Organisms Diversity and Evolution

Interrelationships of Nemertodermatida

--Manuscript Draft--

Manuscript Number:	ODAE-D-15-00083R1	
Full Title:	Interrelationships of Nemertodermatida	
Article Type:	Original Article	
Keywords:	Nemertodermatida; LSU; SSU; molecular phylogeny; cryptic species; Approximately Unbiased test	
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Funding Information:	Vetenskapsrådet (2009-5147)	Ulf Jondelius
	Vetenskapsrådet (2012-3913)	Ulf Jondelius
	Riksmusei Vänner (stipend 2011)	Dr Inga Meyer-Wachsmuth
	Stiftelsen Lars Hiertas Minne (FO2011-0248)	Dr Inga Meyer-Wachsmuth
	Royal Swedish Academy of Sciences (FOA11H-352)	Dr Inga Meyer-Wachsmuth
Abstract:	<p>Nemertodermatida is a small taxon of microscopic marine worms, which were originally classified within Platyhelminthes. Today they are hypothesized to be either an early bilaterian lineage or the sister group to Ambulacraria within Deuterostomia. These two hypotheses indicate widely diverging evolutionary histories in this largely neglected group. Here, we analyze the phylogeny of Nemertodermatida using nucleotide sequences from the ribosomal LSU and SSU genes and the protein coding Histone 3 gene. All currently known species except Ascoparia neglecta and A. secunda were included in the study in addition to several yet undescribed species. Ascopariidae and Nemertodermatidae are retrieved as separate clades, although not in all analyses as sister groups. Non-monophyly of Nemertodermatida was rejected by the Approximately Unbiased test. Nemertodermatid nucleotide sequences deposited in Genbank before 2013 were validated against our dataset; some of them are shown to be chimeric implying falsification of prior hypotheses about nemertodermatid phylogeny: other sequences should be assigned new names. We also show that the genus Nemertoderma needs revision.</p>	
Response to Reviewers:	Answers will be uploaded in a separate file.	

Reviewer #1: The manuscript is summarising a good piece of work and is addressing an important problem of internal phylogeny of Nemertodermatida. The methods are state of the art. I have no comments or suggestions for changes to this manuscript. I suggest publication as it is.

Dear Reviewer #1,

Thank you very much for your favorable view on our work; we are delighted to hear this.

Reviewer #2: The manuscript deals with the phylogeny of Nemertodermatida. The authors have done an important sequencing effort, the phylogenetic analyses are well done, and the trees seem informative enough. I think the ms needs, however, some serious re-writting to better emphasize the findings, specially with regards the higher-level classification and implications. Other than that, I suggest publication, although I have some minor comments and corrections to be addressed.

Dear Reviewer #2,

First of all I would like to thank you for your thorough review of our manuscript. It is good to see such detailed comments. And thank you also for pointing out these nasty small mistakes such as missing italics, double words, wrong spellings etc. One just doesn't seem to see them after some time anymore. Otherwise we went with nearly all your suggestions but for those on Figure 1, since the sequences do not blast to any specific species with high identity. Table 2 was hard to understand, because we missed to add a note that the new accession numbers had not been added yet, or placeholders for them. We hope that the addition of those and the changes of the asterisks make this table much better to understand now. Please find point-by-point answers below:

Minor comments

Abstract

I suggest to include a bit of background of Nemertodermatida and why it is important to decipher its phylogeny. The authors have done a good job in the introduction but the abstract goes directly to results.

We added a few sentences on the importance of Nemertodermatida.

Page 3, lines 70, 71. "not part of Platyhelminthes or Acoelomorpha". Change to something like "not part of Platyhelminthes, and not sister-group to Acoela".

Changed as suggested, thanks.

Page 3, line 73. Ruitort, change to "Riutort"

Changed in manuscript and in Papers.

Page 3, line 83. "i) is Acoelomorpha monophyletic, i.e., is Nemertodermatida the

sister group of Acoela? ". I suggest changing to : i) is Nemertodermatida the sister group of Acoela forming the Acoelomorpha?"

Changed to be easier to read and be more precise.

Material and methods

Please specify exactly which are the specimens collected. It is not clear at all. Are there the one without asterisk in table 2. If so, I suggest to depict them by asterisk instead.

We changed this as you suggested, the asterisk highlighting the new sequences now. Since we did not have accession numbers for new sequences, the table was possibly even more confusing; we should have added placeholders for the review process.

Page 6, lines 150-151. "Meara sp. and species of the genus Ascoparia and could not be collected". Maybe deleted "and"?

Done!

Page 6, lines 151-152. "About 45 new specimens of...were sequenced". Which 45 specimens? Please clarify. Maybe with a table?

The dataset was built in steps; many of the sequences of the 45 specimens are not being published here. We nevertheless mention the process of how we arrived at the current dataset in order to forestall criticism of the dataset built on such a small number of specimens per species.

Page 6, lines 155-156. "highly similar sequences of Meara, Flagellophora and Nemertoderma were discarded in order to reduce the size of the dataset.". How similar? how many of them were similar? similar for which genes? Please clarify.

We specified the similarity (99.9% identity) in the text and that we did this in all three genes. We believe that it is not important for this manuscript, and in contrast will rather introduce more confusion, to distinguish for example in the case of Nemertoderma different lineage and describe how many specimens each had in the preliminary analyses. Flagellophora sequences were more variable than previously expected and sequences should only be published with a proper discussion of population genetics and species delimitation, which is beyond the scope of this manuscript.

Page 6, line 164. "new sequences are published in GenBank (Tab. 2)." which are the accession numbers? The taxa without asterisk does not have GenBank accession numbers. Are those the new sequences? Again, I think table 2 and the taxon sampling is not clear at all.

The accession numbers have been added now that they were available. We also changed the use of the asterisk according to your remarks, they now are directly in front of the accession numbers and highlight sequences published new in this manuscript.

Page 8. I do not find the explanation of Nucleotide frequencies that relevant. I suggest to take most of that paragraph out.

We agree, it is a lot of detail. We took most of the paragraph (we only left the first sentence) and added it to the table in the online resources 1.

Page 10. Including the nodal support in figure 4 will allow a better understanding of this part.

Nodal support has been added.

Page 13, lines 389-392. I do not see the LSU tree (Figure 4) recovering (Nemertoderma 391 (Meara, (Nemertinoidea + Sterreria))). Please clarify.

In Figure 4, the addition of chimeric sequences changed the topology of the tree. Figure 4 only exemplifies the effect that these two chimeric sequences had on the phylogenetic inference of this dataset. The tree(s) referred to (lines 389-392) are the concatenated tree of Fig. 3 (references added to the manuscript) and the gene trees in the supplementary material (as referred to in the same sentence).

Page 14-15. I think the authors could re-write this part a bit to make the findings and the implications more clear. I also think a note should be done here for the need of additional taxon sampling, specially with regards Ascopariidae.

We edited these paragraphs to make them clearer and added a call for collecting more data. We refrained from using the word taxon sampling, since we believe that what is needed is a wider geographical sampling that might very well turn out to be the same as enhanced taxon as shown in Meyer-Wachsmuth et al. 2014, but we do not know that.

Table 1. I suggest including the sequences of the primers. This way the table would be more complete and informative.

We added the sequences of the primers as well as the references.

Figures

Figure 1 is hard to understand. I would suggest including the sequence of the ones they blast to (the gastrotrich species, the unidentified platyhelminth species, and the copepod).

The ID and sometimes query coverage of the blasted sequences is often very bad, as reference in the text. Adding these sequences into the figure would be an arbitrary choice in 3 out of the 4 cases and more pointedly add further confusion with mismatches. Had clear

Figure legend 1. Italicize "Meara stipochi".

Done

Figure 3. Some of the lines of the coloured rectangles interfere with a good visual of the nodal support. Consider removing them (change by colour labels on the right--there is enough space if species names are situated at the tip of the branches) or make them without lines.

We chose to keep the coloured boxes, which make colours less ambiguous than only lines, which have to have a certain weight for colours to be easily distinguishable. But we removed the lines around the boxes and added arrows where nodal support conflicted with the tree.

Figure 4. Why there are not nodal support values? If possible, include labels "LSU" and "SSU" below each tree. I also suggest including labels on the right for each group.

Nodal support values have been added as well as the labels LSU and SSU, but instead of a) and b) above the tree. We also added group labels next to the trees.

We are very happy that this manuscript has been regarded favorably.

With kind regards,

Inga Meyer-Wachsmuth on behalf of all authors

Interrelationships of Nemertodermatida

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Abstract:

Nemertodermatida is a small taxon of microscopic marine worms, which were originally classified within Platyhelminthes. Today they are hypothesized to be either an early bilaterian lineage or the sister group to Ambulacraria within Deuterostomia. These two hypotheses indicate widely diverging evolutionary histories in this largely neglected group. Here, we analyze the phylogeny of Nemertodermatida, a group of microscopic marine worms, was analysed using nucleotide sequences from the ribosomal LSU and SSU genes and the protein coding Histone 3 gene. All currently known species except *Ascoparia neglecta* and *A. secunda* were included in the study in addition to several yet undescribed species. Ascopariidae and Nemertodermatidae, are retrieved as separate clades, although not in all analyses as sister groups. Non-monophyly of Nemertodermatida was rejected by the Approximately Unbiased test. Nemertodermatid n Nucleotide sequences deposited in Genbank before 2013 ~~as nemertodermatid~~ were validated against our dataset; some of them are shown to be chimeric implying falsification of prior hypotheses about nemertodermatid phylogeny: other sequences should be assigned new names. We also show that the genus *Nemertoderma* needs revision.

Keywords: Nemertodermatida; LSU; SSU; molecular phylogeny; cryptic species;

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31 Approximately Unbiased test

32 **Acknowledgements**

33 We would like to thank Ms Keyvan Mirbakhsh for her work in the molecular lab. We are
34 also grateful to the staff at the Sven Lovén Centre for Marine Sciences, Dr Ana Amaral at
35 CCMAR, Faro, Ms Lisa Del Monte, ISZN, Naples with colleagues and Ms Margret Krüß,
36 BAH, Helgoland with colleagues for help with our fieldwork. We would also like to
37 express our gratitude to Prof. Mark Martindale then of the University of Hawaii Kewalo
38 Marine Lab. Furthermore are we indebted to the Professor Philippe Bouchet of the
39 Muséum National d'Histoire Naturelle for organizing sampling in Papua New Guinea.
40 Collections in Papua Guinea took place during the Our Planet Reviewed Papua Niugini
41 Expedition in November–December 2012, organized by the Muséum National d'Histoire
42 Naturelle (MNHN), Pro Natura International, the Institut de Recherche pour le
43 Développement (IRD), and the University of Papua New Guinea. The principal
44 investigators of this expedition were Philippe Bouchet, Sarah Samadi (MNHN) and Claude
45 Payri (IRD) and funding was provided by the Total Foundation, Prince Albert II of
46 Monaco Foundation, Fondation EDF, Stavros Niarchos Foundation and Entrepose
47 Contracting, with support from the Divine Word University and operated under a permit
48 delivered by the Papua New Guinea Department of Environment and Conservation.
49 Financial support from the Swedish Research Council to UJ is gratefully acknowledged
50 (grant numbers 2009-5147 and 2012-3913) as are the stipends by Föreningen Riksmusei
51 Vänner (stipend 2011), Stiftelsen Lars Hiertas Minne grant FO2011-0248 and the Royal
52 Swedish Academy of Sciences grant FOA11H-352 to I. Meyer-Wachsmuth.

Introduction

The phylogenetic position of Nemertodermatida, a group of microscopic marine worms, has been a matter of contention ever since the first species was described by Steinböck (1930), who classified it within the Platyhelminthes and considered it close to the ancestor of the flatworms in terms of morphology. Westblad (1937) placed nemertodermatids in the group Acoela, at that time considered an order within Platyhelminthes. Nemertodermatida differ from acoels in a number of morphological characters including the presence of a gut with an epithelial lining and the possession of a statocyst with two statoliths; these differences led Karling (1940) to recognise ~~the order~~ Nemertodermatida as separate from Acoela. Ultrastructural studies revealed similarities between acoels and nemertodermatids in the epidermal cilia, and the two groups were hypothesised to form the monophylum Acoelomorpha (Ehlers 1985). Analyses of nucleotide sequence data resulted in conflicting phylogenetic hypotheses regarding the position of Nemertodermatida. Initially, SSU rDNA sequences reported from the species *Nemertinoides elongatus* Riser, 1987 placed Nemertodermatida within rhabditophoran flatworms (Carranza, Baguña, & Ruitort 1997; Littlewood, Rohde, Bray, & Herniou 1999). Subsequent analyses using sequences from additional species placed Nemertodermatida as a separate early branching clade predating the split into Deuterostomia and Protostomia, i.e., not part of Platyhelminthes or sister group to Acoela, and demonstrated that the original “*Nemertinoides*” sequences were either contaminated or originating from a misidentified specimen (Jondelius, Ruiz-Trillo, Baguña, & Riutort 2002; Wallberg, Curini-Galletti, Ahmadzadeh, & Jondelius 2007). The nemertodermatid position as a separate early bilaterian clade, i.e., rejecting Acoelomorpha, was further supported by several protein coding nuclear genes (Paps, Baguña, & Riutort 2009; Ruiz-Trillo et al. 2002). The first phylogenomic study including nemertodermatid sequences (Hejnal et al. 2009) on the other hand supported Acoelomorpha, i.e. Nemertodermatida and Acoela, as the sister group of Nephrozoa (the remaining Bilateria). However, a contradictory result was found in a second phylogenomic study that upheld Acoelomorpha, but placed this group nested within Deuterostomia (Philippe et al. 2011).

To summarise: the phylogenetic position of Nemertodermatida is highly controversial on two levels: i) is Nemertodermatida the sister group of Acoela, thus forming the monophyletic Acoelomorpha? and ii) are nemertodermatids a primarily simple early bilaterian clade or are they drastically reduced deuterostomes? -In either case a strongly

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corroborated hypothesis regarding the interrelationships of nemertodermatids is of great interest, as it would allow modelling of character evolution and ancestral character states within the group and, by extension, either during early bilaterian evolution or within deuterostomes.

The current classification of Nemertodermatida is based on Sterrer (1998) and Lundin (2000) who recognised two families and six genera to accommodate the eight nemertodermatid species described between 1930 and 1998 (Faubel & Dörjes 1978; Riedl 1983; Riser 1987; Steinböck 1930; Sterrer 1998; Westblad 1937; 1949). Recently, nine new species were named and one junior synonym reinstated using molecular delimitation methods, raising the nominal species count to 18, and there is evidence of further, as of yet undescribed, species (Meyer-Wachsmuth, Curini-Galletti, & Jondelius 2014).

There are two conflicting hypotheses regarding the phylogenetic relationships within Nemertodermatida. The first, which is derived from a cladistic analysis of light microscope and ultrastructural characters (Lundin 2000), retrieved a basal dichotomy splitting the taxon into two groups, Nemertodermatidae and Ascopariidae, in concordance with Sterrer's classification (1998). The second hypothesis ~~is was~~ based on analyses of the large and small ribosomal subunit genes with the primary goal of testing the monophyly of Acoelomorpha (Wallberg et al. 2007), which was found to be non-monophyletic. In the latter study *Flagellophora apelti* Faubel and Dörjes, 1978 and *Meara stichopi* (Bock) Westblad 1949 formed a monophyletic group, thus rendering Nemertodermatidae and Ascopariidae non-monophyletic.

Here we use nucleotide sequences from three molecular markers, large and small subunit rDNA and histone H3 to generate the most comprehensive phylogenetic hypothesis of Nemertodermatida to-date, including all but two of the known as well as several yet undescribed species. The phylogenetic hypothesis provides a framework for the interpretation of the evolution of such features as the nemertodermatid nervous system (~~Raikova et al., dealt with in a separate publication in this issue~~), and for an updated classification of Nemertodermatida, including a test of the monophyly of nemertodermatid families and genera. Additionally, we also validate nemertodermatid sequences currently available on GenBank.

Material and Methods

Collection of Specimens

Marine meiofauna was extracted from sandy sediments using isotonic magnesium chloride solution (Sterrer 1968) and from muddy sediments by ~~the~~ siphoning off the uppermost layer of settled mud through a 125µm sieve. Specimens were then sorted and identified under a dissecting and compound microscope, if possible equipped with differential interference contrast optics, before fixation in ethanol or RNAlater®. Whenever feasible microphotographs were taken of live specimens to serve as vouchers, because DNA is extracted from whole specimens.

DNA extraction, amplification and sequencing

DNA was extracted using the Qiagen Micro Tissue Kit. Limited availability of sequence data and low yield of DNA, owing to the microscopic size of the worms, restricted testing of new primers and therefore choice of genetic markers. We were able to amplify and sequence part of the large and small ribosomal subunit genes (LSU and SSU, respectively), as well as a part of the nuclear protein coding Histone 3 gene (H3). All markers were amplified and sequenced using several different primer combinations (Tab. 1) and, in case of SSU, a nested PCR approach. Sequences were edited, aligned and, in case of H3, translated into amino acids and checked for open reading frames (standard genetic code), using the commercial Geneious Pro 7.1.5. software package created by Biomatters (<http://www.geneious.com>). Alignments were conducted using the MAFFT algorithm (Katoh, Misawa, Kuma, & Miyata 2002).

Dataset assembly and outgroup choice

Outgroup taxa were chosen from various groups based on the different phylogenetic hypotheses for Nemertodermatida. Early branching species of Acoela (Tab. 2) were added due to their hypothesized sister group relationship to Nemertodermatida (Edgecombe et al. 2011; Hejnol et al. 2009; Philippe et al. 2011). In order to account for the unknown phylogenetic position we also added species of hemichordates and crinoids (Deuterostomia, Ambulacraria) and early branching molluscs (Protostomia). To test for different rates of evolution between the in- and outgroups Tajima's relative rate test

(Tajima 1993) was conducted using Mega 5.2.2. (Tamura et al. 2011).

The final dataset was assembled step-by-step. First, a preliminary dataset was built covering 24 out of 28 nominal or putative species of Nemertodermatida (Meyer-Wachsmuth et al. 2014; Sterrer 1998). *Meara* sp. and species of the genus *Ascoparia* could not be collected. About 45 new specimens of *Nemertoderma*, *Meara* and *Flagellophora* were sequenced for all three genes (not all data included in final dataset). For all species of *Nemertinoides* and *Sterreria* one specimen each was chosen for this dataset from a previously published dataset based on high gene coverage (Meyer-Wachsmuth et al. 2014). Secondly, and Aafter preliminary analyses, ~~highly similar~~ sequences of *Meara*, *Flagellophora* and *Nemertoderma* that were 99.9% or more identical in any gene were discarded and only one exemplary specimen left, based on gene coverage, in order to reduce the size of the dataset. In case of the genus *Nemertoderma* similar sequences were not discarded where there was conflict between morphological identification and genetic placement. Our eleven *Flagellophora* specimens always formed a monophyletic group separate from Nemertodermatidae in preliminary analyses but the sequence variation between specimens was higher than in other nemertodermatids, indicating potential presence of more than one *Flagellophora* species (a matter that cannot be studied until more specimens have been collected). To avoid confusion we chose to include only the sequences from a single individual in our analyses. Finally, Aall nemertodermatid sequences uploaded to GenBank prior to 2013 were then added to this reduced dataset for validation. Four of those appeared chimeric in the alignments and were subsequently excluded again (s. Tab. 2, Fig. 1). The effects of these sequences on phylogenetic analyses were tested in separate analyses.

The final dataset consists of an ingroup of ~~4129~~ terminals covering 24 of the 28 nominal or putative species of Nemertodermatida including nemertodermatid sequences downloaded from GenBank for validation and outgroup taxa, with 28 sequences for each LSU and SSU, and 21 sequences for H3, New sequences, two of them for the outgroup species *Paratomella rubra* Rieger & Ott, 1971, are published in GenBank (marked with an asterisk in Tab. 2). Ten, twelve and one sequence(s) for LSU, SSU and H3, respectively, were downloaded from GenBank and included for validation (Tab. 2). Additional to the sequences downloaded from GenBank for the outgroup taxa new sequences were produced for the acol species *Paratomella rubra* Rieger & Ott, 1971 (Tab. 2). For the concatenated dataset, sequences downloaded from GenBank were combined to ~~form~~ “terminals” based

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either on submitted specimen collection codes (e.g. *Ascoparia* sp. with collection code “AWHel-24” stated in GenBank), similarity of accession number, or taxon, ~~and coverage of at least two genes~~ (Tab. 2).

Data properties

Mega 5.2.2. (Tamura et al. 2011) was used to calculate the nucleotide frequencies for each specimen, gene and codon position as well as Tajima’s D for H3 (Tajima 1989) and the Disparity Index (Kumar & Gadagkar 2001) for the final dataset. The GC-content per lineage and gene was calculated in Excel. Alignments were tested for random similarity with the program Aliscore (Kück et al. 2010; B. Misof & Misof 2009) using the default settings. jModeltest v. 2.1.1. (Darriba, Taboada, Doallo, & Posada 2012) was used with a BioNJ starting tree to test for the importance of the proportion of invariable sites (I, Pinvar) and the rate variation across sites (G), and to obtain values to set useful priors, based on the Bayes Information Criterion (BIC).

Phylogenetic informativeness profiling and saturation

Phylogenetic informativeness profiles were produced with the PhyDesign webserver (López-Giráldez & Townsend 2011) available at <http://phydesign.townsend.yale.edu/> with site rates being calculated by the inbuilt DNAr8es algorithm (unpublished). The ultrametric tree was created from the concatenated dataset using the chronos algorithm (lambda=0.1, model=correlated) of the splits package in R. Saturation plots were created by plotting the pairwise uncorrected p-distances against the phylogenetic distance of the best tree inferred from the concatenated dataset with RAxML using R (Klopfstein, Vilhelmsen, Heraty, Sharkey, & Ronquist 2013).

Phylogenetic analyses

Gene trees and species trees were calculated using two different methods of phylogenetic inference. Maximum Likelihood trees were calculated with the RAxMLGUI (Silvestro & Michalak 2011; Stamatakis 2006) using the GTR+G+I model (Tab. 3), and the ML + rapid bootstrapping algorithm (Stamatakis, Hoover, & Rougemont 2008) with 1000 replicates. Bayesian estimations were conducted with the program MrBayes v.3.2. (Ronquist et al.

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7 215 2012) with no model set and the program being allowed to sample the entire model space
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9 216 of the GTR model. Priors for the proportion of invariable sites and G were set according to
10 217 the results of jModeltest (Tab. 3). To ensure sufficient convergence, analyses were run
11 218 until the standard deviation of split frequencies reached values below 0.02 and the values
12 219 for Potential Scale Reduction Factor (PSRF+) were close to 1.0. Trees were visualized
13 220 using FigTree v1.3.1. (Rambaut 2009).

14 221 We also tested different topological hypotheses discussed here and indicated in preliminary
15 222 analyses with the Approximately Unbiased (AU) test. The test is implemented in the
16 223 program consel (Shimodaira & Hasegawa 2001) and calculates the overall likelihoods of a
17 224 set of topologies based on the per-site log likelihoods (Shimodaira 2002). For this we
18 225 conducted ML analyses (RAxML 7.2.8) of the final dataset with the following ~~three~~four
19 226 topological constraints: i) non-monophyly of Nemertodermatida (Ascopariidae
20 227 (Nemertodermatidae + Acoela)), ii) Lundin's (2000) Nemertodermatidae with a luterious
21 228 and a psammicolous clade (Ascopariidae, ((*Meara* + *Nemertoderma*), (*Nemertinoides* +
22 229 *Sterreria*))), and iii) Wallberg et al.'s (2007) hypothesis clustering *Meara* and
23 230 *Flagellophora* and rejecting the two-family hypothesis (Acoela, ((*Flagellophora* + *Meara*),
24 231 (*Nemertoderma*, (*Nemertinoides* + *Sterreria*))). Topological hypotheses were only tested
25 232 down to genus level. Species relationships have to be addressed by increased
26 233 taxon/geographical sampling.

36 235 Results

37
38 236 The final datasets consists of 44 (LSU), 46 (SSU) and 26 (H3) sequences, including ten,
39 237 twelve and one sequence(s), respectively, downloaded from GenBank for validation
40 238 (Tab. 2) and 16 sequences of outgroup taxa, six each for LSU and SSU, and four for H3.

44 240 Data properties

45 241 The relative rate test did not show significantly different rates between tested lineages,
46 242 allowing the use of the chosen outgroups.

47 243 Nucleotide frequencies within the ingroup are close to equilibrium, only Thymine can
48 244 reach frequencies as low as 15.2% in H3, and Guanine frequencies of 34.2% in LSU
49 245 (summary in Tab. 4, complete table as online resource 1). ~~The observed small biases are~~

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~~mostly consistent across the ingroup, for example Thymine has the lowest frequency of all~~
~~four nucleotides in LSU across all lineages. There are also lineage specific biases.~~
~~*Nemertoderma*, for example, has slightly lower frequencies of Cytosine and Guanine and~~
~~subsequently higher levels of Adenine and Thymine than *Meara*, *Nemertinoidea* and~~
~~*Sterreria* in the LSU data (Tab. 4). These lineage specific biases, however, are overall~~
~~small. The GC content of LSU and H3 is slightly elevated between 51.8% and 58.7%,~~
~~whereas in SSU it is balanced between 48.1% and 52.2%. The~~ disparity indices reveal
homogenous substitution patterns throughout the whole LSU dataset but not the SSU and
H3 datasets (online resource 2). The main deviations in the SSU data are shown between
lineages, for example *N. bathycola* Steinböck, 1930 and *N. westbladi* Steinböck, 1937
versus pacific *Sterreria* species, *Flagellophora* versus *S. psammicola* and *S. lundini*, and
between two subgroups within *Sterreria*. Substitutions patterns in the H3 dataset are
overall heterogeneous.

Aliscore identified ambiguously aligned sites in both the LSU and SSU gene datasets but
not in the coding Histone 3 gene. In the LSU dataset overall 845bp out of 3751bp (22.5%)
were considered potentially randomly aligned, whereas in SSU it was 184 out of 2399 sites
(7.7%). Most of the ambiguity was detected in the beginning and end of the alignments,
where coverage is lower. In the LSU dataset several other areas, often only a few base
pairs long, were highlighted as problematic. These were in highly variable areas, probably
the loops of stem-loop secondary structures, where indels resulted in local low coverage.
ML tree topologies estimated from the Aliscore reduced datasets did not differ from the
original topologies in LSU and only in one node in SSU (~~8~~[online resource: 3](#)).
Nemertinoidea sp. N1 was retrieved as earliest branch within the genus *Nemertinoidea* in
the original dataset but as sister taxon to a clade (*Nemertinoidea* (*Meara* + *Sterreria*)) in the
Aliscore-reduced dataset. In both datasets the majority of nodes stayed stable or changed
only little (online resource [43a-f](#)). Given the rule of thumb that any node below 75%
bootstrap support (BS) should not be considered reliable, the datasets did not change
substantially: two nodes in the LSU and none in the SSU dataset passed this threshold due
to the exclusion of randomly aligned sites, none of the nodes in either dataset decreased
below it. For both genes we decided to use the original datasets due to negligible effects of
the data exclusion.

With $D = 3.100814$ Tajima's D was insignificant. The results of jModeltest strongly suggested the use of the proportion of invariable sites (Pinvar, I) and rate variation across sites (G) (Tab. 3).

Phylogenetic informativeness (PI) profiling and saturation

Absolute measures of PI are not possible. Due to a lack of nemertodermatid fossils and widely varying phylogenetic hypotheses, reliable time calibration of the ultrametric tree used for PI profiling is not ~~feasible~~possible. Relative comparisons of the genes are, however, achievable (Fig. 2). Overall, the LSU gene is the most informative gene across the depth of the tree, while Histone 3 is the least informative one, partially due to its short length. The SSU gene is comparatively little informative especially in the shallow nodes but reaches its peak informativeness only in deeper nodes and stays informative over the depth of most of the ingroup.

Saturation plots indicate that H3 is saturated with the third position being the most variable and thus the most saturated, while the second position is the most conserved and least saturated (online resource [54](#)). LSU and SSU do not show an increase of uncorrected p-distance with increased phylogenetic distance and thus no clear signs of saturation.

Phylogeny

The gene trees of LSU and SSU support the same clusters corresponding to the morphologically delimited known genera (online resource [45a-d](#)). Furthermore, both datasets fully support Nemertodermatidae and Ascopariidae as monophyletic clades; the latter is never nested within the former. However, only the LSU and concatenated analyses recover Ascopariidae and Nemertodermatidae as sister groups, i.e. with BS values above 75% and Bayesian posterior probability (BPP) above 0.95 ~~with high support~~ (online resource [45a, b](#), Fig. 3) whereas SSU does not resolve the relationships between Acoela, Nemertodermatidae (72% BS, 0.84 ~~Bayesian posterior probability (BPP)~~) and Ascopariidae (84% BS, 1.0 BPP; online resource [45c, d](#)). The Histone 3 gene does not recover any of the genera but *Nemertinoidea* (online resource [45e, f](#)). Most deeper nodes are not resolved and bootstrap values overall very low.

The species relationships within the genera are inconsistent between the gene trees. In the

LSU analyses the cluster *S. boucheti* Meyer-Wachsmuth et al., 2014 + *S. variabilis* Meyer-Wachsmuth et al., 2014 is retrieved as sister group to the clade that includes *S. psammicola* (Faubel, 1976) but in the SSU analyses as sister group to the clade that includes *S. ylva* Meyer-Wachsmuth et al., 2014 in (online resource 45a-d). Similarly are the relationships within *Nemertinoidea* inconsistent between the two datasets. These variances are the same as observed by Meyer-Wachsmuth et al. (2014). Within the genus *Nemertoderma*, both LSU and SSU show two or more clusters, which, however, do not support the two morphological species *N. bathycola* and *N. westbladi*.

On genus level, there are differences in topology within Nemertodermatidae. Analyses of the LSU and the concatenated data recovered *Sterreria* and *Nemertinoidea* as sister groups, a clade also supported by Lundin (2000) and Wallberg (2007). In the SSU analyses, however, this group is not recovered but *Meara* included into it.

Addition of the chimeric sequences to the final datasets changed the estimated topologies. In the LSU dataset the relationships between *Nemertoderma*, *Meara* and the clade *Nemertinoidea* + *Sterreria* was not resolved anymore. The changes estimated in the SSU dataset were more dramatic with *Meara* being retrieved as sister group to a clade *Flagellophora* + *Ascoparia*, the topology suggested by Wallberg et al. (2007) and tested here as hypothesis 3 with the AU test.

The AU test rejected all three alternative hypotheses, i.e. the non-monophyly of Nemertodermatida (hypothesis 1), the monophyly of *Meara* + *Nemertoderma* (hypothesis 2, Lundin 2000), and the monophyly of *Meara* + *Flagellophora* (hypothesis 3, Wallberg et al. 2007) (Tab. 5).

GenBank sequence validation

Four sequences downloaded from GenBank appear to be chimeric and another four are registered under incorrect species names (Tab. 2).

The first about 700bp of the LSU sequence with the accession number AM747472 (Wallberg et al. 2007), registered as *F. apelti*, are more similar to the sequence of the gastrotrich species *Chaetonotus neptuni* registered in GenBank under the accession number JQ798610. Despite a high coverage (99%) and an E-value of 0 the identity is only 90%, i.e. species identity is not clear (Fig. 1a). The rest of the sequence is most similar to the

sequence of *Ascoparia* sp. registered as FR837762 in GenBank (E-value 0, query cover 79%, identity 93%). The sequence AM747478 (Wallberg et al. 2007) is registered as *N. bathycola* in GenBank but the first 678bp appear different in the alignment (Fig. 1b). Blast returned several sequences with low E-values, query coverage of about 61% and identities of around 82% all belonging to rhabdocoel species.

The SSU sequence AM747471 (Wallberg et al. 2007) is registered as *F. apelti* but only the first about 970bp align well with newly sequenced *Flagellophora* sequences. The last about 840bp are more similar to *M. stichopi*, by eye in the alignment (Fig. 1c) as well as in BLAST, where they show E-values of 0 and 99% coverage and identity to several sequences of *M. stichopi*, including the one registered as AM747473. Of the sequence AF051328 (Telford, Lockyer, Cartwright-Finch, & Littlewood 2003), registered as *M. stichopi*, only the last about 390bp match other *M. stichopi* sequences in our preliminary dataset and GenBank (e.g. AF119085, E-value 0, coverage 97%, identity 100%). The first about 1410bp of the sequence BLAST as several different species of copepods, with e-values of 0, coverage of about 96% and identities around 91% (Fig. 1d).

The LSU sequences AM747476 and AM747480 (Wallberg et al. 2007) are registered in GenBank as *Nemertinoidea elongatus* and *Sterreria psammicola*, respectively. In our dataset, however, these sequences cluster with *Sterreria rubra* Meyer-Wachsmuth et al., 2014.

The SSU sequence AM747475 (Wallberg et al. 2007) is registered as *N. elongatus* but is recovered in all analyses as *S. psammicola*, whereas sequence AM747479 (Wallberg et al. 2007) is registered as *S. psammicola* and clusters with *S. rubra*.

Discussion

Data properties

In this dataset the LSU was found to be the most informative of the three genes used, in the shallower (more recent) as well as the deeper nodes. The often used more conserved SSU gene appears less informative according to the PI profiles even in the deeper nodes, where it would have been expected to show higher resolution power. The low resolution power of SSU in the shallower nodes is also confirmed in the topology of the gene trees, where it shows only two clades in the genus *Nemertoderma* while LSU recovers three distinct

clades (by eye). This confirms a previously noted lower resolution power and subsequent underestimation of species diversity in several groups of meiofauna (Tang et al. 2012) and shows that SSU is not a suitable marker for biodiversity studies. The comparatively poor performance of SSU versus LSU is also evident in the PI profiles per site (data not shown).

Our analyses of the ribosomal and concatenated datasets support the same supraspecific groups that were previously identified on the basis of morphological characters (Lundin 2000; Meyer-Wachsmuth et al. 2014; Sterrer 1998). Analysis of the Histone 3 nucleotide sequences failed to recover any genus level or deeper clusters.

Species identities

Relationships within the genera *Sterreria* and *Nemertinoidea* are not fully resolved and in some cases they differ between the gene trees and estimation method. The position of *S. martindalei* Meyer-Wachsmuth et al., 2014, for example, is unresolved as it shifts between being sister group to all other *Sterreria* species in the SSU tree with very low support (48% BS, 0.81 BPP) and sister to the Pacific *Sterreria* subgroup including *S. monolithes* Meyer-Wachsmuth et al., 2014 in the LSU trees, again with very poor support (53% BS, 0.66 BPP). Genetic distances between *S. martindalei* species and any other species are large (17.1% LSU, 14.6% SSU, online resource 6). This is arguably due to incomplete taxon sampling in our dataset. Knowledge of nemertodermatid diversity and distribution in the Pacific is extremely limited, with the whole Pacific being represented in our dataset by only three different collection areas, all of them tropical (Hawaii, Papua New Guinea and New Caledonia). The Indian Ocean is even less known in terms of Nemertodermatida: only one record of Nemertodermatida has ever been published (Toldt 2009) and no specimens from the Indian Ocean could be incorporated into this dataset. More complete geographical and taxon sampling would surely help to resolve species relationships. This is true ~~also even~~ for Europe, arguably the best studies area in terms of nemertodermatid diversity, where no nemertodermatid sequences are available from e.g. the Eastern Mediterranean or the British Isles.

According to current classification, the genus *Nemertoderma* comprises two species, *N. bathycola* and *N. westbladi*. The latter was said to be “one of the best defined species” (Sterrer 1998) of Nemertodermatida. The clusters observed on the LSU and SSU datasets, however, are not congruent with the two morphologically delimited species (Fig. 3). Given

the unresolved position of the nominal species and the apparent presence of at least one additional species level taxa even within the small geographic sampling area on the Swedish west coast, it is clear that the genus *Nemertoderma* requires further study.

Supraspecific groups

In the trees derived from analyses of the LSU and the concatenated datasets the relationships within the family Nemertodermatidae are resolved as (*Nemertoderma* (*Meara*, (*Nemertinoidea* + *Sterreria*))), while analyses of the SSU dataset did not resolve this group (Fig. 3, online resource 45a-d). *Nemertinoidea* and *Sterreria* share five ultrastructural apomorphies (Lundin 2000), with *Meara* they share the apomorphies “testes longitudinal follicles” and “ovaries paired”. In his analyses of morphological data Lundin (2000) recovered *Meara* and *Nemertoderma* as sister groups (hypothesis 2) supported by the two apomorphies “intracellular tonofilament bundles present” and “thick terminal web”. However, this topology was not recovered in any of our gene trees or the concatenated analyses (Fig. 3) and it was rejected by the AU test (Tab. 5). We suggest that the two apomorphies hypothesised by Lundin (2000) for *Meara* + *Nemertoderma* may not be particularly strong as intracellular monofilament bundles should occur in all epithelial cells with desmosomes (Lane 1982) and they may have gone undetected in the micrographs used for coding other nemertodermatid species. The terminal web was scored using the three states “weak”, “distinct, but narrow” or “thick” with all other nemertodermatids scored as “distinct, but narrow”. As the appearance of the terminal web is affected by sample preparation, and as much of the data came from several different studies in the literature, the distinction between the character states used by Lundin (2000) may be vague.

Higher level groups

Wallberg et al. (2007) retrieved a topology in which ~~((*Meara* + *Flagellophora*) + (*Sterreria* + *Nemertinoidea*)) where the position of *Flagellophora* and as sister taxon of *Meara* are sister groups. This~~ conflicts with the classification of Sterrer (1998) and ~~The we never *Flagellophora* + *Meara* group was not recovered it~~ in any of our analyses. Furthermore, nor do these species *Flagellophora* and *Meara* do not share any derived character states in the cladistic analysis by (Lundin, 2000). This group topology was also

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rejected by our AU test (hypothesis 3). Both the LSU and SSU “*Flagellophora*” sequences as well as one of the *Meara* sequences used by Wallberg et al. (2007) can be shown to be chimeric (Fig. 1). The hypothesis of Wallberg et al. (2007) regarding the phylogenetic position of *Flagellophora apelti* and *Meara stichopi* can therefore be rejected as caused by chimeric input sequences (Fig 4).

~~In Sterrer’s (1998) classification the two family level groups, Ascopariidae and Nemertodermatidae, have been introduced based on several light microscopic differences and the same groups were~~ retrieved by Lundin’s cladistic analysis (2000) of morphological and ultrastructural characters. ~~The divergence between these two clades is supported in all our analyses, but the H3 gene analyses. –were recovered as separate clades in our concatenated analysis.~~ However, ~~they Ascopariidae and Nemertodermatidae~~ are only retrieved as sister groups in the LSU dataset (91% BS, 0.99 BPP). ~~In the analyses of the~~ SSU, H3 and concatenated datasets ~~do not resolve~~ the relationships between Nemertodermatidae, Ascopariidae and Acoela ~~are not resolved~~. This prompted us to test for non-monophyly of Nemertodermatida, which, however, was rejected by the AU test. The monophyly of Nemertodermatida has not previously been tested with a comprehensive dataset including representatives of Nemertodermatidae and Ascopariidae as well as Acoela and an appropriate outgroup. Most morphological and molecular studies of Nemertodermatida used only species of Nemertodermatidae, ~~e.g. (e.g. Hooge 2001; Jimenez-Guri, Paps, Garcia-Fernandez, & Salo 2006; Jondelius et al. 2002; Lundin 2001; Lundin & Hendelberg 1998; Raikova, Reuter, Jondelius, & Gustafsson 2000; Ruiz-Trillo et al. 2002; Todt 2009). Only three studies also included Ascopariidae (Boone, Bert, Claeys, Houthoofd, & Artois 2011; Rieger & Ladurner 2003; Wallberg et al. 2007). A striking synapomorphy of Nemertodermatida is the statocyst, which differs from the acoel statocysts not~~ Morphological synapomorphies of the Nemertodermatida to the exclusion of Acoela is e.g. the built of the statocyst. Not only in the number of statoliths ~~in the statoliths differ between Acoela and Nemertodermatida~~ but also ~~in~~ its ultrastructure. In Acoela the lithocyte, the statolith-forming cell, contains the statolith and dorsal to that a nucleus, which can sometimes be seen under the microscope as round structure on the statocyst (Ehlers 1985; Ferrero 1973; Ferrero & Bedini 1991). In Nemertodermatidae the lithocyte dissolves and merges with the extracellular matrix of the vacuole containing the statolith and the lithocyte nucleus is attached to the statolith, ~~and~~ visible as a “blister cap” (Sterrer

1998). In Ascopariidae the statoliths appear smooth, the ultrastructure of the statocyst, however, has not been studied yet.

The present study is the first to focus on the interrelationships of nemertodermatids with molecular means. The classification of Nemertodermatida in the two main groups Ascopariidae and Nemertodermatida (Sterrer 1998) is corroborated by our results. However, both previous phylogenetic hypotheses (Lundin 2001, Wallberg et al. 2007) are falsified. There are still unresolved issues in nemertodermatid evolution: species identities in *Nemertoderma* and *Flagellophora* are unclear and there are indications that these taxa are more diverse than current classification suggests. Overall geographic sampling of Nemertodermatida is very limited (see taxon map at acoela.myspecies.info), and the diversity is likely to be drastically underestimated (Meyer-Wachsmuth et al., 2014). Our phylogenetic hypothesis enables analyses of character evolution and ancestral features within Nemertodermatida, e.g. of nervous system, musculature and reproductive organs. This is of great interest in the study of bilaterian evolution.

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Fig 1 Parts of alignments showing the transition in the chimeric sequences (shown in grey). **a** LSU sequence AM747472 is registered as *Flagellophora apelti* in GenBank. The 5'-part is similar to gastrotrich species, the 3'-part can be identified as *Meara stichopi*. **b** LSU sequence AM747478 registered as *Nemertoderma bathycola* in GenBank but the 3'-part blasts as an unidentified platyhelminth species. **c** SSU sequence AM747471 is registered as *Flagellophora apelti* in GenBank the 3'-part of the sequence shows the same pattern as *Meara stichopi*. **d** SSU sequence AF051328 is registered as *M. stichopi* but the 5'-part BLASTs as copepod species

Fig 2 Phylogenetic informativeness profiles (net) of all three genes used in this study. The X-axis indicates time, but is in absence of calibration points relative to node depths. The profile is drawn over the ultrametric tree from which it was calculated

Fig 3 Majority rule consensus tree estimated using RAxML from the concatenated dataset. Bootstrap support values and Bayesian posterior probabilities are plotted on those nodes retrieved in both analyses. Coloured boxes correspond to genera of the ingroup, asterisks indicate species downloaded from GenBank (ingroup only)

Fig 4 LSU-(a) and SSU-(b) gene trees of the final datasets plus the chimeric sequences. The colours correspond to those in Fig. 3, chimeric sequences are highlighted in grey/blue. In both the LSU and SSU datasets the genera *Flagellophora* and *Meara* are pulled artificially closer together by the chimeric sequences (cf. gene trees)

Table 1 Primers used in this study for sequencing of LSU, SSU and H3 markers. TimA and TimB are outer primers spanning the length of the whole fragment, the primer pairs S30/5FK and 4FB/1806R are internal primer for the 5'- and 3'-part, respectively

Gene	Name	dir.	taxon	sequence	reference
28S	U178	for	<i>Nemertoderma, Meara</i>	GCACCCGCTGAAYTTAAG	Telford et al. 2003
	L1642	rev	<i>Nemertoderma, Meara</i>	CGAGCGCCATCCATTTTCA	Telford et al. 2003
	1200F	for	<i>Nemertoderma, Meara</i>	CCCGAAAGATGGTGAACATATGC	Telford et al. 2003
	R2450	rev	<i>Nemertoderma, Meara</i>		Telford et al. 2003
	UJ2176	for	<i>Nemertoderma, Meara</i>	TAAGGGAAGTCGGCAAATTAGATCCG	Wallberg et al 2007
	L3449	rev	<i>Nemertoderma, Meara</i>	ATTCTGACTTAGAGGCGTTCA	Telford et al. 2003
	U1846	for	<i>Nemertoderma, Meara</i>	AGGCCGAAGTGGAGAAGG	Telford et al. 2003
	L2984	rev	<i>Nemertoderma, Meara</i>		
	28SP1F5 Ster	for	<i>Nemertinoidea, Sterreria</i>	CTGAGAAGGGTGTGAGACCCGTAC	Meyer-Wachsmuth et al. 2014
	28SP1R1 Ster	rev	<i>Nemertinoidea, Sterreria</i>	TCCCGTAGATCCGATGAGCGTC	Meyer-Wachsmuth et al. 2014
18S	TimA	for	all	AMCTGGTTGATCCTGCCAG	Norén & Jondelius 1999
	TimB	rev	all	TGATCCATCTGCAGGTTACCT	Norén & Jondelius 1999
	S30	for	all except <i>Flagellophora</i>	GCTTGTCTCAAAGATTAAGCC	Norén & Jondelius 1999
	18SF1_apelti	for	<i>Flagellophora</i>		this publication
	5FK	rev	all	TTCTTGCCAAATGCTTTCGC	Norén & Jondelius 1999
	4FB	for	all except <i>Flagellophora</i>	CCAGCAGCCGCGTAATTCCAG	Norén & Jondelius 1999
	4FB_apelti	for	<i>Flagellophora</i>	CGCTCGTAGTTGGATCTCTCG	this publication
H3	1806R	rev	all	CCTTGTTACGACTTTTACTTCCTC	Norén & Jondelius 1999
	H3 AF	for	all	ATGGCTCGTACCAAGCAGACVGC	Colgan et al. 1998
	H3 AR	rev	all	ATATCCTTRGGCATRATRTGTGAC	Colgan et al. 1998
	H3FNem	for	all	ATGGCTCGTACCAAGCAGACG	Meyer-Wachsmuth et al. 2014
	H3RNem	rev	all	TCCTTGGGCATGATGGTGAC	Meyer-Wachsmuth et al. 2014

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Table 2 Sequences used in the phylogenetic analyses, rows indicate how GenBank sequences were combined for concatenated analyses. Asterisks indicate ~~new previously published~~ sequences published first in this study~~downloaded from GenBank~~, † chimeric sequences and # sequences ~~which that~~ cluster with a different species than specified in GenBank

species	accession number		
	LSU	SSU	H3
outgroup			
<i>Diopisthoporus longitubus</i> (06006)	FR837775.1	FR837692.1	
<i>Paraphanostoma submaculatum</i>			AJ849506.1
<i>Paratomella rubra</i> (11085)	*KT698949	*KT698957	
<i>Chaetoderma nitidulum</i>		AY377658.1	AY377763.1
<i>Chaetoderma</i> sp.	AY145397.1		
<i>Dumetrocrinus antarcticus</i>		KC616753.1	
<i>Balanoglossus clavigerus</i>			1/FN908667.1
<i>Ptychodera flava</i>	2/AF212176.1		
<i>Ptychodera bahamensis</i>		2/AF236802.1	
<i>Saccoglossus kowalevskii</i>	1/AF212175.1		
<i>Lepidopleurus cajetanus</i>	FJ445776.1	AF120502.1	KF527282.1
ingroup			
<i>Ascoparia</i> sp. (AWHel-24)	FR837762.1	FR837678.1	
<i>Flagellophora apelti</i>	†AM747472.1	†AM747471.1	
<i>Meara stichopi</i> (03052)	FR837797.1	FR837714	
<i>Meara stichopi</i> (1)	AY157605	AF100191.2	
<i>Meara stichopi</i> (2)	AY218125	†AF051328	AY218147.1
<i>Meara stichopi</i> (3)	AM747474.1	AM747473.1	
<i>Nemertinoides elongatus</i> (1)	AF021326	AY078381.1	
<i>Nemertinoides elongatus</i> (2)	#AM747476.1 (<i>S. rubra</i>)	#AM747475.1 (<i>S. psammicola</i>)	
<i>Nemertoderma bathycola</i> (1)		AF327725.1	
<i>Nemertoderma bathycola</i> (2)	†AM747478.1	AM747477.1	
<i>Nemertoderma</i> sp. (06016)	FR837800.1	FR837717.1	
<i>Nemertoderma westbladi</i> (1)	AM747482.1	AM747481.1	
<i>Nemertoderma westbladi</i> (2)		AF327726.1	
<i>Sterreria psammicola</i>	#AM747480.1 (<i>S. rubra</i>)	#AM747479.1 (<i>S. rubra</i>)	
<i>Sterreria psammicola</i> (07006)	KM062708	KM062542	
<i>Nemertinoides wolfgangi</i> (08096)	KM062733	KM062568	KM194628
<i>Nemertinoides</i> sp. N3 (08098)	KM062734	KM062569	KM194629
<i>Nemertinoides</i> sp. N1 (08102)	KM062736	KM062571	
<i>Nemertinoides</i> sp. N2 (08123)	KM062748	KM062583	
<i>Sterreria ylvae</i> (10058)	KM062773	KM062607	KM194650
<i>Sterreria martindalei</i> (10060)	KM062774	KM062608	KM194651
<i>Sterreria lundini</i> (10076)	KM062779	KM062613	
<i>Nemertoderma bathycola</i> (12116)	*KT698950	*KT698958	
<i>Nemertoderma westbladi</i> (12122)	*KT698951	*KT698959	
<i>Nemertoderma bathycola</i> (12220)	*KT698952	*KT698960	

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<u>Nemertoderma westbladi (12222)</u>	<u>*KT698953</u>	<u>*KT698961</u>	<u>*KT698965</u>
<u>Nemertoderma sp. (12231)</u>	<u>*KT698954</u>	<u>*KT698962</u>	<u>*KT698966</u>
<u>Flagellophora apelti (13110)</u>	<u>*KT698955</u>	<u>*KT698963</u>	<u>*KT698967</u>
<u>Sterreria rubra (13148)</u>	<u>KM062809</u>	<u>KM062643</u>	<u>KM194672</u>
<u>Sterreria psammicola (13155)</u>			<u>KM194673</u>
<u>Sterreria variabilis (13156)</u>	<u>KM062811</u>	<u>KM062645</u>	<u>KM194674</u>
<u>Sterreria sp. S2 (13157)</u>	<u>KM062812</u>	<u>KM062646</u>	<u>KM194675</u>
<u>Nemertinoides elongatus (13180)</u>	<u>KM062816</u>	<u>KM062649</u>	<u>KM194677</u>
<u>Nemertinoides glandulosum (13181)</u>	<u>KM062817</u>	<u>KM062650</u>	<u>KM194678</u>
<u>Nemertoderma sp. (13330)</u>	<u>*KT698956</u>	<u>*KT698964</u>	<u>*KT698968</u>
<u>Meara stichopi (A)</u>	<u>KM062846</u>	<u>KM062678</u>	<u>KM194693</u>
<u>Nemertinoides sp. N4 (MCG09)</u>	<u>KM062839</u>	<u>KM062672</u>	<u>KM194689</u>
<u>Sterreria sp. S3 (MCG12)</u>	<u>KM062842</u>	<u>KM062675</u>	
<u>Sterreria papuensis (PNG52)</u>	<u>KM062853</u>	<u>KM062685</u>	<u>KM194696</u>
<u>Sterreria sp. P3 (PNG60)</u>	<u>KM062858</u>	<u>KM062690</u>	<u>KM194699</u>
<u>Sterreria sp. S7 (PNG67)</u>	<u>KM062861</u>	<u>KM062693</u>	<u>KM194703</u>
<u>Sterreria monoliths (PNG84)</u>	<u>KM062870</u>	<u>KM062702</u>	<u>KM194710</u>
<u>Sterreria boucheti (PNG87)</u>	<u>KM062872</u>	<u>KM062704</u>	<u>KM194712</u>

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Table 3 Values of parameters estimated with jModeltest based on BIC for the proportion of invariable sites (I) and gamma distribution of rates across sites (G) as well as their importance. The priors used for MrBayes analyses based on the given estimates are shown

	I	importance (I/I+G)	G	importance (G/I+G)	MrBayes
LSU	0.2250	0/1.0	0.5269	0/1.0	shapepr=Uniform(0.10,0.70) pinvarpr=Uniform(0.01,0.5)
SSU	0.2957	0/1.0	0.4802	0/1.0	shapepr=Uniform(0.10,0.70) pinvarpr=Uniform(0.01,0.5)
H3	0.5181	0/1.0	1.0902	0/1.0	shapepr=Uniform(0.40,1.30) pinvarpr=Uniform(0.01,0.8)

Table 4 Observed nucleotide frequencies and GC-content averaged per lineage for all three genes used in this study

	T	C	A	G	GC-content
LSU					
<i>Sterreria</i>	21.58	23.56	23.08	31.77	55.34
<i>Nemertinoidea</i>	21.26	24.36	22.51	31.87	56.23
<i>Meara</i>	20.60	24.12	22.63	32.65	56.77
<i>Nemertoderma</i>	23.55	22.49	23.93	30.03	52.52
<i>Flagellophora</i>	20.7	25.3	21.6	32.4	57.8
<i>Ascoparia</i>	20.5	26.3	20.8	32.4	58.7
SSU					
<i>Sterreria</i>	24.44	22.46	25.25	27.86	50.32
<i>Nemertinoidea</i>	21.8	24.1	22.8	31.3	50.23
<i>Meara</i>	22.3	24.3	23.0	30.5	51.6
<i>Nemertoderma</i>	25.38	21.53	25.92	27.17	48.7
<i>Flagellophora</i>	24.1	24.3	23.7	27.9	52.2
<i>Ascoparia</i>	24.5	23.6	25.3	26.6	50.2
H3					
<i>Sterreria</i>	19.0	28.7	26.9	25.4	54.1
<i>Nemertinoidea</i>	17.5	28.8	27.1	26.5	55.3
<i>Meara</i>	17.1	28.4	29.5	25.0	53.4
<i>Nemertoderma</i>	21.9	28.9	24.3	24.9	53.8
<i>Flagellophora</i>	26.8	26.8	20.4	26.0	52.8
<i>Ascoparia</i>	X	X	X	X	X

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Table 5 Results of the Approximately Unbiased test for the comparison of topologies performed by the program Consel, including other statistical tests of tree comparison. The best tree inferred from the concatenated dataset without constraint was compared to the best trees calculated from the same dataset with alternative topological constraints. (AU: Approximately Unbiased test; BP: Bootstrap probability of the selection; PP: Bayesian Posterior Probability calculated with the Bayesian Information Criterion)

topological constraint	AU	BP	PP
no constraint	0.942	0.905	1.0
non-monophyly of Nemertodermatida	0.008	0.006	0
<i>Meara</i> + <i>Nemertoderma</i> (Lundin 2000)	0	0	0
<i>Meara</i> + <i>Flagellophora</i> (Wallberg et al. 2007)	0	0	0

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7 674 **Online Resource 1** Observed nucleotide frequencies and GC-content per specimen for all
8 675 three genes used in this study and the codon positions of the protein-coding gene Histone
9 676 3. Species names highlighted in red have greatly varying sequence lengths, which may
10 677 have affected the observed nucleotide bias
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14 679 **Online Resource 2** The estimates of the disparity index for all three genes. The Disparity
15 680 Indices per-site are shown for each sequence pair above the diagonal. Below, the p-values
16 681 of the Disparity Indices are shown, those smaller than 0.05 are considered significant.
17 682 Evolutionary analyses were conducted in MEGA5.2.2.
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21 684 **Online Resource 3** Comparison of the topologies of trees estimated from the original (left)
22 685 and Aliscore-reduced (right) SSU datasets. The differing nodes are marked
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24 686
25 687 **Online Resource 4** Saturation plots for all three genes used in this study and for the codon
26 688 positions of H3. Histone 3, and especially its 3rd codon position, show patterns indicating
27 689 saturation
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31 691 **Online Resource 5** Gene trees for LSU, SSU and H3. Maximum Likelihood (ML) trees
32 692 were estimated with RAxML and Bayesian inferences (MB) were performed with
33 693 MrBayes. The colours correspond to genera, and in case of Ascopariidae to the family. a)
34 694 ML LSU. b) MB LSU. c) ML SSU. d) MB SSU. e) ML H3. f) MB H3
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38 696 **Online Resource 6** Pairwise distances across the a) LSU, b) SSU and c) Histone 3 gene
39 697 datasets
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