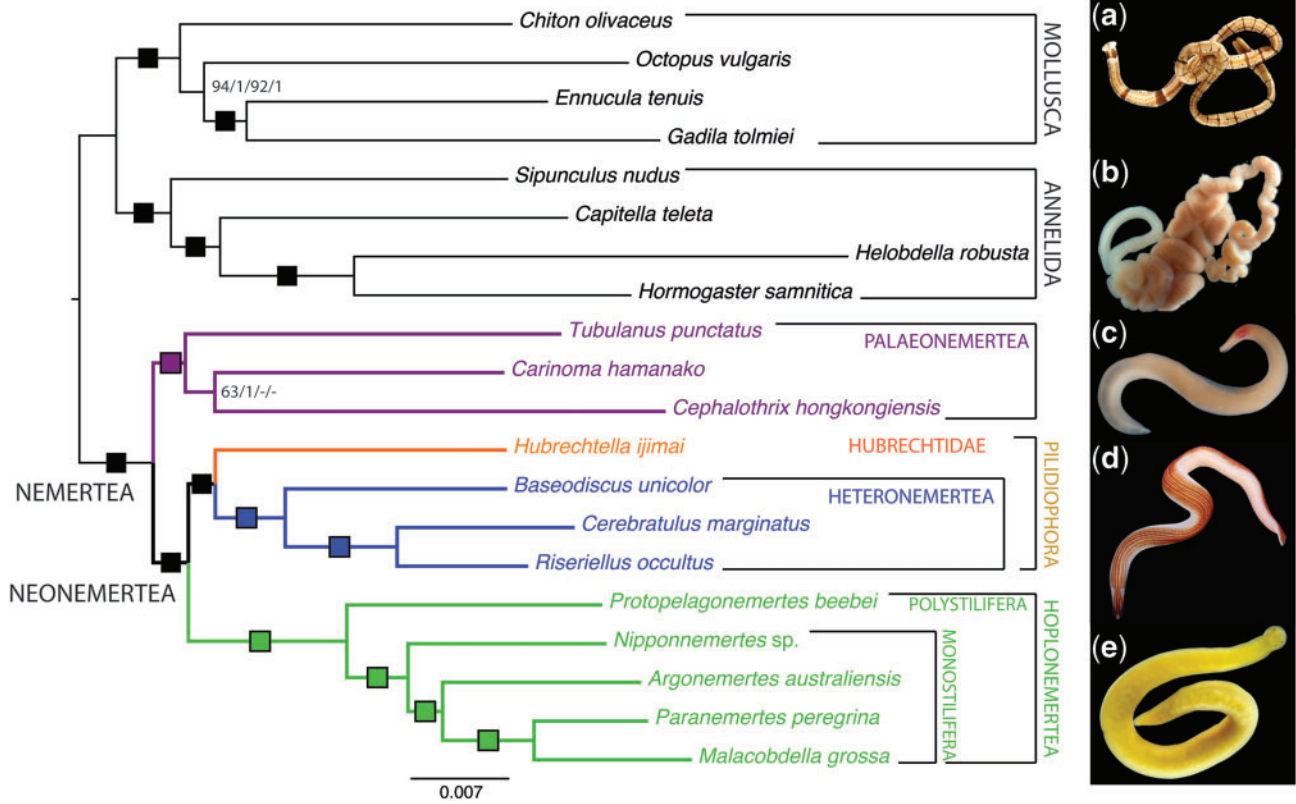


FIG. 2. Phylogenetic hypothesis based on the large data matrix analyzed in RAXML ($-\ln L = -5,973,224.55$) with support values (bootstrap values or posterior probabilities) plotted as follows: Large matrix RAXML/large matrix ExaBayes/small matrix RAXML/small matrix ExaBayes. Squares indicate maximum support in all four analyses. Nemertean lineages shown in color/shades. Photos are of representatives of the different lineages of nemerteans: (a) Palaeonemertea (*Tubulanus rhabdotus*), (b) Hubrechtidae (*Hubrechtella ijimai*), (c) Heteronemertea (*Cerebratulus leucopsis*), (d) Hoplonemertea, Polystilifera (*Drepanophorus spectabilis*), and (e) Hoplonemertea, Monostilifera (*Tetrastemmatidae* sp.).

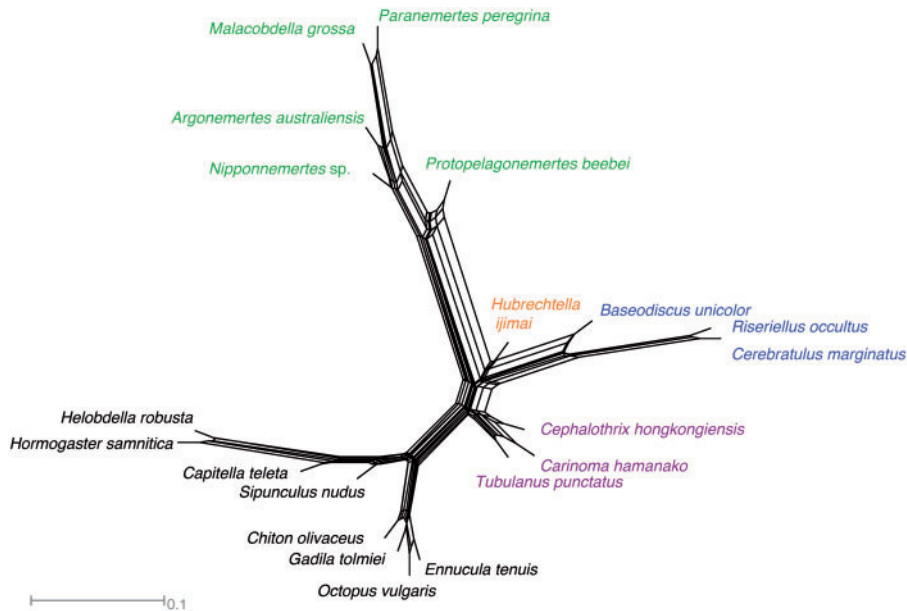


FIG. 3. Unrooted SuperQ ML splits network for the large data matrix. Colors/shades are as in figure 2.

Smith et al. 2011; Struck et al. 2011; Hartmann et al. 2012; von Reumont et al. 2012; Fernández, Hormiga, et al. 2014; Fernández, Laumer, et al. 2014). This is not without controversy, and several aspects have been identified that negatively

impact phylogenomic reconstructions, perhaps foremost, gene occupancy (missing data) (Roure et al. 2013), taxon sampling (Pick et al. 2010), and quality of data (both for paralogy, ortholog prediction, and exogenous contamination)

(Philippe et al. 2011; Salichos and Rokas 2011). In addition, issues of concatenation (Salichos and Rokas 2013) and model selection (Lartillot and Philippe 2008) have also been identified as possible pitfalls for phylogeny reconstruction in a phylogenomics framework. Finally, it has been shown that conflict may exist between classes of genes and some have proposed the use of slow-evolving genes to resolve deep metazoan splits (Nosenko et al. 2013). These issues have been recently analyzed in detail in two studies on arachnid phylogeny (Fernández, Hormiga, et al. 2014; Sharma et al. 2014), and we follow the same basic strategy explored there. First, we have minimized the amount of missing data and worked with one of the most complete phylogenomic matrices for nonmodel organisms, with gene completeness between 78% and 95%. Given our levels of missing data and matrix completeness, it is unlikely that our well-supported results are a consequence of a problem with gene occupancy or missing data. Likewise, taxon sampling has been optimized to represent all major nemertean lineages, including a representative of each of the three main groups of Palaeonemertea, Hubrechtidae, the two main clades of Heteronemertea, and within Hoplonemertea, both Polystilifera and Monostilifera. No major hypothesis on nemertean phylogenetics thus remains untested with our sampling, although it would be desirable to add a second hubrechtid species to further test the Pilidiophora hypothesis (Thollessen and Norenburg 2003), a clade supported by the pilidium larva and the striking way the juvenile worm develops inside the larva from a series of isolated rudiments, called the imaginal discs (Maslakova 2010a, 2010b), and other synapomorphies, such as proboscis musculature (Chernyshev et al. 2013) and caudal cirrus and dermal musculature (Chernyshev et al. 2013). Orthology prediction is another important issue in phylogenomic analysis, and we have followed an automated methodology (see Materials and Methods). Given the strong similarity of our results to Sanger-based phylogenies, we have no reason to suspect that the support obtained for our relationships is artifactual.

An issue remains untested with our data set, the effects of concatenation, and gene incongruence among data partitions (Jeffroy et al. 2006; Nosenko et al. 2013; Salichos and Rokas 2013). These authors question the exclusive reliance on concatenation, and argue that selecting genes with strong phylogenetic signals and demonstrating the absence of significant incongruence are essential for accurately reconstructing ancient divergences. This idea contrasts with the basic premises of phylogenetic inference and the additive nature of signal versus the nonadditive signal of noise (Wenzel and Siddall 1999), and it has been suggested that the conclusions reached by Salichos and Rokas (2013) about the higher levels of incongruence and lower phylogenetic signal reported for conserved genes are due to sampling error and not necessarily due to conflict (Betancur-R. et al. 2014).

Detecting incongruence between large numbers of genes is empirically difficult, and gene tree approaches (Edwards et al. 2007; Liu et al. 2008, 2009) can account for the discordance between gene trees and species trees (Degnan and Rosenberg 2006). These methods are now routinely applied to

multilocus data sets as an alternative to concatenation: However, in both empirical and theoretical applications of this paradigm, data sets analyzed are almost always for closely related species or multiple individuals per species (Degnan and Rosenberg 2006; Heled and Drummond 2010; McCormack et al. 2011; Satler et al. 2011). This is not our case, where hundreds or thousands of species could be placed between any two terminals included in our phylogeny, and thus the expectation that genes would coalesce prior to the bifurcating event tends to zero. In addition, our protein-encoding, transcriptomic-based phylogeny is highly similar to previous hypotheses on nemertean phylogenetics using a much smaller set of genes (Thollessen and Norenburg 2003; Andrade et al. 2012; Kvist et al. 2014)—none of which were included in this study—thus supporting the idea that our results are not artifactual. Our hypothesis is also 100% compatible with a mitogenomics hypothesis, although with much more limited sampling (Chen et al. 2012), again, stressing the congruence between such three disparate data sets.

In summary, our analytical approach using the presented tools seems appropriate for resolving the phylogenetic relationships among nemerteans. We found strong support for the monophyly of the phylum, as well as for its constituent clades Palaeonemertea, Neonemertea, Pilidiophora, Heteronemertea, Hoplonemertea, Monostilifera, Cratenemertea, and Distromatonemertea, all with maximal support, stability to model and method selection, and with high gene support frequency. Hubrechtidae and Polystilifera are represented by a single species and therefore their monophyly is untested in this study, but unlikely to be disrupted according to prior morphological and molecular work. With a phylogenomic approach we are able to infer a stable tree for nemerteans in contrast to Sanger-sequencing approaches, which failed to resolve some relationships, demonstrating the feasibility and utility of phylogenomics for neglected, but nonetheless unique, phyla.

Materials and Methods

Species Selection

After identifying all the major nemertean lineages, we obtained live specimens for approximately 30 nemertean species and selected 12 of these for sequencing after examination of RNA quality and cDNA library quality. RNA was extracted from specimens frozen in liquid nitrogen or from RNAlater-preserved specimens. Tissues preserved in RNAlater were processed as soon as possible to avoid RNA degradation.

Outgroup selection was based on recent phylogenomic studies (Dunn et al. 2008; Hejnol et al. 2009; Struck et al. 2014) which placed Nemertea in a clade with Brachiopoda, Annelida, and Mollusca in the larger Trochozoa (Hejnol et al. 2009). Based on this evidence and data availability, the following eight representatives were selected as outgroups: Four molluscs (*Chiton olivaceus*, *Octopus vulgaris*, *Gadila tolmiei*, and *Ennucula tenuis*) and four annelids, including a sipunculan (*Sipunculus nudus*, *Hormogaster samnitica*, *Helobdella robusta*, and *Capitella teleta*).

Specimen vouchers, leftover tissues, as well as RNA and DNA are deposited in the Museum of Comparative Zoology, either in the collections of the Department of Invertebrate Zoology or in the Cryogenic collection. Specimen details are available online at MCZbase (<http://mczbase.mcz.harvard.edu>, last accessed September 7, 2014). Accession numbers for the sequence data specimens are provided on the supplementary table S1, [Supplementary Material](#) online.

Molecular Techniques

RNA Extraction

Tissues were preserved in at least ten volumes of RNAlater soon after the animals were collected; if sent to the laboratory alive, animals were flash-frozen in liquid nitrogen. All samples were stored at 80 °C until RNA was extracted. Tissues were cut into pieces ranging from 0.25 to 0.5 cm in thickness, except for tissues of *Cep. hongkongiensis*, *P. beebei*, and *Hu. ijimai*, which were not subsampled due to small size. Total RNA was extracted using Tri-Reagent (Ambion), following the manufacturer's protocol. Subsequent mRNA purification was performed with the Dynabeads mRNA Purification Kit for mRNA (Invitrogen). Purification from Total RNA preps followed the manufacturer's instructions. Further details of the RNA extraction and purification protocols can be found in [Riesgo, Andrade, et al. \(2012\)](#). For *Tubulanus punctatus*, total RNA was extracted from tissue fragments of one specimen following the same procedure above, processed by Roche (454 Life Sciences, a Roche company, CT), and sequenced on the 454 Genome Sequencer FLX Titanium.

Quantity and Quality Control of mRNA

Quantity and quality (purity and integrity) of mRNA were assessed by two different methods. Quantity of mRNA was measured with the fluorometric quantitation performed by the Qubit Fluorometer (Invitrogen, CA). Also, capillary electrophoresis in an RNA Pico 6000 chip was done using an Agilent Bioanalyzer 2100 System with the "mRNA pico Series II" assay (Agilent Technologies, CA). Integrity of mRNA was estimated by the electropherogram profile and lack of rRNA contamination (based on rRNA peaks for 18S and 28S rRNA given by the Bioanalyzer software).

Illumina Sequencing

Next-generation sequencing was performed using the Illumina platform Genome Analyzer GAII (Illumina, Inc., San Diego, CA). Each library was run in a full lane at the FAS Center for Systems Biology at Harvard University. mRNA concentrations between 20.1 and 53.4 ng/μl were used for cDNA synthesis, which was performed following methods published elsewhere ([Riesgo, Pérez-Porro, et al. 2012](#)). The Illumina samples were prepared with the NEBNext mRNA Sample Prep kit (New England BioLabs, Ipswich, MA). cDNA was ligated to Illumina adapters, as described earlier ([Riesgo, Andrade, et al. 2012](#)). Size-selected cDNA fragments of around 300 bp excised from a 2% agarose gel were amplified using Illumina polymerase chain reaction (PCR) primers for paired-end reads (Illumina, Inc.) and 18 cycles of the PCR program consisting of 98 °C—30 s, 98 °C—10 s, 65 °C—30 s, and 72 °C—30 s, followed by an extension step of 5 min at 72 °C. The

concentration of the cDNA libraries was measured with the Qubit dsDNA High Sensitivity (HS) Assay Kit using the Qubit Fluorometer (Invitrogen, Carlsbad, CA). The quality of the library and size selection were checked using the "HS DNA assay" in a DNA chip for an Agilent Bioanalyzer 2100 (Agilent Technologies). All samples sequenced on Illumina GAII had 150 bp read length. Details on the sequencing method (if paired or single-end) and number of raw and processed reads are presented in [table 1](#).

Sequence Processing, Orthology Prediction, and Alignment

All filtered reads generated for this study are deposited in the National Center for Biotechnology Information Sequence Read Archive ([supplementary table S1, Supplementary Material](#) online).

Roche 454 data for *Tubulanus* were assembled with the NewblerGS de novo assembler (version 2.3, Roche) with the flags "-cdna -nrm -nosplit." In cases in which multiple splice variants (isotigs in Newbler terminology) were produced for a gene (an isogroup in Newbler terminology), a single exemplar splice variant was selected.

Reads were trimmed at the 5'-end when needed and the ones that did not have an average quality score of at least 32 based on a Phred scale were removed using the python scripts from [Dunn et al. \(2008\)](#). Illumina data were assembled with Velvet v.1.1.06 ([Zerbino and Birney 2008](#)) and Oases v. 0.2.06 ([Schulz et al. 2012](#)). Insert lengths for Oases were estimated based on the observed graph from the 2100 Agilent Bioanalyzer. We examined the assemblies over a range of *k* values (41–71, in increments of 10), which were merged by Oases on the final step. In order to select a minimum of splice variants (or transcripts in Oases terminology) for each gene (locus in Oases terminology), [Smith et al.'s \(2011\)](#) procedure was adopted: Only transcripts that contained at least 150 nucleotides, had a length of at least 85% of the longest transcript for the gene, and had the highest read coverage were chosen. Loci with more than 50 transcripts were filtered out, as these often appeared to be the result of misassembly. *Capitella teleta* and *He. robusta* translated assemblies were obtained from a publicly available database (JGI).

Assembled data were compared with NCBI's nr protein database with the BLASTX tool, with an *e* cutoff of 1e-5. The BLASTx comparison was performed reducing the search field to the NCBI Taxon ID 33154 database (Fungi/Metazoa group). Only sequences with a hit to the database were retained for subsequent analyses; sequences with hits to rRNA sequences were excluded. Nucleotide sequences were translated with the prot4EST v. 3.1b pipeline ([Wasmuth and Blaxter 2004](#)).

Orthology assignment for the data set assemblies was performed with OrthoMCL v2.0.9 ([Li et al. 2003](#)). All-by-all comparisons were conducted with BLASTP following OrthoMCL guideline and using the 10⁻²⁰ *e* value threshold. The MCL inflation parameter was varied in increments of 0.2 ranging from 1.4 to 2.6. The final cluster composition was not particularly sensitive to different inflation values in this range;

therefore, an inflation value of 2.0 was selected, which is within the range of inflation parameters used in similar studies. Clusters with at least 15 taxa (> 75% gene occupancy) were aligned by using MAFFT L-INS-i v.7.149b (Katoh et al. 2005; Katoh and Toh 2008), followed by trimming with TrimAl v1.2 to account for alignment uncertainty, with gap threshold of 80% and conserving a minimum of 20% of the original alignment (Capella-Gutiérrez et al. 2009). After trimming, we obtained, for each partition, one ML phylogenetic tree with RAxML v.7.7.5 (Stamatakis 2006). In this analysis, we applied 50 rapid bootstraps and PROTGAMMALG as evolution model.

Monophyly masking was conducted to reduce the number of monophyletic sequences from the same taxon to one sequence. The resultant 2,779 phylogenies from the previous step were then analyzed by an iterative paralogy pruning procedure using PhyloTreePruner (<http://sourceforge.net/projects/phyloreepruner/>, last accessed September 7, 2014), by which maximally inclusive subtrees with no more than one sequence per taxon were pruned and retained. FASTA-formatted files were generated from subtrees that were produced by the paralogy pruning procedure. These files were then aligned with MAFFTL-INS-i v.7.149b, trimmed with trimAl, and concatenated into the final matrices.

Phylogenetic Analyses

We conducted the phylogenetic analyses with two matrices: 1) A large matrix with 2,779 orthogroups (genes) including 411,138 aligned amino acid positions and with a 78% gene occupancy and 2) a reduced matrix with 464 genes, 82,012 amino acid positions, and 95% gene occupancy. Both matrices (summarized in fig. 1) were analyzed using the same methods.

An ML analysis was conducted using RAxML v.7.7.5 (Stamatakis 2006). The best-fit model of amino acid evolution per partition was estimated by ProtTest 3.4 (Darriba et al. 2011), using the corrected Akaike Information Criterion. The most frequent model of evolution, LG (2,255 of 2,779 partitions in the large matrix and 401 of 464 in the reduced matrix) was used as base model as long with the “partition.txt” input for the ML analysis. Due to the outperformance of the flexible LG4X model, which can provide gains of up to hundreds of log-likelihood units and seems better adjusted for the complexity of amino acid replacements and more efficient than models which use single replacement matrices (Le et al. 2012), we ran two preliminary tests with the large matrix, using LG and LG4X as main models. Best-scoring ML trees were inferred for each gene under the selected model (with the gamma model of rate variation, but no invariant term) from 100 replicates of parsimony starting trees. In total, 160 traditional (nonrapid) bootstrap replicates for the large matrix and 200 for the reduced matrix were also inferred. To draw the bipartition information on the best tree given by RAxML, we used its function “-f b” along with “-t” based on multiple trees (provided by the bootstrap output file).

Bayesian inference was conducted with ExaBayes version 1.3 (The Exelixis Lab, <http://sco.h-its.org/exelixis/web/soft>

[ware/exabayes/](#), last accessed September 7, 2014). ExaBayes is a Bayesian phylogenetic tool that implements Markov chain Monte Carlo (MCMC) sampling approach similar to the one implemented in MrBayes (Ronquist et al. 2012). It is however better adapted for large data sets by its ability to parallelize each independent run, each chain, and the data (i.e., unique site patterns of the alignment). We used the revMat model prior, which integrates over amino acid general time reversible matrices (189 free parameters). Two and five independent MCMC chains, for 1,000,000 generations each, were run for the reduced and large matrices, respectively. The first 100,000 trees (10%) were discarded as burn-in for each MCMC run prior to convergence (i.e., when maximum discrepancies across chains <0.1).

Gene Tree Analyses

To investigate potential incongruence between individual gene trees, we followed three different approaches for both matrices. First, Salichos and Rokas (2013) stressed the need of choosing genes with strong phylogenetic signals for accurately reconstructing ancient divergences. These can be derived from the bootstrap support from the inferred trees, a measurement called “internode certainty,” which estimates the level of conflict among internodes. To calculate the “internode certainty,” the function -L from RAxML was implemented and the relative tree certainty (from now on, TC) was calculated. TC is the sum of all internode certainty (IC) scores for all trees, which is computed by taking all conflicting bipartitions that have $\geq 5\%$ support into account, where a TC = 1 means no conflict among internodes. Second, we employed SuperQ v.1.1 (Grünwald et al. 2013) to visualize predominant intergenic conflict. Here the gene trees were decomposed into quartets, and a supernet network assigning edge lengths based on the quartet frequencies was inferred from these quartets selecting the “balanced” edge-weight optimization function, with no filter (see Fernández, Laumer, et al. 2014). Finally, we employed Conclustador v.0.4a (Leigh et al. 2011), using the default settings as an automated incongruence-detection algorithm using the bootstrap tree files from ML. In order to visualize the networks from the latter two approaches results, we used SplitsTree v.4.13.1 (Huson and Bryant 2006).

Supplementary Material

Supplementary table S1 is available at *Molecular Biology and Evolution* online (<http://www.mbe.oxfordjournals.org/>).

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