Molecular phylogenetic analyses of Bryozoa, Brachiopoda, and Phoronida

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"The affinities of all the beings of the same class have sometimes been represented by a great tree. [...] As buds give rise by growth to fresh buds, and these, if vigorous, branch out and overtop on all sides many a feebler branch, so by generation I believe it has been with the great Tree of Life, which fills with its dead and broken branches the crust of the earth, and covers the surface with its ever branching and beautiful ramifications."

—Charles Darwin, *On The Origin Of Species* (1959)

Summary

The present thesis focuses on molecular and computational analyses to elucidate the phylogenetic position of the lophophorate lineages, i.e., ectoproct bryozoans, brachiopods, and phoronids. Its main section is organized in chapters corresponding to manuscripts that have been published in or submitted to scientific journals.

For the first manuscript, "*Multigene analysis of lophophorate and chaetognath phylogenetic relationships*", seven nuclear housekeeping gene fragments of seven representatives of ectoproct bryozoans, brachiopods, phoronids, and chaetognaths were PCR amplified and sequenced. According to phylogenetic analyses based on this dataset — and strongly supported by topology tests — the lophophorate lineages are more closely related to molluscs and annelids than to deuterostomes. While this study also suggests that they are polyphyletic, the data was neither sufficient to place chaetognaths, nor to robustly resolve the phylogenetic relations among lophophorates or among lophotrochozoans in general.

Consequently, this approach was abandoned in favour of EST sequencing. More than 4000 expressed sequence tags (ESTs) of the cheilostome ectoproct *Flustra foliacea* were incorporated into a second study, "*Spiralian phylogenomics supports the resurrection of Bryozoa comprising Ectoprocta and Entoprocta.*" Accessing additional EST projects and public archives, a super-alignment derived from 79 ribosomal protein gene sequences of 38 metazoan taxa was compiled. Maximum likelihood and Bayesian inference analyses based on this dataset indicate the monophyly of Bryozoa including ectoprocts and entoprocts — two taxa that have been separated for more than a century due to seemingly profound morphological differences. These and other findings suggest that classical developmental and morphological key characters such as cleavage pattern, coelomic cavities, gut architecture and body segmentation are subject to greater evolutionary plasticity than traditionally assumed.

This dataset was further complemented by 2000 ESTs each of the craniiform brachiopod *Novocrania anomala* and the phoronid *Phoronis muelleri*, leading to the publication of the third study, "*Phylogenomic analyses of lophophorates (brachiopods, phoronids and bryozoans) confirm the Lophotrochozoa concept.*" According to this analysis, all three lophophorate lineages are clearly to be placed within

Lophotrochozoa. Their monophyly, however, was not recovered; instead, ectoprocts and entoprocts presumably branch off at the lophotrochozoan base, while brachiopods and phoronids, robustly united to Brachiozoa, appear to be more closely allied to molluscs, annelids, and nemertines. These results are congruent with recent and careful re-evaluations of morphological characters traditionally used to unite lophophorate taxa with deuterostomes, e.g., archimery, possession of a mesodermal tentacular apparatus and the mode of mesoderm formation.

With robust interphyletic resolution still lacking, additional EST projects were performed to improve the taxon sampling within Lophotrochozoa. A total of 2000 ESTs each of the cyclostome bryozoan *Tubulipora sp.* and the ctenostome bryozoan *Alcyonidium diaphanum* were generated for the study "*Reducing compositional heterogeneity improves phylogenomic inference of lophotrochozoan relationships.*" Again, ribosomal protein sequences were retrieved and supplemented by all data available of bryozoan, brachiopod, and phoronid taxa to date. To mitigate the potential impact of compositional heterogeneity displayed by metazoan taxa, several approaches were applied to reduce this trait. Among these, recoding amino acids into groups of functional interchangeability proved to be the most efficient, and provides further evidence for the monophyly of Bryozoa and Brachiozoa. Although internal relations of both taxa could also be elucidated, most interphyletic relationships within Lophotrochozoa remain nevertheless poorly supported, nourishing the idea that this group underwent a rapid series of cladogenetic events in the Precambrium.

As paralogy has been identified as another pitfall of phylogenetic inference, a novel, phylogenetic approach to evaluate gene homology relations is finally proposed in „*Tree-based orthology assessment illustrated by the evaluation of ribosomal protein genes.*" By reconstructing gene trees of ribosomal proteins gathered from genomic datasets using an automated pipeline, and assigning each gene to one of three categories representing varying degrees of evidence for orthology or paralogy, most ribosomal protein genes were identified as suitable for the reconstruction of bilaterian phylogeny. A final, comprehensive phylogenetic analysis restricted to these genes confirms the central results of the previous phylogenetic studies, emphasising that these were not misled by artefacts related to paralogy.

Zusammenfassung

Ziel der vorliegenden Arbeit ist mittels molekulargenetischer Analysen die phylogenetische Stellung der lophophoraten Linien, d.h. der ektoprokten Bryozoen, der Brachiopoden und der Phoroniden, aufzudecken. Der zentrale Forschungsbericht ist in Kapitel gegliedert, die in Fachzeitschriften publizierten oder zur Veröffentlichung eingereichten Manuskripten entsprechen.

Im Rahmen der ersten Studie, "*Multigene analysis of lophophorate and chaetognath phylogenetic relationships*", wurden partielle Sequenzen von sieben nukleären Haushaltsgenen mittels PCR in sieben Vertretern der ektoprokten Bryozoen, Brachiopoden, Phoroniden und Chaetognathen bestimmt. Den phylogenetischen Analysen dieses Datensatzes zufolge — und gut gestützt durch Topologie-Tests — sind die lophophoraten Linien näher mit Mollusken und Anneliden verwandt als mit Deuterstomiern. Zwar legt die Studie auch die Polyphylie dieser Taxa nahe, jedoch erwiesen sich die Daten sowohl als ungenügend, die phylogenetische Position der Chaetognathen zu bestimmen, als auch die verwandtschaftlichen Beziehungen zwischen den Lophophoraten oder den Lophotrochozoen im Allgemeinen aufzuklären.

Infolgedessen wurde dieser Ansatz zugunsten der EST-Technik verworfen. Mehr als 4000 "Expressed Sequence Tags" (ESTs) des cheilostomen Ektoprokten *Flustra foliacea* flossen in eine zweite Studie ein, "*Spiralian phylogenomics supports the resurrection of Bryozoa comprising Ectoprocta and Entoprocta.*" Unter Einsatz zusätzlicher EST-Projekte und Zugriff auf öffentliche Datenbanken wurde ein Alignment erstellt, das Sequenzen von 79 ribosomalen Proteinen aus 38 Taxa enthielt. Maximum-Likelihood und Bayes'sche Analysen basierend auf diesem Datensatz zeigen die Monophylie der Bryozoa einschließlich Ectoprocta und Entoprocta, zweier Taxa, die aufgrund scheinbar tief greifender morphologischer Unterschiede vor über einem Jahrhundert getrennt wurden. Diese und andere Ergebnisse legen nahe, dass klassische ontogenetische und morphologische Schlüssel-Merkmale wie Furchungsmuster, Coelomräume, Architektur des Darms und Segmentierung im Lauf der Evolution Gegenstand größerer Plastizität sind als traditionell angenommen.

Die Erweiterung dieses Datensatzes um jeweils 2000 ESTs des craniiformen Brachiopoden *Novocrania anomala* und des Phoroniden *Phoronis muelleri* führte zur Publikation einer dritten Studie, "*Phylogenomic analyses of lophophorates (brachiopods, phoronids and bryozoans) confirm the Lophotrochozoa concept.*" Dieser Untersuchung zufolge müssen alle drei lophophoraten Linien eindeutig innerhalb der Lophotrochozoa platziert werden. Deren Monophylie konnte jedoch nicht bestätigt werden; stattdessen zweigen Ekto- und Entoprokten vermutlich an der Basis der Lophotrochozoen ab, während die robust zu Brachiozoa vereinigten Brachiopoden und Phoroniden näher mit Anneliden, Mollusken und Nemertinen verwandt zu sein scheinen. Diese Ergebnisse sind kongruent zu sorgfältigen Neubewertungen jener morphologischer Merkmale, die traditionell verwendet werden, um die nähere Verwandtschaft der Lophophoraten zu den Deuterostomiern zu untermauern, z.B. Archimerie, der Besitz eines mesodermalen Tentakel-Apparats und der Modus der Mesoderm-Bildung.

Nachdem eine robuste Auflösung zwischen den Stämmen noch immer nicht erreicht wurde, wurden weitere EST-Projekte durchgeführt, um die Zahl der Taxa zu erhöhen. Insgesamt jeweils 2000 ESTs des cyclostomen Bryozoen *Tubulipora sp.* und des ctenostomen Bryozoen *Alcyonidium diaphanum* wurden für die Studie "*Reducing compositional heterogeneity improves phylogenomic inference of lophotrochozoan relationships*" erhoben. Wie zuvor wurden ribosomale Protein-Sequenzen erfasst und durch entsprechende Daten aller bis dato verfügbaren Bryozoen, Brachiopoden und Phoroniden ergänzt. Um den potentiellen Einfluss heterogener Aminosäure-Zusammensetzung zu mindern, wurden mehrere Ansätze verfolgt. Am effizientesten erwies sich die Rekodierung der Aminosäuren in Gruppen funktioneller Ähnlichkeit, wodurch weitere Belege für die Monophylie der Bryozoen und der Brachiozoen erbracht werden konnten. Obwohl Verwandtschaftsverhältnisse innerhalb beider Taxa ebenfalls beleuchtet werden konnten, bleiben die Beziehungen zwischen den Stämmen der Lophotrochozoen dennoch schlecht unterstützt, was die Vorstellung nährt, dass diese Gruppe im Präkambrium durch eine schnelle Folge kladogenetischer Ereignisse entstand.

Da Paralogie eine weiteres Problem in der phylogenetischen Rekonstruktion darstellt, wurde in „*Tree-based orthology assessment illustrated by the evaluation of ribosomal protein genes*" ein neuartiger, phylogenetischer Ansatz zur Evaluation von Homologie-Verhältnissen von Genen vorgestellt. Mithilfe

eines automatisierten Arbeitsablaufs wurden Gen-Bäume ribosomaler Proteine rekonstruiert, und jedes Gen einer von dreien Kategorien zugeteilt, die Grade unterschiedlicher Beweiskraft für Orthologie oder Paralogie repräsentieren. Dadurch konnte der Großteil der ribosomalen Proteine als geeignet identifiziert werden, die Stammesgeschichte der Bilateria zu untersuchen. Eine abschließende, umfassende phylogenetische Analyse, die sich auf diese Gene beschränkt, bestätigt die zentralen Ergebnisse der vorherigen Studien und zeigt, dass diese nicht durch paraloge Genkopien beeinflusst wurden.

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1. Introduction

Resolving the phylogenetic relationships of the animal phyla is an important prerequisite to understand many aspects central to modern biology. Knowledge of the animal kingdom's evolutionary history will provide insights into underlying internal and external processes, e.g., how the diversification of body plans has been shaped by genetic innovation, embryonic development, and palaeoecological conditions. The present work aims to contribute to this goal by investigating the phylogenetic position of Bryozoa, Brachiopoda, and Phoronida, collectively known as lophophorate lineages. Below, general information about these enigmatic taxa is provided, followed by an introduction to the conflicting views concerning their phylogeny, and a list of data collected for this study. Finally, the objectives of this work are formulated.

1.1. The lophophorate lineages

1.1.1. Ectoproct Bryozoa

Ectoproct bryozoans or moss animals comprise a moderately speciose phylum of aquatic, sessile animals that are organized exclusively in colonies. The group is predominantly marine, although it includes a minority of freshwater and estuarine species. Bryozoans are surprisingly common animals occurring worldwide, and form a notable part of the hard substratum epifauna on most rocky shores. Although they are especially abundant in shallow sublittoral habitats, some species have been found to inhabit deep-sea environments as well. Many species form encrusting sheets on stones, shells or kelp blades, while others develop erect, dendritic or lobate colonies reminiscent of corals or algae (Fig. 1a–b). Each colony consists of minuscule individuals — the zooids — that arise through budding from an ancestral zooid resulting from a sexually produced, metamorphosed larva. The number of zooids that make up a colony can reach millions. While colonies range in size from millimetres to metres, a single zooid is usually less than a millimetre long. Each zooid is encased in a gelatinous, chitinous or calcareous exoskeleton, the zooecium, which can be intricately structured by pores, ridges and spines, and in its entirety forms the

colonial skeleton. Individual zooids consist of two parts: the cystid, a box- or tube-shaped lower body encased in a usually rather stiff body wall, and a retractable upper body or polypide. The latter is composed of the U-shaped gut and a ciliated ring of tentacles surrounding the mouth opening, the lophophore. This current-producing structure is used by the animals to filter food particles including diatoms and other unicellular algae from the water column, but also serves respiratory functions. Many bryozoan species are characterized by zooid polymorphism, and display a bewildering variety of heterozooids specialized in reproduction, defence or cleaning, which are dependant on food-gathering autozooids for nourishment. Over 5000 extant species of ectoproct bryozoans are known, and there is an extensive fossil record dating back to the Lower Ordovician. However, the actual number of species is supposed to be twice as big (Hayward and Ryland, 1998). Living bryozoans can be classified into the following major groups (Ax, 2001): Phylactolaemata, a small group of putatively primitive freshwater forms, Stenolaemata, whose only extant member Cyclostomata is characterized by cylindrical, calcified zooids, and the speciose Eurystomata, which can be further divided into the uncalcified Ctenostomata and the typically box-shaped, calcified Cheilostomata.

1.1.2. Brachiopoda

Brachiopods or lamp shells are a small phylum of exclusively marine, sessile and solitary invertebrates. Superficially, most resemble clams due to their two-valved calcareous shell, but in contrast to the molluscs' lateral symmetry, brachiopods possess a dorsal and a ventral valve of usually different shape. The valves are lined and secreted by the mantle folds, and are held together by muscles and a hinge in most species („articulate" brachiopods). Apart from some burrowing species, they live attached to rocky substrate or coarse sediment by means of a fleshy stalk, the pedicle, and filter food particles by opening their valves and drawing water into a cavity enclosing the lophophore, a coiled pair of tentacle-bearing arms. No habitual predators of brachiopods are known, although their shells are often damaged by boring carnivorous gastropods, or boring or encrusting sponges and bryozoans seeking habitation substrate. Brachiopods reproduce exclusively sexually, and possess predominantly discrete genders; fertilization outside the body is the norm, as are free-swimming, highly derived larvae. The animals can

be found from polar seas to tropical reefs, and from intertidal environments to abyssal depths. However, in terms of abundance and species diversity they reach a peak at the continental slopes. While there are only about living 300 species described, tens of thousands of fossil forms dating back to the Lower Cambrian are known, underlining that brachiopods constituted an important faunal element of many palaeozoic ecosystems (Brunton and Curry, 1979). The group has been divided into three subphyla (Nielsen, 2001): the primitive, burrowing Linguliformea, the cemented Craniformea (Fig. 1d), and the largest and most diverse group, the articulate Rhychonelliformea.

1.1.3. Phoronida

Phoronids or horseshoe worms are marine, sedentary, worm-like animals that occupy tubes buried vertically in mud, sand or borings in hard substrate. These chitinous tubes are secreted and often covered by incorporated sand grains and fragments of other materials. Posteriorly, the millimetre-thin body widens into a bulb used for anchorage, while the anterior end bears the conspicuous lophophore (Fig. 1d). This organ varies considerably between species, ranging from simple, oval designs carrying but few tentacles to intricate helicoidal structures supporting thousands of tentacles, and can be spread for feeding or folded when the animal retracts into its tube. Like the other lophophore-bearing lineages, phoronids feed on phytoplankton and detritus particles, which are transported by ciliary action through the mouth opening at the bottom of the lophophoral cavity into the eponymous, U-shaped digestive tract. Conversely, they are probably preyed upon by fishes, gastropods and nematodes. Phoronids reproduce sexually, either as hermaphrodites, or dioeciously. Different types of development are known, the most prominent including a prolonged pelagic life stage as a characteristic actinotroch larva that ends with a rapid, „catastrophic“ metamorphosis. Phoronids constitute one of the smallest animal phyla, with about 20 species known today and virtually no reliable fossil record. However, most species are probably cosmopolitan, and can become very abundant in favourable conditions of the intertidal zone to about 200 m depth, with thousands of individuals per m^2 (Emig, 1979).

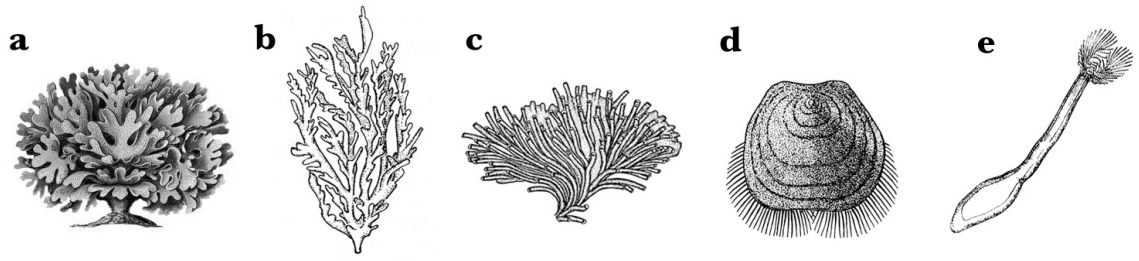

Figure 1. The lophophorate lineages illustrated by species used in this study to generate EST data: the ecotproct bryozoans *Flustra foliacea* (a), *Alcyonidium diaphanum* (b), and *Tubulipora sp.* (c), as well as the brachiopod *Novocrania anomala* (d) and the phoronid *Phoronis muelleri* (e). Drawings are not to scale, and were taken from Haeckel (1904) and Hayward and Ryland (1995).

1.2. Phylogenetic hypotheses

1.2.1. Traditional perspective

Ectoproct bryozoans, brachiopods, and phoronids have early been grouped together as Tentaculata (Hatschek, 1891) or Lophophorata (Hyman, 1959) based on morphological and embryological similarities. These characters include the eponymous lophophore, a ciliated tentacular feeding apparatus shared by all lophophorate taxa, a putatively tripartite body organization with three distinct coelomic cavities, namely protocoel, mesocoel and metacoel (archimery), and mesoderm formation by enterocoely. The same characters are supposed to be autapomorphies of Radialia, a group uniting deuterostomes and the lophophorate lineages, with the latter constituting either the sister or paraphyletic stem group of the former (Ax, 1995; Lüter and Bartolomaeus, 1997; Brusca and Brusca, 2003). This assumption is sustained by the radial cleavage pattern observable in brachiopods and phoronids, an allegedly plesiomorphic character state of Deuterostomia (Lüter and Bartolomaeus, 1997). Nielsen (2001) also followed this argumentation, and retains brachiopods plus phoronids within Radialia. However, he claims lophophorate polyphyly by placing ectoproct bryozoans next to entoprocts among protostome animals on the basis of cleavage pattern, ciliary structure and larval morphology. Ultimately, the mixture of protostome and deuterostome features displayed by lophophorate taxa, particularly ectoprocts, makes it unlikely that the origin of these lineages can be inferred by traditional, morphological characters alone.

1.2.2. Modern view

The advent of molecular tools in phylogenetics twenty years ago has unveiled a scenario of animal evolution profoundly at conflict with the traditional perspective. Besides refuting the concepts of Articulata and Coelomata, one of the most striking rearrangements brought by this new animal phylogeny concerns the position of the lophophorate lineages (Halanych, 2004). Using 18S ribosomal DNA sequences, Halanych et al. (1995) first provided evidence for a closer relationship of the lophophorate taxa to molluscs and annelids than to deuterostomes. Based on these results, the node-based name Lophotrochozoa was proposed for the group comprising „the last common ancestor of the three traditional lophophorate taxa, the mollusks, and the annelids, and all of the descendants of that common ancestor". Later studies employing 18S and/or 28S rDNA sequences confirmed the existence of this clade to the exclusion of taxa placed within Ecdysozoa (moulting animals, i.e., arthropods, nematodes, and kin; Aguinaldo et al., 1997) or Deuterostomia (Mackey et al, 1996; Giribet et al., 2000; Peterson and Eernisse, 2001; Mallatt and Winchell, 2002; Passamaneck and Halanych, 2006). A range of independent data sources including *hox* genes (de Rosa et al., 1999; Passamaneck and Halanych, 2004), *myosin* (Ruiz-Trillo et al., 2002), *ATPase* (Anderson et al., 2004) and mitochondrial protein sequences (Stechmann and Schlegel, 1999; Helfenbein and Boore, 2004; Waeschenbach et al., 2006) leading to the same conclusion have further increased confidence into the lophotrochozoan affinities of the lophophorate lineages. However, although molecular evidence for the Lophotrochozoa concept is unequivocal, lack of resolution and incongruency plague the exploration of lophotrochozoan relationships and the position of the lophophorates (e.g., Passamaneck and Halanych, 2006). The majority of molecular analyses using various markers have argued against lophophorate monophyly, but do not agree on the exact relationships except for usually favouring the monophyly of brachiopods and phoronids to the exclusion of bryozoans (e.g., Cohen, 2000; Giribet et al., 2000; Anderson et al., 2004; but see Ruiz-Trillo et al., 2002; Passamaneck and Halanych, 2006).

In conclusion, the precise phylogenetic relationships of ectoproct Bryozoa, Brachiopoda and Phoronida were unknown at the beginning of this study, although the uncertainty surrounding their deuterostome or protostome affinities makes them pivotal for the understanding of animal evolution. The

incomplete resolution of this and other parts of the animal tree of life has made it increasingly clear that both traditional morphological characters and single genes lack the resolving power to robustly infer phylogenetic relationships at the depth of phyla (Adoutte et al., 2000). In this study, efforts were therefore made to procure and analyse a larger number of genes from lophophorate and other taxa. Approaches to do so included the targeted amplification of multiple genes by PCR, and the generation of EST data from selected taxa, which is to date the most economical method to obtain large amounts of data for the purpose of molecular systematics (Philippe and Telford, 2006).

1.3. Study taxa and data collection

Representatives of each lophophorate lineage and — where applicable — its major higher-level taxa were selected to study the phylogenetic position of these groups. Table 1 displays all species for which ESTs were generated in the course of this study. For initial analyses, genetic data was also collected from the phylactolaemate bryozoan *Plumatella repens*, and the rhynchonelliform brachiopod *Terebratulina retusa* (not shown).

Table 1. Details of the EST projects conducted during this study, including the higher-level taxa represented by the study species, the number of single reads generated, the number of contigs assembled from these reads, and the number of ribosomal protein genes that could be retrieved from each dataset (all phylogenetic analyses in this study using EST data were based on this class of genes). Illustrations of the study species can be found in Figure 1.

Species	Taxon	No. Reads	No. Contigs	No. RP
Flustra foliacea	Ectoprocta (Cheilostomata)	4075	1755	75
Alcyonidium diaphanum	Ectoprocta (Ctenostomata)	2331	1369	74
Tubulipora sp.	Ectoprocta (Stenolaemata)	2040	1375	49
Novocrania anomala	Brachiopoda	2247	1699	43
Phoronis muelleri	Phoronida	2315	1467	55

1.4. Objectives

The objectives of this study can be summarized as follows:

- Clarify whether ectoproct bryozoans, brachiopods and phoronids are more closely related to deuterostomes (Radialia concept) or to molluscs, annelids, and allies (Lophotrochozoa concept)
- Investigate whether „Lophophorata“ is a valid monophyletic taxon, or a para- or polyphyletic grouping
- Identify the sister taxon of each of the three lophophorate lineages, and their exact phylogenetic position in the animal tree of life
- Contribute to resolving the internal phylogeny of ectoproct bryozoans and brachiopods
- Improve the general resolution of bilaterian phylogeny, especially within Lophotrochozoa
- Develop strategies to reduce the impact of systematic errors on deep phylogenetic analyses, e.g., due to compositional bias and paralogy

2. Research report

The following chapters reproduce the major research results that have been obtained during this study. Each corresponds to an article that has been published in a scientific journal, or a manuscript that has recently been submitted for publication. The final chapter summarizes the results of these articles.

2.1. Multigene analysis of lophophorate and chaetognath phylogenetic relationships

Helmkampf M., Bruchhaus I., and Hausdorf B. 2008. Multigene analysis of lophophorate and chaetognath phylogenetic relationships. *Molecular Phylogenetics and Evolution* 46: 206–214.

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Multigene analysis of lophophorate and chaetognath phylogenetic relationships

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Abstract

Maximum likelihood and Bayesian inference analyses of seven concatenated fragments of nuclear-encoded housekeeping genes indicate that Lophotrochozoa is monophyletic, i.e., the lophophorate groups Bryozoa, Brachiopoda and Phoronida are more closely related to molluscs and annelids than to Deuterostomia or Ecdysozoa. Lophophorates themselves, however, form a polyphyletic assemblage. The hypotheses that they are monophyletic and more closely allied to Deuterostomia than to Protostomia can be ruled out with both the approximately unbiased test and the expected likelihood weights test. The existence of Phoronozoa, a putative clade including Brachiopoda and Phoronida, has also been rejected. According to our analyses, phoronids instead share a more recent common ancestor with bryozoans than with brachiopods. Platyhelminthes is the sister group of Lophotrochozoa. Together these two constitute Spiralia. Although Chaetognatha appears as the sister group of Priapulida within Ecdysozoa in our analyses, alternative hypothesis concerning chaetognath relationships could not be rejected.

Keywords: Lophophorata; Lophotrochozoa; Bryozoa; Brachiopoda; Phoronida; Chaetognatha; Metazoa; Phylogeny

1. Introduction

In the past two decades, the predominating ideas about animal evolution have changed radically based mainly on analyses of 18S rDNA sequences (Halanych, 2004). The major new hypotheses concerning the relationships of the larger metazoan phyla like the subdivision of Protostomia into two main groups, Lophotrochozoa and Ecdysozoa, have also been corroborated by studies of nuclear-encoded protein sequences (e.g., Ruiz-Trillo et al., 2002; Anderson et al., 2004; Peterson et al., 2004; Philippe et al., 2005). However, so far only few nuclear-encoded protein sequences are available from some smaller taxa like Lophophorata or Chaetognatha. In such cases, more information from additional markers is necessary to corroborate the new hypotheses based on rDNA sequence analyses.

The position of the lophophorate taxa assessed by rDNA studies is particularly conflicting with the traditional perspective. Lophophorata includes Bryozoa (Ectoprocta), Brachiopoda and Phoronida, and is characterized by the eponymous lophophore, a ciliated, tentacular feeding apparatus surrounding the mouth opening which is shared by these taxa. Based on embryological and morphological characters Lophophorata was traditionally considered the sister or paraphyletic stem group of Deuterostomia (Hennig, 1979; Schram, 1991; Ax, 1995; Lüter and Bartolomaeus, 1997; Lüter, 2000; Brusca and Brusca, 2002). However, Nielsen (2001) challenged the homology of the lophophore of Bryozoa and Brachiopoda plus Phoronida and considered Lophophorata polyphyletic. Analyses of rDNA (Halanych et al., 1995; Mackey et al., 1996; Littlewood et al., 1998; Cohen, 2000; Giribet et al., 2000; Peterson and Eernisse, 2001; Mallatt and Winchell, 2002; Halanych, 2004; Passamaneck and Halanych, 2006), *Hox* genes (de Rosa et al., 1999; Passamaneck and Halanych,

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2004) and mitochondrial protein sequences (Stechmann and Schlegel, 1999; Helfenbein and Boore, 2004; Waeschenbach et al., 2006) consistently indicated that Bryozoa, Brachiopoda and Phoronida are more closely related to protostome phyla than to Deuterostomia. More precisely, these studies showed that Lophophorata is presumably polyphyletic and that the lophophorate lineages are more closely related to Trochozoa, i.e., Annelida, Mollusca, and related groups than to other protostomes (i.e., Ecdysozoa). Halanych et al. (1995) therefore united Lophophorata and Trochozoa to Lophotrochozoa. There is also one total evidence analysis combining morphological and rDNA data which assigned all lophophorate lineages to Lophotrochozoa (Peterson and Eernisse, 2001). However, a similar study placed Bryozoa basal to the main group of protostomes including Trochozoa, Platyzoa and Ecdysozoa (Giribet et al., 2000). Brachiopoda and Phoronida cluster in total evidence analyses either with Deuterostomia (Zrzavý et al., 1998) or with Trochozoa (Giribet et al., 2000).

Chaetognatha is another minor phylum with uncertain phylogenetic relationships. Based on embryological and morphological characters, it has been supposed that Chaetognatha is more closely related to Deuterostomia than to Protostomia (Ghirardelli, 1981; Brusca and Brusca, 2002). However, other morphological investigations indicated closer relationships to some "aschelminth" groups (Schram, 1991; Nielsen, 2001). The first analyses of 18S rDNA sequences already rejected the hypothesis that Chaetognatha is more closely allied to Deuterostomia than to Protostomia (Telford and Holland, 1993; Wada and Satoh, 1994). In later analyses of 18S rDNA, chaetognaths formed a monophyletic group with nematodes (Halanych, 1996; Littlewood et al., 1998) or nematomorphs (Peterson and Eernisse, 2001). In the total evidence analysis of Zrzavý et al. (1998) and Peterson and Eernisse (2001) chaetognaths also clustered with ecdysozoan phyla, whereas they appeared as the sister group of Nemertodermatida at the base of Protostomia in another total evidence analysis (Giribet et al., 2000). Giribet et al. (2000) therefore concluded, "the position of the phylum Chaetognatha continues to be one of the most enigmatic issues in metazoan phylogeny". More recent investigations of chaetognath relationships based on mitochondrial protein-coding genes (Papillon et al., 2004) and an EST derived dataset (Matus et al., 2006) indicated that chaetognaths are more closely related to lophotrochozoans than to ecdysozoans. In contrast, a second analysis of mitochondrial protein-coding genes (Helfenbein et al., 2004) and another EST dataset (Marlétaz et al., 2006) provide support for a placement of Chaetognatha as sister group of Lophotrochozoa plus Ecdysozoa. The contradictory outcomes of phylogenetic analyses concerning the position of chaetognaths are probably mainly the result of increased substitution rates and consequential long branch attraction effects.

In order to provide a more robust basis for the resolution of the phylogenetic relationships of these controversial taxa, we compiled a data set of seven nuclear protein-coding genes covering all major lophophorate lineages and a chaetognath representative.

2. Materials and methods

2.1. Material

Samples of *Flustra foliacea* (Bryozoa, Gymnolaemata), *Alcyonidium diaphanum* (Bryozoa, Gymnolaemata) and *Phoronis muelleri* (Phoronida) were obtained from the Biologische Anstalt Helgoland (Germany). Specimens of *Terebratulina retusa* (Brachiopoda, Rhynchonelliformea) from Stömstad (Sweden) and from Norway were purchased from the Tjärnö Marine Biological Laboratory (Sweden) or supplied by G. Jarms (University of Hamburg), respectively. *Novocrania anomala* (Brachiopoda, Craniiformea), collected offshore Gothenburg (Sweden) and from Ramsøy, Hjeltefjord (Norway), were respective gifts of M. Obst (Kristineberg Marine Research Station, Sweden) and C. Schander (University of Bergen, Norway). H. Kapp (Deutsches Zentrum für Marine Biodiversität sforschung, Hamburg) kindly provided specimens of *Sagitta setosa* (Chaetognatha) from Helgoland. Specimens of *Plumatella repens* (Bryozoa, Phylactolaemata) were collected in lake Zotzensee near Mirow (Mecklenburg-Vorpommern, Germany). Voucher specimens were deposited in the Zoological Museum Hamburg.

2.2. Molecular techniques

Total RNA was extracted from tissue fixed in RNAlater (Sigma) or from living animals using TRIzol (Invitrogen) and purified by precipitation or column-based methods (Quiagen RNeasy or Invitrogen TRIzol Plus). First-strand cDNA was synthesized from 0.3–1.0 μg total RNA by reverse transcription using the SuperScript III system (Invitrogen). To increase cDNA yield, a subsequent PCR targeting adaptor sequences attached to cDNA molecules during first-strand synthesis was performed, thereby obtaining amplified cDNA from even minute amounts of RNA (Schramm et al., 2000). Fragments of seven nuclear-encoded genes, namely aldolase, methionine adenosyltransferase, ATP synthase β, elongation factor 1-α, triosephosphate isomerase, phosphofructokinase and catalase, were amplified with GoTaq polymerase (Promega) via touchdown style PCR using universal primers designed by Peterson et al. (2004). To minimize replication errors, proof-reading *Pwo* polymerase (Roche) was added to the reaction mix. In the case of *T. retusa*, a fragment of elongation factor 1-α could only be obtained after using a nested primer pair (nETf 5′-ATHTAYAARTGYGGNGGNAT-3′ and nETr 5′-AYTTRCANGCDATRTGNGC-3′). PCR fragments of the expected sizes were excised from agarose gel and purified (Macherey-Nagel NucleoSpin Extract). If no visible amounts of amplificates of the expected size were produced, a second amplification using DNA purified from

gel slices excised at the appropriate height as template was performed. Each purified fragment was ligated into the pCR2.1-TOPO cloning vector (Invitrogen) and transformed into *Escherichia coli* TOP10 cells (Invitrogen). Clones containing inserts of the correct size were sequenced in both directions on an ABI 377 automated sequencer (Applied Biosystems) using BigDye sequencing chemistry (Applied Biosystems). In those cases where we could not confidently span the gap with both reads, specific internal primers were designed. Usually, multiple clones were sequenced per fragment and organism. Sequences were translated and aligned with orthologous sequences of other taxa obtained from GenBank employing the ClustalW algorithm implemented in MacVector 9.0.2 (MacVector, Inc.). The resulting alignments were inspected and adjusted manually. The concatenated alignment has been deposited in TreeBASE (http://www.treebase.org) under the study accession number S1855.

2.3. Phylogenetic analysis

The appropriate likelihood model of protein evolution was determined for each gene fragment as well as for the complete data set by ProtTest (Abascal et al., 2005) using the "slow" optimization strategy and the AICc criterion. The goodness of fit of the model to the data of separate models for each of the gene fragments was compared to that of the best uniform model for the complete dataset using Treefinder (Jobb et al., 2004; Jobb, 2007).

The phylogenetic information content of the alignment was visualized by likelihood-mapping (Strimmer and von Haeseler, 1997) as implemented in Tree-Puzzle 5.2 (Schmidt et al., 2002).

Maximum likelihood (ML) analyses were conducted with Treefinder (Jobb et al., 2004; Jobb, 2007). Confidence values for the edges of the ML tree were computed by bootstrapping (Felsenstein, 1985) (100 replications).

To test predefined phylogenetic hypotheses we used constrained trees and the 'resolve multifurcations' option of Treefinder to obtain the ML tree for a specified hypothesis. Then we investigated whether the ML trees for these hypotheses are part of the confidence set of trees applying the approximately unbiased test (Shimodaira, 2002) and the expected likelihood weights method (Strimmer and Rambaut, 2002).

Bayesian inference (BI) analyses were performed using the parallel version of MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001). Two independent runs were carried out simultaneously for 1,000,000 generations starting from randomly chosen trees. Each run employed one cold and five heated chains set to a heating parameter of 0.5. Trees were sampled every 250 generations, resulting in 4000 trees collected in total. Both runs reached convergence after 260,000 generations as defined by the average standard deviation of split frequencies dropping below 0.1. Chain equilibrium was also analysed using Tracer v1.3 (Rambaut and Drummond, 2004). To allow for burn-in of the Markov chains, the first 26% of all sampled trees were discarded before calculating a 50% majority rule consensus tree from the remaining 2960 trees. The frequency of a clade among the sampled trees was interpreted as its posterior probability.

3. Results

Fragments of seven nuclear genes coding for ATP synthase β (427 amino acids), catalase (264 aa), elongation factor 1-α (411–423 aa), fructose-bisphosphate aldolase (196–199 aa), methionine adenosyltransferase (319 aa), phosphofructokinase (172 aa) and triosephosphate isomerase (210–213 aa) were sequenced from six lophophorate taxa and a chaetognath. GenBank accession numbers of these sequences are listed in Table 1. A few gene fragments could not be amplified by PCR. The concatenated data set was complemented by orthologous sequences of 31 additional taxa obtained from GenBank and encompasses 2033 amino acid positions.

The likelihood-mapping analysis shows that the concatenated alignment has a high phylogenetic information content and is suitable for phylogenetic reconstruction, since 96.0% of the quartets (in the corner areas of attraction in Fig. 1) were fully resolved. When analysing the seven genes individually, 69.5–85.0% of the quartets were fully resolved (mean ± SD 79.2 ± 6.0%). The phylogenetic information content of the individual gene fragments was correlated with their length ($r = 0.77$; $p = 0.04$).

The use of separate models of protein evolution for each of the seven gene fragments improved the goodness of fit of the model to the data in comparison to the best uniform model for the complete dataset according to the AICc criterion. Thus, all phylogenetic analyses were based on the partitioned dataset with separate models for each of the seven gene fragments.

The results of maximum likelihood and Bayesian inference analyses of this dataset are shown in Figs. 2 and 3, respectively. Both analyses recover the main bilaterian clades, i.e., Deuterostomia, Ecdysozoa and Spiralia including Lophotrochozoa and Platyhelminthes. The lophophorate groups Bryozoa, Brachiopoda and Phoronida are more closely related to nemerteans, molluscs and annelids than to deuterostomes or ecdysozoans. Thus, Lophotrochozoa is monophyletic, although the support for this clade is not strong. Platyhelminthes appears as the sister group of Lophotrochozoa.

Lophophorata does not constitute a monophyletic group: bryozoans and phoronids apparently share a more recent common ancestor with annelids and molluscs than with brachiopods. While articulate and inarticulate brachiopods are sister to each other, bryozoans also do not appear as a monophyletic taxon. Instead, phylactolaemate bryozoans seem to be more closely related to Phoronida than to gymnolaemate bryozoans.

Chaetognatha emerges within Ecdysozoa as the sister group of Priapulida, while nematodes turn up more closely related to arthropods than to priapulids. Thus Cycloneura-

Table 1
GenBank accession numbers of the nuclear-encoded protein-coding gene fragments sequenced from lophophorate and chaetognath representatives

Group	Species	Aldolase	Methionine adenosyltransferase	ATP synthase β	Triosephosphate isomerase	Elongation factor 1-α	Phosphofructokinase	Catalase
Bryozoa, Gymnolaemata, Ctenostomata	*Alcyonidium diaphanum* (Hudson, 1778)	EU074260	EU074287	EU074267	EU074297	EU074281	—	EU074274
Bryozoa, Gymnolaemata, Cheilostomata	*Flustra foliacea* (Linnaeus, 1758)	EU074261	EU074288	EU074268	EU074298	EU074282	EU074293	EU074275
Bryozoa, Phylactolaemata	*Plumatella repens* (Linnaeus, 1758)	EU074262	EU074289	EU074269	EU074299	EU074283	EU074294	EU074276
Brachiopoda, Craniiformea	*Novocrania anomala* (Müller, 1776)	EU074263	—	EU074270	EU074300	EU074284	EU074295	EU074277
Brachiopoda, Rhynchonelliformea	*Terebratulina retusa* (Linnaeus, 1758)	EU074266	EU074292	EU074273	EU074303	—	EU074296	EU074280
Phoronida	*Phoronis muelleri* (Selys-Lonchamps, 1903)	EU074264	EU074290	EU074271	EU074301	EU074285	—	EU074278
Chaetognatha	*Sagitta setosa* (Müller, 1847)	EU074265	EU074291	EU074272	EU074302	EU074286	—	EU074279

Gene fragments indicated by a dash (—) could not be amplified by PCR.

Fig. 1. Likelihood-mapping analysis of the concatenated alignment of the seven nuclear-encoded housekeeping genes used in this study.

lia, as represented by Priapulida and Nematoda, is paraphyletic.

The only inconsistency between the trees reconstructed with maximum likelihood and Bayesian inference is that Annelida is monophyletic in the ML tree, whereas it is paraphyletic with respect to Phoronida and phylactolaemate Bryozoa in the BI reconstruction.

The hypotheses stating that Lophophorata is more closely related to Deuterostomia than to Protostomia, that it is monophyletic, and that Bryozoa is sister to Spiralia and Ecdysozoa have been significantly rejected with both the approximately unbiased test and the expected likelihood weights method (Table 2). The monophyly of the segmented phyla, Annelida plus Arthropoda, i.e., the Articulata hypothesis, and the monophyly of Neotrochozoa including Annelida and Mollusca, have also been significantly rejected with both tests. All other tested phylogenetic hypotheses could not be ruled out with the approximately unbiased test. However, the expected likelihood weights method did reject hypotheses stating the monophyly of Phoronozoa (Brachiopoda plus Phoronida), a sister group relationship between Phoronozoa and Mollusca, and the Eubilateria hypothesis (claiming Platyhelminthes is sister to all other bilaterians). Neither topological test could reject the other phylogenetic hypotheses listed in Table 2 on the basis of our protein data set.

4. Discussion

The results of maximum likelihood (Fig. 2) as well as Bayesian inference analyses (Fig. 3) of seven concatenated fragments of nuclear-encoded housekeeping genes show that the lophophorate lineages Bryozoa, Brachiopoda and Phoronida do not form the sister group or the paraphyletic stem group of Deuterostomia as has been supposed based on embryological and morphological characters (Hennig, 1979; Schram, 1991; Ax, 1995; Lüter

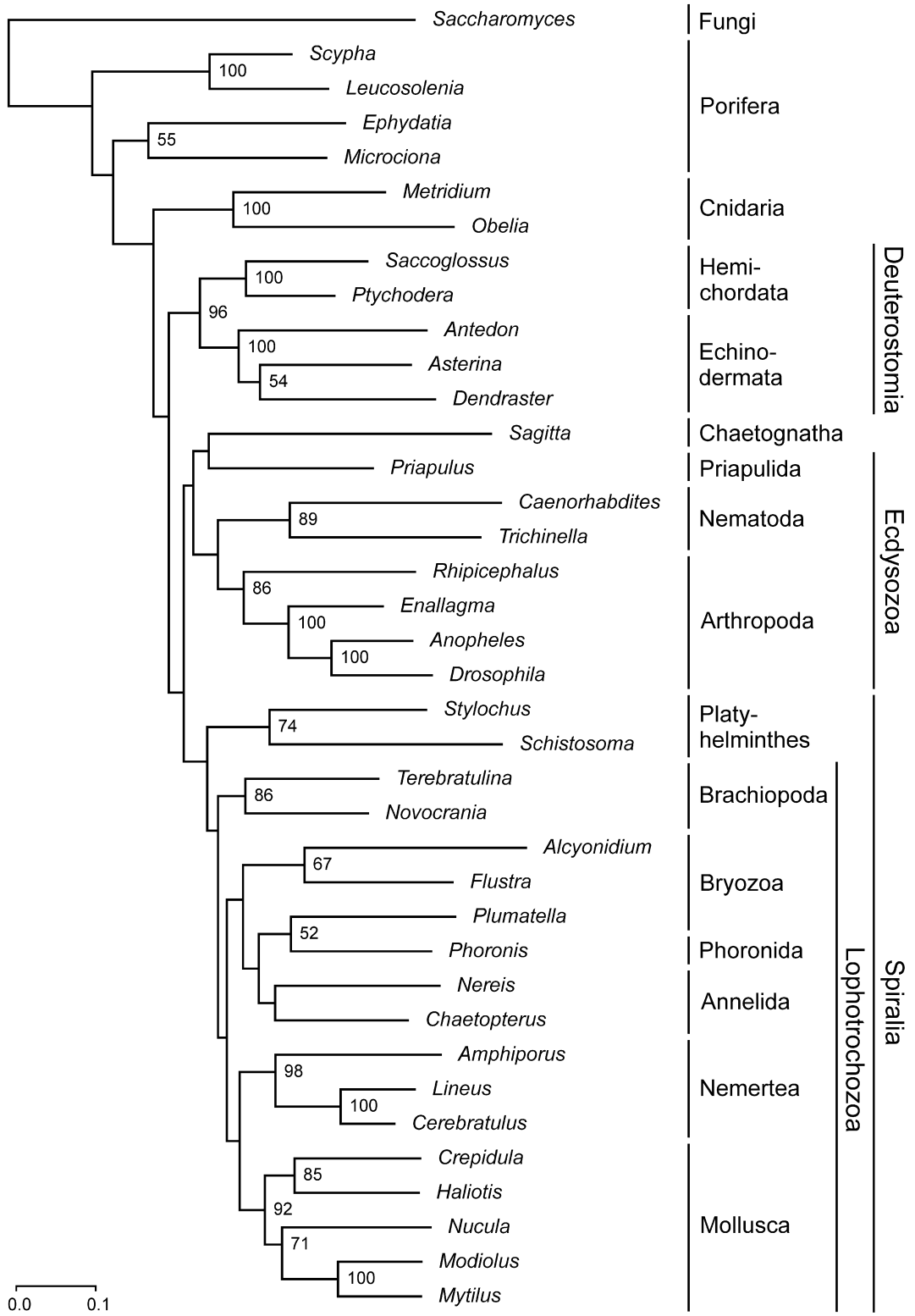

Fig. 2. Maximum likelihood tree based on the analysis of approximately 2000 amino acids derived from the seven concatenated housekeeping genes listed in Table 1. Bootstrap support values larger than 50% are shown to the left of the nodes.

and Bartolomaeus, 1997; Lüter, 2000; Brusca and Brusca, 2002). The hypothesis that lophophorates are more closely related to Deuterostomia than to Protostomia has been significantly rejected with both the approximately unbiased test and the expected likelihood weights method (Table 2).

Instead, the analyses based on our protein data set (Figs. 2 and 3) indicate that the lophophorate groups Bryozoa, Brachiopoda and Phoronida share a more recent common ancestor with molluscs and annelids than with deuterostomes or ecdysozoans. Even though the

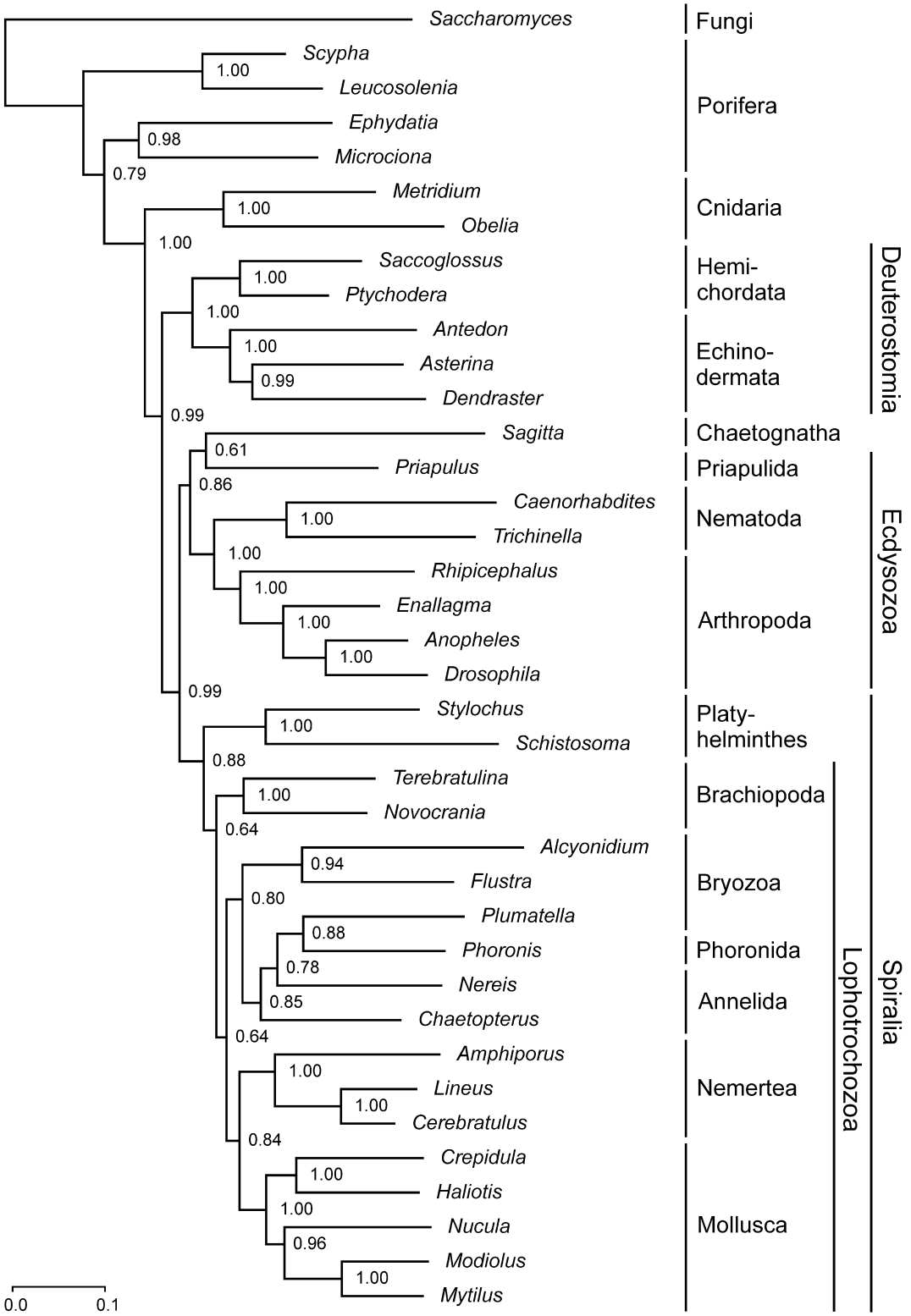

Fig. 3. Bayesian inference reconstruction based on the analysis of approximately 2000 amino acids derived from the seven concatenated housekeeping genes listed in Table 1. Bayesian posterior probabilities are shown to the left of the nodes.

support for this clade is not strong, this confirms the monophyly of Lophotrochozoa and corroborates the results of studies based on rDNA (Halanych et al., 1995; Littlewood et al., 1998; Cohen, 2000; Peterson and Eernisse, 2001; Mallatt and Winchell, 2002; Halanych, 2004; Passamaneck and Halanych, 2006), *Hox* genes (de Rosa et al., 1999; Passamaneck and Halanych, 2004) and mitochondrial protein sequences (Stechmann and Schlegel, 1999; Helfenbein and Boore, 2004; Waeschenbach et al., 2006).

Table 2
Topology test results

Phylogenetic hypothesis	References	AU	ELW
ML tree		0.8893*	0.3239*
Lophophorata + Deuterostomia	Hennig (1979), Schram (1991), Ax (1995), Lüter and Bartolomaeus (1997), Lüter (2000), Brusca and Brusca (2002)	0.0000	0.0000
Lophophorata monophyly		0.0000	0.0024
Phoronozoa (Brachiopoda + Phoronida)	Mackey et al. (1996), Cohen et al. (1998), Littlewood et al. (1998), Zrzavý et al. (1998), Cohen (2000), Giribet et al. (2000), Nielsen (2001), Peterson and Eernisse (2001), Mallatt and Winchell (2002), Anderson et al. (2004), Halanych (2004), Cohen and Weydmann (2005)	0.0725*	0.0101
Phoronozoa + Mollusca	Halanych (2004)	0.0682*	0.0042
Bryozoa monophyly	Hennig (1979), Giribet et al. (2000), Nielsen (2001), Brusca and Brusca (2002)	0.3385*	0.0714*
Bryozoa sister to Spiralia	Halanych et al. (1995), Halanych (2004)	0.3784*	0.1503*
Bryozoa sister to Spiralia + Ecdysozoa	Giribet et al. (2000)	0.0000	0.0004
Articulata (Annelida + Arthropoda)	Hennig (1979), Schram (1991), Nielsen (2001), Brusca and Brusca (2002)	0.0000	0.0000
Neotrochozoa (Annelida + Mollusca)	Zrzavý et al. (1998), Giribet et al. (2000), Peterson and Eernisse (2001)	0.0362	0.0050
Eutrochozoa (Neotrochozoa + Nemertea)	Zrzavý et al. (1998), Giribet et al. (2000), Peterson and Eernisse (2001)	0.1593*	0.0241*
Parenchymia (Platyhelminthes + Nemertea)	Nielsen (2001)	0.4472*	0.1296*
Eubilateria	Hennig (1979), Ax (1985)	0.1018*	0.0203
Chaetognatha + Deuterostomia	Ghirardelli (1981), Brusca and Brusca (2002)	0.3220*	0.0421*
Chaetognatha + Spiralia	Papillon et al. (2004), Matus et al. (2006)	0.6219*	0.1164*
Chaetognatha + (Spiralia + Ecdysozoa)	Giribet et al. (2000), Halanych (2004), Helfenbein et al. (2004), Marlétaz et al. (2006)	0.5395*	0.0998*

AU, approximately unbiased test (p-values); ELW, expected likelihood weights. Values for the topologies included in the 0.95 confidence set are indicated by an asterisk (i.e., p-values above 0.05 for the approximately unbiased test and expected likelihood weights of the trees with the highest confidence levels that add up to 0.95 for the expected likelihood weights method).

The Articulata hypothesis (Hennig, 1979; Schram, 1991; Nielsen, 2001; Brusca and Brusca, 2002), i.e., the monophyly of the segmented phyla Annelida and Arthropoda as an alternative to Lophotrochozoa, has been rejected with both topology tests (Table 2), indicating that segmentation originated independently in these phyla.

As rDNA and mtDNA analyses have shown before (Halanych et al., 1995; Littlewood et al., 1998; Giribet et al., 2000; Peterson and Eernisse, 2001; Halanych, 2004; Passamaneck and Halanych, 2006; Waeschenbach et al., 2006), our multigene analyses also indicate that Lophophorata is polyphyletic. The monophyly of this group has been rejected with both topology tests (Table 2), suggesting that lophophore structures originated several times independently during animal evolution.

Moreover, our results (Figs. 2 and 3, Table 2) question the existence of Phoronozoa, a putative clade including Brachiopoda and Phoronida. Phoronozoa was found in analyses based on rDNA (Mackey et al., 1996; Cohen et al., 1998; Littlewood et al., 1998; Cohen, 2000; Mallatt and Winchell, 2002; Halanych, 2004; Cohen and Weydmann, 2005; but see Passamaneck and Halanych, 2006), sodium–potassium ATPase α-subunit (Anderson et al., 2004), and in total evidence analyses (Zrzavý et al., 1998; Giribet et al., 2000; Peterson and Eernisse, 2001). However, this clade could not be recovered by the present investigation and has been rejected by the expected likelihood weights method (Table 2). The same applies to the hypothesis of Halanych (2004) suggesting that Phoronozoa is the sister group of Mollusca.

The analyses of the protein data set presented herein (Figs. 2 and 3) further indicate that ectoproct bryozoans are polyphyletic. Phylactolaemate bryozoans seem to be more closely related to phoronids than to gymnolaemate bryozoans. Actually, Mundy et al. (1981) have proposed such a relationship based on similarities in lophophore architecture and other morphological features. However, the support for the clade including phylactolaemate bryozoans and phoronids is not strong and the monophyly of bryozoans could not be rejected by topological tests (Table 2). The two bryozoan lineages and Phoronida form a clade also comprising Annelida. Nevertheless, a sister group relation of Bryozoa and all other Lophotrochozoa (or Spiralia, according to our trees) as advocated by Halanych et al. (1995) and Halanych (2004) can not be ruled out according to the topology tests (Table 2). In contrast, the hypothesis that Bryozoa is the sister group of Spiralia and Ecdysozoa (Giribet et al., 2000) has been rejected with the expected likelihood weights method (Table 2).

Peterson and Eernisse (2001) proposed several clades within Lophotrochozoa, e.g., Neotrochozoa that includes Annelida, Mollusca, Echiura and Sipuncula, and Eutrochozoa that comprises Neotrochozoa and Nemertea. So far these clades were found only in total evidence analyses (Zrzavý et al., 1998; Giribet et al., 2000; Peterson and Eernisse, 2001). We did not recover them in the analyses of our protein data set (Figs. 2 and 3). The Neotrochozoa hypothesis could be rejected with topology tests, whereas the Eutrochozoa hypothesis could not (Table 2).

Platyhelminths (as the only members of Platyzoa represented in our analysis) are the sister group of Lophotrocho-

zoa according to our analyses (Figs. 2 and 3), consistent with some rDNA analyses (Littlewood et al., 1998; Peterson and Eernisse, 2001; but see Mallatt and Winchell, 2002; Halanych, 2004; Passamaneck and Halanych, 2006). However, a sister group relationship between Platyhelminthes and Nemertea (Parenchymia in the sense of Nielsen, 2001) could not be rejected with topology tests (Table 2). Nonetheless, both maximum likelihood and Bayesian inference analyses indicate that Platyhelminthes do not belong to Lophotrochozoa, which is defined as the last common ancestor of the three traditional lophophorate taxa, the molluscs, and the annelids, and all descendants of that ancestor (Halanych et al., 1995). Assuming that the spiral-quartet cleavage of plathyhelminths is homologous to that of nemerteans, annelids, and molluscs, we use the name Spiralia for the clade including platyhelminths (and possibly other Platyzoa) and lophotrochozoans as has been done by Garey and Schmidt-Rhaesa (1998) and Giribet et al. (2000). The analyses based on our protein data set thus contradict the result of a combined analysis of 18S and 28S rDNA sequences that suggested a topologically derived position of Platyzoa within Lophotrochozoa (Passamaneck and Halanych, 2006). However, we cannot rule out that Platyhelminthes indeed belong to Lophotrochozoa, because the topology tests did not reject a position of the bryozoan lineages as sister to Spiralia (Table 2). Since platyhelminths are the only representatives of Platyzoa in our data set, a denser sampling of Platyzoa is required for conclusions that are more robust.

The Eubilateria hypothesis (Hennig, 1979; Ax, 1985), according to which Platyhelminthes is not related to Lophotrochozoa, but is instead the sister group of all other bilaterians, has been rejected with the expected likelihood weights method (Table 2).

Chaetognaths appear as the sister group of Priapulida within Ecdysozoa in our phylogenetic analyses (Figs. 2 and 3). Actually, a relationship of chaetognaths with ecdysozoans has been proposed several times based on 18S rDNA sequences (Littlewood et al., 1998; Zrzavý et al., 1998; Peterson and Eernisse, 2001). However, there are several alternative hypotheses concerning the relationships of Chaetognatha. Firstly, a relationship of Chaetognatha with Deuterostomia has been supposed based on embryological and morphological data (Ghirardelli, 1981; Brusca and Brusca, 2002). Secondly, Chaetognatha has been placed basal to the remaining protostomes in a total evidence analysis (Giribet et al., 2000), an analysis of mitochondrial protein sequences (Helfenbein et al., 2004), and an EST analysis (Marlétaz et al., 2006). Thirdly, another analysis of mitochondrial protein sequences (Papillon et al., 2004) and a second EST analysis (Matus et al., 2006) placed chaetognaths and Spiralia in a clade. Unfortunately, none of these hypotheses can be ruled out according to topological tests based on this multigene analysis (Table 2).

Although we were able to recover the main clades within Bilateria, namely Deuterostomia, Ecdysozoa and Spiralia including Lophotrochozoa and Platyhelminthes, the sequences of seven gene fragments were not sufficient for a robust resolution of the phylogenetic relationships of the lophophorate groups and chaetognaths. This indicates that still more data are necessary. We thus plan EST projects to obtain information on a genomic scale to shed further light on the relationships of the lophophorate lineages.

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2.2. Spiralian phylogenomics supports the resurrection of Bryozoa comprising Ectoprocta and Entoprocta

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Spiralian Phylogenomics Supports the Resurrection of Bryozoa Comprising Ectoprocta and Entoprocta

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Phylogenetic analyses based on 79 ribosomal proteins of 38 metazoans, partly derived from 6 new expressed sequence tag projects for Ectoprocta, Entoprocta, Sipuncula, Annelida, and Acanthocephala, indicate the monophyly of Bryozoa comprising Ectoprocta and Entoprocta, 2 taxa that have been separated for more than a century based on seemingly profound morphological differences. Our results also show that bryozoans are more closely related to Neotrochozoa, including molluscs and annelids, than to Syndermata, the latter comprising Rotifera and Acanthocephala. Furthermore, we find evidence for the position of Sipuncula within Annelida. These findings suggest that classical developmental and morphological key characters such as cleavage pattern, coelomic cavities, gut architecture, and body segmentation are subject to greater evolutionary plasticity than traditionally assumed.

Introduction

With the establishment of Lophotrochozoa and Ecdysozoa (Halanych et al. 1995; Aguinaldo et al. 1997), molecular data have substantially changed our view of animal evolution. Recent phylogenomic approaches have generally sustained these hypotheses (Philippe et al. 2005; Philippe and Telford 2006; Baurain et al. 2007), but adequate genomic data are still lacking for many minor phyla whose affinities are still in dispute (Giribet et al. 2000; Halanych 2004). Two of the most enigmatic minor animal phyla are the moss animals, that is, Ectoprocta and Entoprocta. When first discovered, entoprocts (Kamptozoa) were treated together with the ectoproct bryozoans because of their sessile life style and ciliated tentacles. Nitsche (1869) pointed to the differences between the position of the anus and the retractability of the tentacle crowns and proposed the names Entoprocta and Ectoprocta for the 2 main groups of bryozoans. Subsequently, the 2 groups have almost unanimously been treated as separate higher taxa, mainly based on the differences in cleavage patterns and body cavities (Hatschek 1891; Korschelt and Heider 1893; Hennig 1979; Emschermann 1982; Schram 1991; Zrzavý et al. 1998; Ax 1999; Giribet et al. 2000; Sørensen et al. 2000; Brusca and Brusca 2002). So far, all analyses of rDNA sequences have supported the assumption that they do not constitute sister taxa (Mackey et al. 1996; Littlewood et al. 1998; Zrzavý et al. 1998; Giribet et al. 2000; Peterson and Eernisse 2001; Passamaneck and Halanych 2006). However, Nielsen (1971, 1985, 2001) and Cavalier-Smith (1998) maintained the monophyly of Bryozoa in the broader sense.

To acquire molecular data sufficient for a resolution of the phylogenetic relationships of ectoprocts and entoprocts, we generated 2,000–4,000 expressed sequence tags (ESTs) from representatives of Ectoprocta, Entoprocta, Sipuncula, Annelida, and Acanthocephala (table 1). The comparison of the 6 analyzed transcriptomes revealed a broad coverage of ribosomal proteins, which are valuable markers for phylogenomic analyses (Veuthey and Bittar 1998; Philippe et al. 2004; Hughes et al. 2006; Marlétaz et al. 2006) because of the rarity of known gene duplications resulting in paralogs and their conservation among eukaryotes. We compiled from our EST projects a data set comprising 79 ribosomal proteins, which we complemented by orthologous sequences of 32 additional taxa obtained from public databases.

Key words: Metazoa, phylogenomics, Bryozoa, Ectoprocta, Entoprocta, Spiralia.

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Materials and Methods

Isolation of RNA and Library Construction

Total RNA of the organisms specified in table 1 was extracted from living or frozen tissue employing TRIzol (Invitrogen, Karlsruhe, Germany) or column-based methods (Qiagen RNeasy Plant Mini Kit). *Flustra* RNA was additionally purified by the RNeasy Mini Kit cleanup procedure (Qiagen, Hilden, Germany), whereas for the purification of *Barentsia* RNA, we applied the NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany). Quality of total RNA was visually checked on agarose gel, and mRNA was subsequently captured by using the polyATract mRNA Isolation System III (Promega, Mannheim, Germany) or Dynabeads (Invitrogen, Karlsruhe, Germany) for *Sipunculus*. All cDNA libraries were constructed at the Max Planck Institute for Molecular Genetics in Berlin by primer extension, size fractioning, and directional cloning applying the Creator SMART cDNA Libraries Kit (Clontech, Heidelberg, Germany) or Invitrogen's CloneMiner technology (*Arenicola* only), using the respective vectors pDNR-LIB or pDONR222. Clones containing cDNA inserts were sequenced from the 5′ end on the automated capillary sequencer systems ABI 3730 XL (Applied Biosystems, Darmstadt, Germany) and MegaBace 4500 (GE Healthcare, München, Germany) using BigDye chemistry (Applied Biosystems). If possible, clones containing ribosomal proteins from the libraries of *Barentsia* and *Sipunculus* were completed by reverse sequencing with polyT- and vector-specific reverse primer to maximize sequence coverage.

EST Processing

EST processing was accomplished at the Center for Integrative Bioinformatics in Vienna. Sequencing

Table 1
List of Investigated Taxa and Data Used in Phylogenetic Analyses

Species	Taxon	Origin	# EST	# RP
Flustra foliacea (Linnaeus 1758)	Ectoprocta	Helgoland, North Sea	4.074	77
Barentsia elongata (Jullien and Calvet 1903)[a]	Entoprocta	Lab culture	2.154	47
Arenicola marina (Linnaeus 1758)	Annelida	Sylt, North Sea	2.199	61
Eurythoe complanata (Pallas 1776)	Annelida	Lab culture	2.257	41
Sipunculus nudus (Linnaeus 1766)	Sipuncula	Roscoff, France	2.329	48
Pomphorhynchus laevis (Müller 1776)	Acanthocephala	Gimbsheim, Germany (from host *Barbus fluviatilis*)	2.207	65

NOTE.—# EST: number of sequenced EST clones; # RP: number of ribosomal proteins retrieved at least partially from the EST data sets. Voucher specimens were deposited at the Zoological Museum, Hamburg.

[a] The data set of *B. elongata* was complemented by 2 sequences derived from 95 ESTs of *Barentsia benedeni* (Foettinger 1886).

chromatograms were first base called and evaluated using the Phred application (Ewing et al. 1998). Vector, adapter, poly-A, and bacterial sequences were removed employing the software tools Lucy (www.tigr.org), SeqClean (compbio.dfci.harvard.edu/tgi/software), and CrossMatch (www.phrap.org). Repetitive elements were subsequently masked with RepeatMasker. Clustering and assembly of the clipped sequences were performed using the TIGCL program package (compbio.dfci.harvard.edu/tgi/software) by first performing pairwise comparisons (MGIBlast) and a subsequent clustering step (CAP3). Low-quality regions were then removed by Lucy. Finally, contigs were tentatively annotated by aligning them pairwise with the 25 best hits retrieved from National Center for Biotechnology Information's nonredundant protein database using the BlastX algorithm (www.ncbi.nlm.nih.gov). Alignment and computation of the resulting match scores on which annotation was based were conducted by GeneWise (Birney et al. 2004) in order to account for frameshift errors. The EST data used in our analyses have been deposited in GenBank under the accession numbers EU139167–EU139243 (*Flustra*), EU116892–EU116936, EU220741 (*Barentsia*), EU116844–EU116891 (*Sipunculus*), EU124931–EU124992 (*Arenicola*), EU124993–EU125033 (*Eurythoe*), and AM849482–AM849546 (*Pomphorhynchus*).

Sequence Analyses and Ribosomal Proteins Alignment

Ribosomal protein sequences were extracted from the newly obtained EST data by their annotation or by using the human ribosomal protein genes retrieved from the Ribosomal Protein Gene Database (ribosome.med.miyazaki-u.ac.jp) as search template during local Blast searches (using the TblastN algorithm and an *e* value $<e^{-10}$ as match criterion). The observed sequences were checked for assembly errors by visual inspection and by comparison with corresponding sequences of related taxa, and translated into amino acid sequences. Orthologous sequences of *Priapulus caudatus*, *Ascaris suum*, *Aplysia californica*, *Idiosepius paradoxus*, *Macrostomum lignano*, *Philodina roseola*, *Flaccisagitta enflata*, and *Strongylocentrotus purpuratus* were obtained from public EST databases using TblastN searches also employing human sequences as query. Additional ribosomal protein data were retrieved from the alignments compiled by Baurain et al. (2007) and provided by H. Philippe (Université de Montréal), and complemented for missing genes. Ribosomal proteins of *Ciona intestinalis*, *Takifugu rubripes*, *Anopheles gambiae*, and, in part, *Apis mellifera* were acquired directly from the Ribosomal Protein Gene Database. Sequences of *Spadella cephaloptera* were provided by F. Marlétaz (Station Marine d'Endoume, Marseille).

All ribosomal protein sequences obtained were aligned by the ClustalW algorithm (Thompson et al. 1994). The resulting 79 ribosomal protein alignments were inspected and adjusted manually. Questionably aligned positions were eliminated with Gblocks (Castresana 2000), applying all less stringent block selection parameters available and thereafter concatenated to a single multiple sequence alignment. This alignments is available at TreeBASE (http://www.treebase.org; accession number S1884).

Phylogenetic Analyses

Maximum Likelihood (ML) analyses were conducted with Treefinder (Jobb et al. 2004; Jobb 2007). The rtRev + G + F model of protein evolution was used for the ML analyses because it was superior to other uniform models for the concatenated data set as well as a mixed model combining separate models as determined by ProtTest (Abascal et al. 2005) for each of the 79 gene partitions according to the Akaike Information Criterion with a correction term for small sample size. Confidence values for the edges of the ML tree were computed by applying expected likelihood weights (ELWs) (Strimmer and Rambaut 2002) to all local rearrangements (LR) of tree topology around an edge (1,000 replications).

To test predefined phylogenetic hypotheses, we used constrained trees and the 'resolve multifurcations' option of Treefinder to obtain the ML tree for a specified hypothesis. Then we investigated whether the ML trees for these hypotheses are part of the confidence set of trees applying the expected likelihood weights method (Strimmer and Rambaut 2002).

Bayesian inference (BI) analyses based on the site-heterogeneous CAT model (Lartillot and Philippe 2004) were performed using PhyloBayes v2.1c (Blanquart and Lartillot 2006). Two independent chains were run simultaneously for 10,000 points each. Chain equilibrium was estimated by plotting the log-likelihood and the alpha parameter as a function of the generation number. The first 1,000 points were consequently discarded as burn-in. According to the

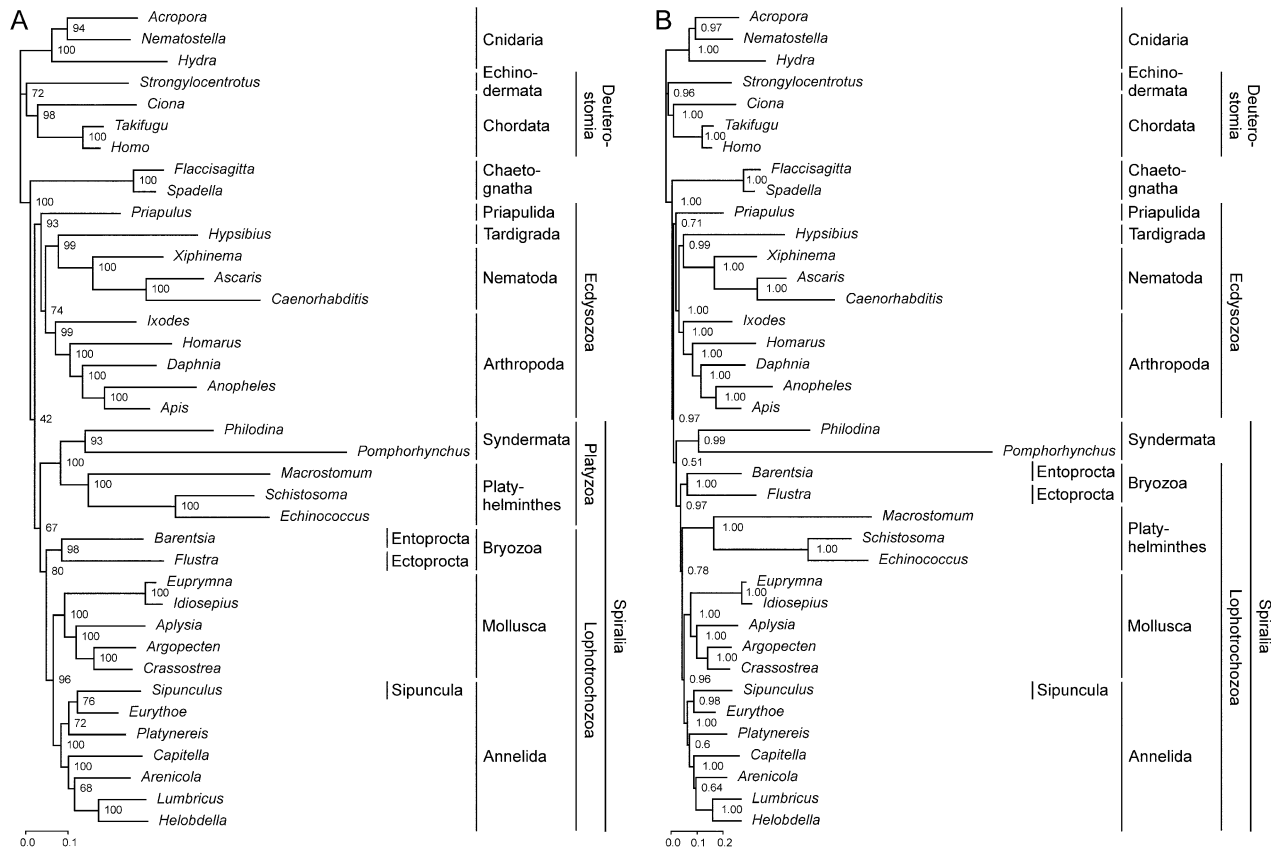

FIG. 1.—Spiralian phylogenomics unites ectoprocts with entoprocts, resurrecting Bryozoa *sensu lato*. Phylogenetic analyses were performed on the basis of 11,428 amino acid positions derived from 79 concatenated ribosomal proteins. (*A*) ML tree. Approximate bootstrap support values (LR-ELW) are shown to the right of the nodes. (*B*) BI reconstruction. Bayesian posterior probabilities are shown to the right of the nodes.

divergence of bipartition frequencies, both chains reached convergence (maximal difference <0.3, mean difference <0.005), supported by the fact that both chains produced the same consensus tree topology. Taking every 10th sampled tree, a 50% majority rule consensus tree was finally computed using both chains.

Results and Discussion

Bryozoa *sensu lato*: A Century-Old Hypothesis Resurrected

Phylogenetic analyses of the concatenated sequences of 79 ribosomal proteins encompassing 11,428 amino acid positions show for the first time Bryozoa as a monophyletic clade comprising Entoprocta and Ectoprocta. The monophyly is supported by strong nodal support values (fig. 1). Therefore, the century-old hypothesis of Bryozoa in the broader sense has to be resurrected.

Ectoprocts have been included in Lophophorata based on similarities of the tentacular apparatus and the radial cleavage they share with phoronids and brachiopods. Lophophorata was traditionally considered the sister or paraphyletic stem group of Deuterostomia (Hennig 1979; Schram 1991; Ax 1995; Brusca and Brusca 2002). However, studies employing rDNA (Halanych et al. 1995; Mackey et al. 1996; Littlewood et al. 1998; Peterson and Eernisse 2001; Mallatt and Winchell 2002; Halanych 2004; Passamaneck and Halanych 2006), *Hox* genes (Passamaneck and Halanych 2004), multiple nuclear genes (Helmkampf et al. forthcoming), and mitochondrial protein sequences (Stechmann and Schlegel 1999; Helfenbein and Boore 2004; Waeschenbach et al. 2006) showed that Ectoprocta as well as Phoronida and Brachiopoda are more closely related to Annelida, Mollusca, and allies than to Deuterostomia or Ecdysozoa. Therefore, Halanych et al. (1995) united them under the name Lophotrochozoa. Some of these studies further demonstrated that Lophophorata is polyphyletic (Halanych et al. 1995; Mackey et al. 1996; Littlewood et al. 1998; Giribet et al. 2000; Halanych 2004; Passamaneck and Halanych 2006; Helmkampf et al. forthcoming). On the basis of our data, the hypotheses that ectoprocts are related to Deuterostomia, that they are sister to all remaining Spiralia (Halanych et al. 1995; Littlewood et al. 1998; Halanych 2004; Passamaneck and Halanych 2006), and that they are sister to all other protostomes except chaetognaths (Giribet et al. 2000) could be rejected by topology tests (table 2, hypotheses 1–3).

Entoprocts exhibit spiral cleavage and trochophora-type larvae, leading to the assumption of closer connections to taxa also possessing these features (Ax 1995, 1999; Zrzavý et al. 1998; Giribet et al. 2000; Peterson and Eernisse 2001). Molecular phylogenetic analyses of 18S rDNA generally confirmed the affiliation of entoprocts with taxa having trochophora larvae, but their exact relationships

Table 2
Topology Test Results

Number	Phylogenetic Hypothesis	References	ELW Test
	ML tree (fig. 1*A*)		0.3452*
1	Lophophorata + Deuterostomia	Hennig (1979); Schram (1991); Ax (1995); Sørensen et al. (2000); Brusca and Brusca (2002)	0.0000
2	Ectoprocta sister to other Spiralia	Halanych et al. (1995); Halanych (2004); Passamaneck and Halanych (2006)	0.0007
3	Ectoprocta sister to other Spiralia + Ecdysozoa	Giribet et al. (2000)	0.0006
4	Lacunifera (=Entoprocta + Mollusca)	Bartolomaeus (1993); Haszprunar (1996); Ax (1999); Haszprunar (2000)	0.0037
5	Entoprocta + Annelida (+Sipuncula)	Emschermann (1982)	0.0094
6	Entoprocta + Platyzoa	Halanych (2004); Passamaneck and Halanych (2006)	0.0262
7	Entoprocta + Neotrochozoa	Zrzavý et al. (1998); Giribet et al. (2000); Peterson and Eernisse (2001)	0.0804*
8	Articulata (=Annelida + Arthropoda)	Hennig (1979); Schram (1991); Ax (1999); Sørensen et al. (2000); Nielsen (2001); Brusca and Brusca (2002)	0.0000
9	Annelida monophyly (exclusive Sipuncula)	Schram (1991); Zrzavý et al. (1998); Ax (1999); Giribet et al. (2000); Sørensen et al. (2000); Nielsen (2001); Brusca and Brusca (2002); Passamaneck and Halanych (2006)	0.1000*
10	Sipuncula + Mollusca	Scheltema (1993); Zrzavý et al. (1998)	0.0000
11	Sipuncula sister to (Annelida + Mollusca)	Giribet et al. (2000)	0.0000
12	Eubilateria	Hennig (1979); Ax (1985)	0.0000
13	Chaetognatha + Deuterostomia	Ghirardelli (1981); Sørensen et al. (2000); Brusca and Brusca (2002)	0.0271*
14	Chaetognatha sister to Spiralia	Matus et al. (2006)	0.2221*
15	Chaetognatha + Ecdysozoa	Littlewood et al. (1998); Zrzavý et al. (1998); Peterson and Eernisse (2001)	0.1847*

NOTE.—Numbers refer to the order of appearance in the text. Values for the topologies included in the 0.95 confidence set are indicated by an asterisk (i.e., ELW of the trees with the highest confidence levels that added up to 0.95).

remained controversial (Mackey et al. 1996; Littlewood et al. 1998; Zrzavý et al. 1998; Giribet et al. 2000; Peterson and Eernisse 2001). Combined analyses of 18S and 28S rDNA data resulted in a placement within Platyzoa, but also without significant support (Passamaneck and Halanych 2006).

With our data, most alternative hypotheses concerning the phylogenetic position of Entoprocta, in particular a sister group relationship between Entoprocta and Mollusca (Bartolomaeus 1993; Haszprunar 1996, 2000; Ax 1999), a neotenic origin of entoprocts from annelids (Emschermann 1982), and their placement within Platyzoa (Halanych 2004; Passamaneck and Halanych 2006) could be ruled out according to the expected likelihood weights test (table 2, hypotheses 4–6). However, a sister group relationship between Entoprocta and Neotrochozoa, which comprises Mollusca, Sipuncula, and Annelida (Zrzavý et al. 1998; Giribet et al. 2000; Peterson and Eernisse 2001), could not be significantly rejected (table 2, hypothesis 7). Nonetheless, our analyses strongly support the monophyly of Bryozoa in the broader sense including Ectoprocta and Entoprocta and thus confirm the morphology-based argumentation of Nielsen (1971, 1985, 2001) and Cavalier-Smith (1998). Morphological data (Funch and Kristensen 1995; Zrzavý et al. 1998; Sørensen et al. 2000) and rDNA sequences (Passamaneck and Halanych 2006) indicate that Entoprocta and Cycliophora are sister groups. Although genomic data for Cycliophora are unfortunately still missing, we suggest to also include Cycliophora in Bryozoa *sensu lato* as has been done by Cavalier-Smith (1998).

Sipuncula as an Annelid Taxon

Both ML (fig. 1*A*) and BI analyses (fig. 1*B*) recovered Neotrochozoa, which comprises Mollusca, Sipuncula, and Annelida, thus confirming studies using morphological and molecular data (Zrzavý et al. 1998; Giribet et al. 2000; Peterson and Eernisse 2001). Based on segmentation, Annelida has traditionally been regarded as sister to Arthropoda (Hennig 1979; Schram 1991; Sørensen et al. 2000; Nielsen 2001; Brusca and Brusca 2002), but this so-called Articulata hypothesis is significantly rejected by topology testing (table 2, hypothesis 8).

In accordance with mitochondrial amino acid sequences and gene order data (Boore and Staton 2002; Staton 2003; Jennings and Halanych 2005; Bleidorn et al. 2006), our analyses indicate with strong support that Sipuncula is more closely related to Annelida than to Mollusca (fig. 1). More precisely, these unsegmented worms appear as a subtaxon of Annelida, which has also been suggested in some previous analyses (Peterson and Eernisse 2001; Bleidorn et al. 2006; Struck et al. 2007). However, the monophyly of Annelida excluding Sipuncula (Schram 1991; Zrzavý et al. 1998; Ax 1999; Giribet et al. 2000; Sørensen et al. 2000; Nielsen 2001; Brusca and Brusca 2002; Passamaneck and Halanych 2006) could not be ruled out by topology testing (table 2, hypothesis 9). On the other hand, the alternative hypotheses that Sipuncula forms a monophyletic group with Mollusca (Scheltema 1993; Zrzavý et al. 1998) and that Sipuncula is sister to Annelida plus Mollusca (Giribet et al. 2000) were rejected (table 2, hypotheses 10 and 11).

Spiralia—Syndermata, Platyhelminthes, and Lophotrochozoa

Our analyses strongly support the clade Syndermata, formed by Rotifera and Acanthocephala (fig. 1). This taxon has been established on the basis of morphological evidence (Ahlrichs 1995a, 1995b, 1997) and has been further

supported by analyses of 18S rDNA sequences (Garey et al. 1996; Garey and Schmidt-Rhaesa 1998; Littlewood et al. 1998; Zrzavý et al. 1998; Giribet et al. 2000; Herlyn et al. 2003).

The position of Platyhelminthes differs in our analyses as either being sister to Syndermata (fig. 1*A*) or to Neotrochozoa (fig. 1*B*). The former confirms the Platyzoa hypothesis. Platyzoa comprise Platyhelminthes, Syndermata, Gastrotricha, and Gnathostomulida (Garey and Schmidt-Rhaesa 1998; Cavalier-Smith 1998; Giribet et al. 2000) and has first been hypothesized by Ahlrichs (1995a) based on sperm morphology. Platyzoa was either corroborated (Giribet et al. 2000; Passamaneck and Halanych 2006) or contradicted (Zrzavý et al. 1998; Peterson and Eernisse 2001) by rDNA and total evidence analyses. The lack of a robust resolution of the phylogenetic relationships of Platyhelminthes within Spiralia despite the large available data set is probably due to increased substitution rates in Platyhelminthes and Syndermata causing long-branch attraction artifacts. However, the Eubilateria hypothesis (Hennig 1979; Ax 1985) can clearly be rejected by topology testing (table 2, hypothesis 12). According to this hypothesis, Platyhelminthes, which do not have an anus, are considered to be the sister group of all other Bilateria possessing a 1-way gut and an anus.

Lophotrochozoa is defined as including the last common ancestor of lophophorates, molluscs, and annelids, and its descendants (Halanych et al. 1995). Because Bryozoa is more closely related to Neotrochozoa than to Syndermata in our analyses (fig. 1), syndermatans (and according to the ML analysis also platyhelminths) are not lophotrochozoans, even though to further substantiate this conclusion genomic data of Phoronida and Brachiopoda are necessary.

For the clade including Lophotrochozoa, Platyhelminthes, and Syndermata, some authors have used the name Spiralia (Garey and Schmidt-Rhaesa 1998; Giribet et al. 2000; Helmkampf et al. forthcoming). We follow this usage because spiral quartet cleavage might be an autapomorphy of that taxon (see below).

Chaetognatha Remain Enigmatic

Chaetognatha, or arrow worms, represents the sister group of Spiralia and Ecdysozoa in our analyses (fig. 1). This confirms previous findings based on analyses of 18S rDNA (Giribet et al. 2000), mitochondrial DNA (Helfenbein et al. 2004), and an EST data set (Marlétaz et al. 2006). However, alternative hypotheses, namely a common ancestry with Deuterostomia (Ghirardelli 1981; Brusca and Brusca 2002) or Ecdysozoa (Littlewood et al. 1998; Zrzavý et al. 1998; Peterson and Eernisse 2001) or a sister group relationship to Spiralia (Matus et al. 2006), could not be excluded (table 2, hypotheses 13–15). The phylogenetic position of chaetognaths thus remains elusive.

Implications for Character Evolution

Cleavage pattern was often considered a key character for the reconstruction of metazoan phylogeny. Typical spiral quartet cleavage with mesoderm formation by the 4d mesoteloblast or one of its daughter cells (Sørensen et al. 2000; Nielsen 2001) is known from several lophotrochozoan groups (Mollusca, Annelida, Nemertea, and Entoprocta), Platyhelminthes, and Gnathostomulida. If we map this character state on our tree (fig. 1) considering the close relationship of Syndermata to Gnathostomulida (Ahlrichs 1995a, 1995b, 1997; Cavalier-Smith 1998; Garey and Schmidt-Rhaesa 1998; Giribet et al. 2000; Sørensen et al. 2000; Nielsen 2001), it turns out to be a possible autapomorphy of the clade including Syndermata, Plathyhelminthes, and Lophotrochozoa, for which we accepted the name Spiralia, although it has been secondarily modified several times within this clade (e.g., in Syndermata, Neoophora, Ectoprocta, Brachiopoda, and Cephalopoda). The sister group relation of ectoprocts and entoprocts demonstrates that the transition from spiral to radial cleavage can happen within a clade without any transitional stages being preserved. After all, the different cleavage types were one of the main reasons that the 2 taxa were classified in different major groups for more than a century.

Often coelomic cavities were considered an autapomorphy of a clade Coelomata (Hennig 1979; Blair et al. 2002; Philip et al. 2005). If the coelomic cavities of lophotrochozoans are considered homologous to those of deuterostomes and to the small coelomic cavities present in some ecdysozoans, our trees would indicate a frequent reduction of coelomic cavities in several bilaterian lineages (e.g., in chaetognaths, priapulids, nematodes, platyzoans, and entoprocts). However, the differing developmental origin of coelomic cavities in the different bilaterian lineages cast doubts on the homology of the coelom across bilaterians (Nielsen 2001).

The significant rejection of the Eubilateria hypothesis and the derived position of platyhelminths within Spiralia indicates that the anus has been secondarily reduced in platyhelminths, in which the mouth is the only opening to the intestinal system.

Finally, the significant rejection of Articulata as well as the derived position of Annelida within Spiralia supports the hypothesis that segmentation originated convergently in annelids and arthropods. The placement of unsegmented worms within Annelida, namely Sipuncula (this study; Peterson and Eernisse 2001; Bleidorn et al. 2006; Struck et al. 2007) and Echiura (McHugh 1997; Bleidorn et al. 2003; Struck et al. 2007), further reveals that segmentation has been secondarily lost in annelid subtaxa. Sipunculans possess a U-shaped gut, a feature already established in Cambrian fossils (Huang et al. 2004). The movement of the anus in the anterior direction requires the disorganization of segmentation, a factor that may have eased inhabiting holes in solid substrates.

The results presented herein, therefore, indicate that several of the supposed key characters of animal phylogeny such as cleavage pattern, coelomic cavities, body segmentation, and gut architecture are much more variable during evolution than previously thought.

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2.3. Phylogenomic analyses of lophophorates (brachiopods, phoronids and bryozoans) confirm the Lophotrochozoa concept

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Phylogenomic analyses of lophophorates (brachiopods, phoronids and bryozoans) confirm the Lophotrochozoa concept

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Based on embryological and morphological evidence, Lophophorata was long considered to be the sister or paraphyletic stem group of Deuterostomia. By contrast, molecular data have consistently indicated that the three lophophorate lineages, Ectoprocta, Brachiopoda and Phoronida, are more closely related to trochozoans (annelids, molluscs and related groups) than to deuterostomes. For this reason, the lophophorate groups and Trochozoa were united to Lophotrochozoa. However, the relationships of the lophophorate lineages within Lophotrochozoa are still largely unresolved. Maximum-likelihood and Bayesian analyses were performed based on a dataset comprising 11 445 amino acid positions derived from 79 ribosomal proteins of 39 metazoan taxa including new sequences obtained from a brachiopod and a phoronid. These analyses show that the three lophophorate lineages are affiliated with trochozoan rather than deuterostome phyla. All hypotheses claiming that they are more closely related to Deuterostomia than to Protostomia can be rejected by topology testing. Monophyly of lophophorates was not recovered but that of Bryozoa including Ectoprocta and Entoprocta and monophyly of Brachiozoa including Brachiopoda and Phoronida were strongly supported. Alternative hypotheses that are refuted include (i) Brachiozoa as the sister group of Mollusca, (ii) ectoprocts as sister to all other Lophotrochozoa including Platyzoa, and (iii) ectoprocts as sister or to all other protostomes except chaetognaths.

Keywords: Brachiopoda; Bryozoa; Lophophorata; Metazoa; Phoronida; phylogeny

1. INTRODUCTION

Phylogenetic analyses of molecular markers have substantially changed our view of animal evolution in the past two decades (Halanych 2004). The new subdivision of Protostomia into two main groups, Lophotrochozoa and Ecdysozoa, originally based on 18S rDNA sequences (Halanych *et al.* 1995; Aguinaldo *et al.* 1997), has been corroborated by sequences of single nuclear protein-encoding genes (e.g. Ruiz-Trillo *et al.* 2002; Anderson *et al.* 2004), datasets combining multiple nuclear protein-encoding sequences (Peterson *et al.* 2004; Helmkampf *et al.* 2008) and phylogenomic approaches (Philippe *et al.* 2005; Philippe & Telford 2006; Baurain *et al.* 2007; Hausdorf *et al.* 2007).

However, the relationships within Lophotrochozoa could not be resolved robustly so far, neither with a large dataset of combined small and large subunit rDNAs (Passamaneck & Halanych 2006), nor with a dataset including several nuclear protein-encoding sequences (Helmkampf *et al.* 2008). Phylogenomic data were able to resolve some disputed relationships within Lophotrochozoa (Hausdorf *et al.* 2007), but such data are still missing for some phylogenetically important phyla such as Brachiopoda and Phoronida.

The placement of the lophophorate taxa within Lophotrochozoa as indicated by molecular phylogenetic studies is particularly inconsistent with the morphological evidence (Lüter & Bartolomaeus 1997). As originally defined based on morphology, Lophophorata consists of Ectoprocta, Brachiopoda and Phoronida, taxa that share a ciliated, tentacular feeding apparatus around the mouth opening called lophophore. Based on embryological and morphological characters, Lophophorata was traditionally considered the sister or paraphyletic stem group of Deuterostomia (Hennig 1979; Schram 1991; Ax 1995; Lüter & Bartolomaeus 1997; Sørensen *et al.* 2000; Brusca & Brusca 2003). However, Nielsen (2001) argued that the lophophore of Bryozoa is not homologous to that of Brachiopoda plus Phoronida, and considered Lophophorata diphyletic. He suggested that ectoprocts are more closely related to entoprocts within Spiralia, whereas he still considered Brachiopoda+Phoronida as the sister group of Deuterostomia *sensu stricto* (his Neorenalia). By contrast, the molecular phylogenetic studies have shown Ectoprocta as well as Brachiopoda and Phoronida to be more closely related to Trochozoa, i.e. Annelida, Mollusca and related groups, than to Deuterostomia; these include analyses that used rDNA (Halanych *et al.* 1995; Mackey *et al.* 1996; Littlewood *et al.* 1998; Cohen 2000; Giribet *et al.* 2000; Peterson & Eernisse 2001; Mallatt & Winchell 2002; Passamaneck & Halanych 2006), *Hox* genes (de Rosa *et al.* 1999; Passamaneck & Halanych 2004), mitochondrial protein genes (Stechmann & Schlegel 1999; Helfenbein & Boore 2004; Waeschenbach *et al.* 2006), single nuclear protein genes (e.g. Ruiz-Trillo *et al.* 2002; Anderson *et al.* 2004) and sets of multiple nuclear

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protein genes (Helmkampf *et al.* 2008). For this reason, Halanych *et al.* (1995) united the lophophorate groups and Trochozoa into Lophotrochozoa.

Yet the morphological similarities between Brachiozoa (Brachiopoda + Phoronida = Phoronozoa) and Deuterostomia seem so strong that they affect the topology of the trees even in some analyses considering both 18S rDNA sequences and morphological characters. In the total-evidence analysis of Zrzavý *et al.* (1998), Brachiozoa clustered with Deuterostomia, while in the analysis of Eernisse & Peterson (2004) deuterostomes were the sister group of Lophotrochozoa. There, the brachiozoans were sister to the remaining lophotrochozoan groups. However, this was not the case in some other total-evidence analyses (Giribet *et al.* 2000; Peterson & Eernisse 2001) in which Brachiopoda and Phoronida were part of Lophotrochozoa (or Trochozoa), and Deuterostomia did not appear as the sister group of Lophotrochozoa. As a caveat to these findings, the above-mentioned studies did not include many genes at all.

To provide a more robust resolution of the relationships of Brachiopoda, Phoronida and Bryozoa, we supplemented a previously compiled dataset of 79 sequences encoding ribosomal proteins with new expressed sequence tag (EST) sequences of a brachiopod and a phoronid.

2. MATERIAL AND METHODS

(a) *EST generation and processing*

Specimens of the brachiopod *Novocrania anomala* (Müller 1776) and the phoronid *Phoronis muelleri* Selys-Longchamps 1903 were collected in the Gullmarsfjord near Kristineberg, Sweden. To minimize potential contamination sources, care was taken to remove epibionts growing on the shells and tubes, respectively. Total RNA was isolated from pools of 20 living adult individuals each with the TRIzol Plus purification system (Invitrogen, Karlsruhe, Germany). The mRNA of *Novocrania* was purified by the Dynabeads mRNA Purification Kit (Invitrogen) before it was transcribed by primer extension. The products were size fractioned and cloned directionally using CloneMiner technology (Invitrogen) to construct a cDNA library. In *Phoronis*, the PolyATtract mRNA Isolation System III (Promega, Mannheim, Germany) was used, followed by transcription and long-distance PCR amplification, size fractioning and directional cloning employing the Creator SMART cDNA Library Construction Kit (Clontech, Heidelberg, Germany).

From these libraries, ESTs were generated by sequencing 2247 (*Novocrania*) and 2315 (*Phoronis*) clones from the 5′ end on the automated capillary sequencer system ABI 3730XL (Applied Biosystems, Darmstadt, Germany) using BigDye chemistry (Applied Biosystems). EST processing was accomplished as described previously (Hausdorf *et al.* 2007), with the addition of a second clustering step after quality clipping to improve contig assembly. The final number of contigs acquired from each organism amounted to 1699 (*Novocrania*) and 1467 (*Phoronis*).

(b) *Extraction and alignment of ribosomal protein sequences*

Ribosomal protein sequences were retrieved from the new datasets using 79 human ribosomal protein sequences as local BLAST search queries. A total of 42 and 54 (at least partial) ribosomal protein sequences were identified in *Novocrania* and *Phoronis*, respectively. These sequences, available in GenBank under the accession nos. EU558289–EU558330 (*Novocrania*) and EU558331–EU558384 (*Phoronis*), were individually aligned to orthologous riboprotein sequences of 36 additional taxa compiled previously (Hausdorf *et al.* 2007) and of a nemertean (Struck & Fisse 2008) using the ClustalW algorithm (Thompson *et al.* 1994). The resulting single-gene alignments were inspected and adjusted manually, and concatenated into a single multiple sequence alignment. Ambiguously aligned positions were automatically removed by Gblocks (Castresana 2000) applying low stringency parameters. The resulting alignment included 5458 amino acids of *Novocrania* (47.8% of the total alignment length minus gap positions) and 7922 amino acids of *Phoronis* (69.3%). More extensive information about the number of genes and amino acids present per taxon is reported in the electronic supplementary material. The final alignment has been deposited at TreeBase (http://www.treebase.org, study accession no. S2050).

(c) *Phylogenetic analyses*

Maximum-likelihood analyses were conducted with Treefinder (Jobb *et al.* 2004; Jobb 2007). The rtRev + G + F model of protein evolution was used for the maximum-likelihood analyses because its fit to the present dataset was superior to other models according to the Akaike information criterion with a correction term for small sample size. Confidence values for the edges of the maximum-likelihood tree were computed by bootstrapping (100 replications; Felsenstein 1985).

To test predefined phylogenetic hypotheses, we used constrained trees and the 'resolve multifurcations' option of Treefinder to obtain the maximum-likelihood tree for a specified hypothesis. Then we investigated whether the maximum-likelihood trees for these hypotheses are part of the confidence set of trees applying the approximately unbiased test (Shimodaira 2002) and the expected likelihood weights method (Strimmer & Rambaut 2002).

Bayesian analyses were performed using PhyloBayes v. 2.3 (Blanquart & Lartillot 2006) based on the site-heterogeneous CAT model (Lartillot & Philippe 2004). Four independent Markov chains, starting from random points of the parameter space, were run simultaneously for 20 000 cycles each. Chain stationarity was evaluated by monitoring key parameters for long-term trends (e.g. log likelihood, alpha parameter). The first 2000 points were consequently discarded as burn-in. Both runs reached convergence, indicated by the maximal and mean difference of split frequencies amounting to 0.21 and 0.007, respectively. Subsampling every tenth tree from each chain, a 50% majority rule consensus tree was finally computed. We accept Bayesian posterior probabilities larger than 95% and bootstrap values larger than 70% as significant.

3. RESULTS AND DISCUSSION

(a) *Deuterostome versus lophotrochozoan relationships of lophophorates*

The results of our maximum-likelihood (figure 1*a*), as well as Bayesian analyses (figure 1*b*), based on concatenated sequences of 79 ribosomal proteins encompassing 11 445 amino acid positions from 39 taxa, demonstrate that the three lophophorate lineages, Ectoprocta, Brachiopoda and Phoronida, are more closely related to trochozoan

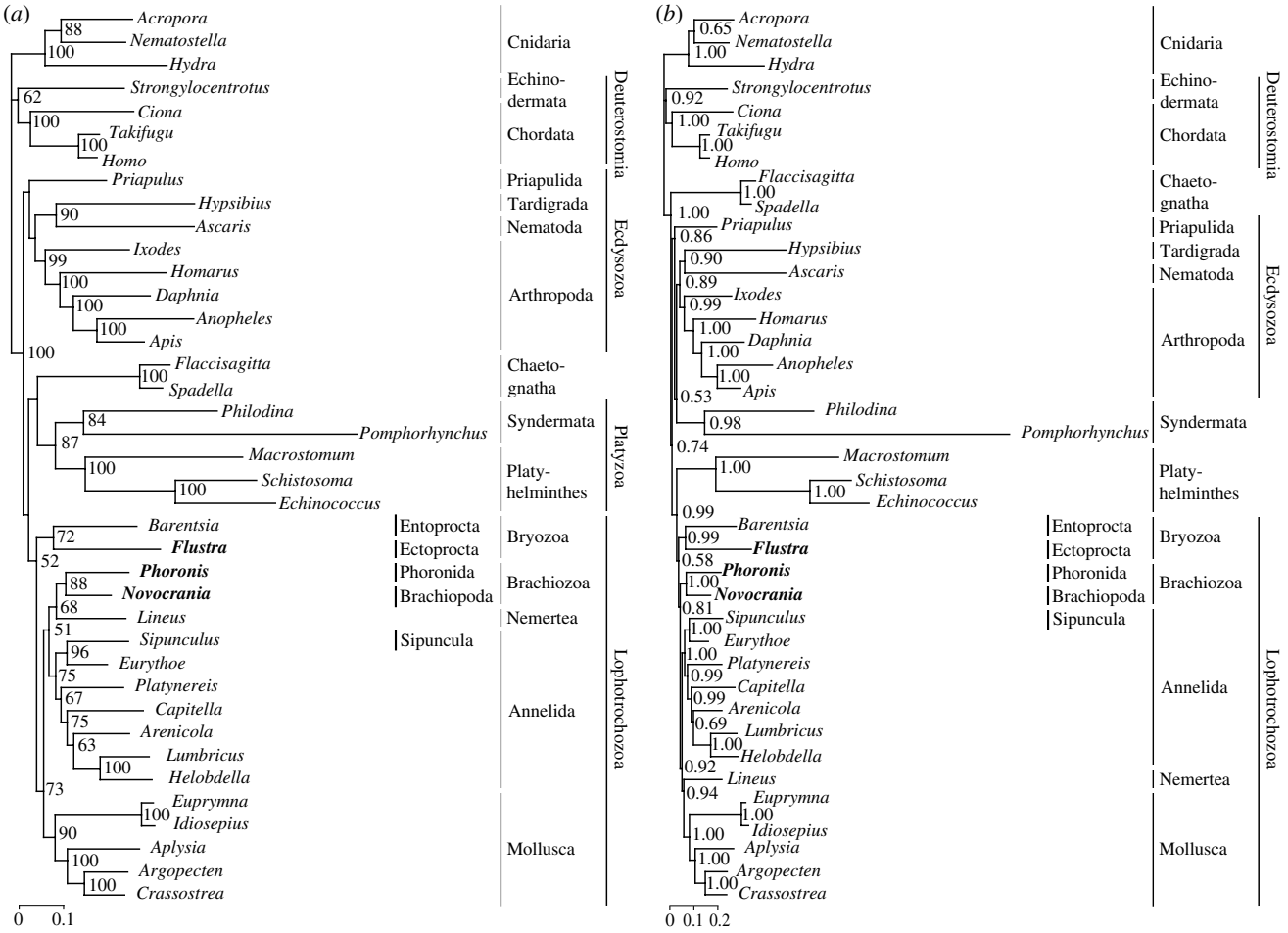

Figure 1. Phylogenetic analyses of lophophorate relationships based on 11 445 amino acid positions derived from 79 concatenated ribosomal proteins. Lophophorate lineages appear in bold. (*a*) Maximum-likelihood tree. Bootstrap support values larger than 50% are shown to the right of the nodes. (*b*) Bayesian inference reconstruction. Bayesian posterior probabilities are shown to the right of the nodes.

phyla than to deuterostomes. They do not form a monophyletic group. Thus, our analyses confirm the results of previous molecular phylogenetic studies (see §1).

Characters that were traditionally used to unite Lophophorata with deuterostomes include the following: a body organization with three distinct coelomic cavities, namely protocoel, mesocoel and metacoel (archimery); a mesosomal tentacular apparatus; entomesoderm derived from the archenteron by enterocoely, larvae with upstream-collecting ciliary bands; and heterogeneously assembled metanephridia (Hennig 1979; Schram 1991; Ax 1995; Lüter & Bartolomaeus 1997; Sørensen *et al.* 2000; Brusca & Brusca 2003). However, the hypothesis that all lophophorate lineages are more closely allied to Deuterostomia than Protostomia can be rejected by topology tests based on our ribosomal protein data (table 1, hypothesis 1). Nielsen (2001) argued that ectoprocts show no trace of archimery and that only Brachiopoda and Phoronida form a monophyletic group with Deuterostomia *sensu stricto* (his Neorenalia). Lüter (2000) suggested that the origin of the coelomic anlage from differentiated archenteral epithelium, which he defined as enterocoely, is a synapomorphy of Brachiopoda and Deuterostomia; he therefore considered these two taxa as sister groups. Consequently, we tested the hypotheses that Brachiozoa or Brachiopoda alone are the sister groups of Deuterostomia. Both possibilities were rejected (table 1, hypotheses 2–3).

The conflicting results concerning the phylogenetic relationships of the lophophorates is a major incongruity between morphological and molecular phylogenetic approaches. However, in the last decade, the morphological evidence for a close relationship between the lophophorate groups and the deuterostomes has become weaker by careful re-examinations of the characters. It has been shown that neither brachiopods nor phoronids possess three coelomic cavities, because a protocoel is lacking in all lophophorate groups (Lüter 2000; Bartolomaeus 2001). Thus, the archicoelomate concept (Siewing 1980) uniting Lophophorata and Deuterostomia, founded on the similarities of three distinct coelomic cavities, lost its basis. Additionally, the finding that Pterobranchia may nest within the enteropneusts (Cameron *et al.* 2000; Peterson & Eernisse 2001; Winchell *et al.* 2002) suggests that the ancestral deuterostome more closely resembled a mobile worm-like enteropneust than a sessile colonial pterobranch. This means that the similar tentacular feeding apparatuses of lophophorates and pterobranchs are not a synapomorphy of lophophorates and deuterostomes as supposed previously (Hennig 1979; Schram 1991; Ax 1995; Lüter & Bartolomaeus 1997), but evolved independently as convergent adaptations to the sessile

Table 1. Topology test results.

no.[a]	phylogenetic hypothesis	references claiming the hypothesis	likelihood	Δlikelihood[b]	AU[c]	ELW[d]
	ML tree (figure 1*a*)		−273 512	0	0.6421	0.5010
1	Lophophorata+Deuterostomia	Hennig (1979), Schram (1991), Ax (1995), Lüter & Bartolomaeus (1997), Sørensen *et al.* (2000) and Brusca & Brusca (2003)	−273 957	445	0.0000*	0.0000*
2	Brachiozoa+Deuterostomia	Nielsen (2001)	−273 977	465	0.0000*	0.0000*
3	Brachiopoda+Deuterostomia	Lüter (2000)	−273 805	293	0.0000*	0.0000*
4	Ectoprocta sister to all other Lophotrochozoa inclusive Platyzoa	Halanych *et al.* (1995), Halanych (2004) and Passamaneck & Halanych (2006)	−273 599	87	0.0000*	0.0052*
5	Ectoprocta sister to all other protostomes except chaetognaths	Giribet *et al.* (2000)	−273 624	112	0.0000*	0.0002*
6	Trochozoa (Entoprocta+Eutrochozoa)	Zrzavý *et al.* (1998), Giribet *et al.* (2000) and Peterson & Eernisse (2001)	−273 592	80	0.0634	0.0107*
7	Eutrochozoa (Neotrochozoa+Nemertea)	Zrzavý *et al.* (1998), Giribet *et al.* (2000) and Peterson & Eernisse (2001)	−273 535	23	0.4114	0.1817
8	Neotrochozoa (Annelida+Mollusca)	Zrzavý *et al.* (1998), Giribet *et al.* (2000) and Peterson & Eernisse (2001)	−273 520	8	0.5929	0.2777
9	Conchozoa (Brachiozoa+Mollusca)	Cavalier-Smith (1998) and Halanych (2004)	−273 571	59	0.0000*	0.0015*
10	Lophophorata monophyly	Emig (1984)	−273 571	59	0.1111	0.0219*

[a] Numbers refer to the order of appearance in the text.
[b] Δlikelihood, differences between the likelihood of a constrained tree and the maximum-likelihood tree.
[c] AU, approximately unbiased test (*p*-values). Values for topologies significantly rejected at the 0.05 level are indicated by an asterisk.
[d] ELW, expected likelihood weights. Values for topologies not included in the 0.95 confidence set are indicated by an asterisk.

lifestyle (Halanych 1996). Moreover, Lüter (2000) argued that the mesoderm does not originate by enterocoely in Ectoprocta and Phoronida, but that this is the case in Brachiopoda and Deuterostomia only. What is more, whether the mesoderm of brachiopods originates by enterocoely is also in dispute. Jenner (2004) tentatively concluded that reports of true enterocoely, i.e. mesoderm origin by epithelial folding, in brachiopods appear unsupported and that no fundamental difference in the source of mesoderm and mode of coelomogenesis exists between brachiopods and various protostomes. To conclude, there are fewer morphological characters arguing against protostome affiliations of brachiopods and phoronids than traditionally assumed.

(b) *Relationships of lophophorates within Lophotrochozoa*

The phylogenetic analyses of our ribosomal protein dataset (figure 1) strongly indicate that Brachiopoda and Phoronida constitute a monophyletic group, Brachiozoa (=Phoronozoa; bootstrap support 88%, Bayesian posterior probability 1.00). This corroborates previous results based on rDNA (Mackey *et al.* 1996; Cohen *et al.* 1998; Littlewood *et al.* 1998; Cohen 2000; Mallatt & Winchell 2002; Halanych 2004; Cohen & Weydmann 2005; but see Passamaneck & Halanych 2006), sodium–potassium ATPase α-subunit (Anderson *et al.* 2004), morphology (Nielsen 2001) and a combination of morphological and 18S rDNA datasets (Zrzavý *et al.* 1998; Giribet *et al.* 2000; Peterson & Eernisse 2001).

Our previous study (Hausdorf *et al.* 2007) recovered Ectoprocta as the sister group of Entoprocta, but this finding was considered tentative until a phoronid and a brachiopod could be added to the analysis, which was done here (figure 1). Indeed Ectoprocta and Entoprocta remain strongly united (bootstrap support 72%, Bayesian posterior probability 0.99). This agrees with the hypothesis that Bryozoa *sensu lato* is monophyletic (Nielsen 1971, 1985, 2001; Cavalier-Smith 1998). Alternative hypotheses concerning the phylogenetic position of ectoprocts, namely that they are sister to all other Lophotrochozoa including Platyzoa, i.e. Platyhelminthes, Syndermata and related groups (Halanych *et al.* 1995; Littlewood *et al.* 1998; Halanych 2004; Passamaneck & Halanych 2006), or that they are sister to all other protostomes except chaetognaths (Giribet *et al.* 2000) could be rejected (table 1, hypotheses 4–5).

Peterson & Eernisse (2001) defined several nested clades within Lophotrochozoa, namely (i) Neotrochozoa, which unites Mollusca and Annelida (with the annelids including Echiura and Sipuncula; see Hausdorf *et al.* (2007) and Struck *et al.* (2007)), (ii) Eutrochozoa, which includes Neotrochozoa and Nemertea, and (iii) Trochozoa, which comprises Eutrochozoa and Entoprocta. This last hypothesis, which we could not rule out with the previous dataset (Hausdorf *et al.* 2007), is now rejected by the expected likelihood weights method relying on the enlarged dataset (table 1, hypothesis 6). Although the more conservative approximately unbiased test is still marginally insignificant, this strengthens the evidence for the monophyly of Bryozoa *sensu lato*. On the other hand,

neither the Neotrochozoa hypothesis nor the Eutrochozoa hypothesis is rejected by either test method (table 1, hypotheses 7–8).

Brachiopods plus phoronids appear as the sister group of nemerteans in the maximum-likelihood tree (figure 1*a*). By contrast, the Bayesian inference analysis shows a sister-group relationship of Brachiozoa and Eutrochozoa (figure 1*b*). The relationships of Brachiozoa within Lophotrochozoa thus remain uncertain. However, we can dismiss the Conchozoa hypothesis (Cavalier-Smith 1998; Mallatt & Winchell 2002), according to which Brachiozoa is the sister group of Mollusca (table 1, hypothesis 9).

As mentioned earlier, the three traditional lophophorate lineages, Ectoprocta, Phoronida and Brachiopoda, did not join into a monophyletic clade in our trees (figure 1). The monophyly of Lophophorata was rejected with the expected likelihood weights method, but not with the approximately unbiased test (table 1, hypothesis 10). If we constrain the monophyly of Lophophorata, it becomes the sister group of Eutrochozoa in the resulting maximum-likelihood tree (not shown). In this tree, Entoprocta is the sister group of Lophophorata plus Eutrochozoa. Even if this topology should prove correct, the radial cleavage of Lophophorata would be a secondary modification derived from spiral cleavage, given that the spiral cleavage of Entoprocta is homologous to that of Annelida and Mollusca.

When we constrain the monophyly of Eutrochozoa (table 1, hypothesis 7), then Brachiozoa and Bryozoa (including Ectoprocta and Entoprocta) form a monophyletic group in the resulting maximum-likelihood tree. The same maximum-likelihood tree results if we constrain the monophyly of Brachiozoa and Bryozoa. Thus, the test results (table 1, hypothesis 7) apply to this hypothesis as well. This extended version of 'Lophophorata' including Entoprocta is therefore part of the confidence set of trees, given our ribosomal protein dataset, a possibility that is especially interesting, because it is in better agreement with morphological data than topologies that suggest independent origins of Ectoprocta and Brachiozoa within Lophotrochozoa. Potential synapomorphies of Brachiozoa and Bryozoa are the transition to a sessile lifestyle accompanied by the evolution of a horseshoe-shaped, tentacular feeding apparatus and a hydrostatic skeleton consisting of a lophophore coelom and a trunk coelom. In this view, both coelomic cavities were connected in the common ancestor of the two bryozoan subgroups and then were lost in Entoprocta. Most potential synapomorphies of Brachiozoa and Bryozoa are characters that were once thought to support a sister-group relationship between Lophophorata and Deuterostomia, but in light of the present evidence that these two groups are unrelated, must have originated by convergence (see above). Hypotheses that suppose that Ectoprocta and Brachiozoa originated independently of each other from different lophotrochozoan ancestors would require additional convergences of these characters.

Despite the progress presented herein, the resolution achieved in our analyses is still insufficient to fully reconstruct the evolutionary history of Lophotrochozoa. This lack of resolution could neither be avoided by the inclusion of many riboprotein genes and all major lophotrochozoan taxa, nor by the use of the CAT model, which has been shown often to overcome long-branch attraction artefacts when other models fail (Baurain *et al.* 2007; Lartillot *et al.* 2007). Actually, the grouping of taxa with the longest branches in the maximum-likelihood tree (figure 1*a*), namely Syndermata and Platyhelminthes, is dissolved in the Bayesian inference reconstruction calculated with the CAT model (figure 1*b*). Further systematic errors unaccounted for by the present tree reconstruction methods, aggravated by the presumably rapid radiation of the lophotrochozoan taxa in the Late Precambrian and the limited taxon sampling within many phyla, might be responsible for the lack of resolution within Lophotrochozoa, which has been observed both here and in other studies (Halanych *et al.* 1995; Giribet *et al.* 2000; Peterson & Eernisse 2001; Mallatt & Winchell 2002; Ruiz-Trillo *et al.* 2002; Anderson *et al.* 2004; Passamaneck & Halanych 2006; Helmkampf *et al.* 2008). Improved models of molecular evolution and further taxonomic sampling within lophophorates and other lophotrochozoans will hopefully solve these issues in the future.

Added in preparation. While our manuscript was submitted, Dunn *et al.* (2008) published an important phylogenomic analysis of a huge number of new metazoan EST data. Regarding the relationships of brachiopods and phoronids, our maximum-likelihood tree (figure 1*a*) corresponds closely with the results presented by Dunn *et al.* (2008). In both analyses, brachiopods and phoronids form a clade with nemerteans (clade A in Dunn *et al.* 2008) that is the sister group of annelids (including sipunculans). These groups together (clade B in Dunn *et al.* 2008) are sister to the molluscs (together called clade C in Dunn *et al.* 2008). However, the results of our analyses differ from those of Dunn *et al.* (2008) with regard to the relationships of ectoprocts and entoprocts. Whereas these two groups form a well-supported clade in our analyses, their position is unstable in the analyses of Dunn *et al.* (2008). In the 77-taxon analysis of Dunn *et al.* (2008; figure 1), ectoprocts are sister to Platyzoa and entoprocts are sister to clade C.

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2.4. Reducing compositional heterogeneity improves phylogenomic inference of lophotrochozan relationships

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Reducing compositional heterogeneity improves phylogenomic inference of lophotrochozoan relationships

Research Article

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Running head: Compositional bias and phylogenomic inference

The amino acid composition of proteins is not homogenous across Metazoa. We studied the influence of compositional heterogeneity on phylogenomic analyses of metazoan relationships. Approaches to reduce this heterogeneity like the exclusion of taxa with strongly differing amino acid composition, the use of LogDet distances or the recoding of amino acids into groups with similar properties diminish artifacts and result in higher confidence values. Given a fixed threshold of accepted significant pairwise differences in the composition of the sequences used, fewer taxa have to be excluded from the recoded data set than from the unmodified amino acid data set. Despite the loss of information that results from employing a reduced amino acid alphabet, the support for Bryozoa and Brachiozoa is higher in the maximum likelihood tree based on the analysis of the recoded data set than in the one resulting from the analysis of the unmodified amino acid data set from which the taxa most strongly differing in amino acid composition have been excluded or the neighbor-joining tree based on LogDet + Γ distances. Thus, using reduced amino acid alphabets is a more efficient method for mitigating artifacts in phylogenetic analyses that arise from compositional heterogeneity than the simple exclusion of taxa with a divergent amino acid composition or the employment of distance methods using LogDet distances. Applying these approaches to reduce compositional heterogeneity on a data set with eleven representatives of the lophophorate lineages and entoprocts, we could confirm the monophyly of Bryozoa and Brachiozoa. This data set includes two new EST data sets of major ectoproct groups not covered so far. Within Bryozoa our analyses validate the monophyly of Ectoprocta and a sister group relationship of Phylactolaemata and Gymnolaemata including Stenolaemata and Eurystomata.

Introduction

In the 1990' the analyses of 18S rDNA sequences profoundly changed our understanding of metazoan phylogeny. The polyphyly of taxa like Articulata, Aschelminthes, Radialia and Coelomata has been recognized, and Lophotrochozoa and Ecdysozoa were newly delimited based on these data (Halanych et al. 1995; Aguinaldo et al. 1997). Analyses of alignments containing large amounts of protein sequences but only few taxa indicated that the new findings might have been artifacts (Blair et al. 2002; Wolf et al. 2004; Philip et al. 2005). These concerns were rejected by investigating protein-coding genes with a better taxon sampling (Ruiz-Trillo et al. 2002; Anderson et al. 2004; Peterson et al. 2004; Helmkampf et al. 2008a). However, the relationships of the phyla within the larger clades could be resolved robustly neither with rDNA, nor with single protein coding genes or small multigene data sets.

The advent of phylogenomics based on large EST data sets or genome projects resulted in alignments that are a magnitude larger than previously available data sets, and promised the resolution of the relationships of metazoan phyla (Philippe et al. 2005; Philippe and Telford 2006; Baurain et al. 2007). Taxon sampling has been improved so that genomic scale data are now available from most metazoan phyla (Hausdorf et al. 2007; Brinkmann and Philippe 2008; Dunn et al. 2008; Helmkampf et al. 2008b; Struck and Fisse 2008; Witek et al. 2008). Despite these progresses, many internal nodes are still poorly supported, and different analyses produce contradictory results. One such example concerns ectoprocts and entoprocts that form a monophylum in some phylogenomic analyses (Hausdorf et al. 2007; Helmkampf et al. 2008b; Struck and Fisse 2008; Witek et al. 2008), whereas ectoprocts are sister to Platyzoa and entoprocts to the remaining lophotrochozoans in the tree of Dunn et al. (2008). One reason for such incongruent results may be violations of the assumptions of the models used for tree reconstruction that lead to systematic errors (Delsuc et al. 2005).

Most models of protein evolution assume that amino acids evolve according to a time-reversible Markov model. Violations of this assumption can result in incorrect topological estimation. Compositional heterogeneity (Lockhart et al. 1994; Foster and Hickey 1999; Foster 2004; Jermiin et al. 2004; Phillips et al. 2004; Collins et al. 2005) or a combination of compositional heterogeneity and rate heterogeneity among lineages (Ho and Jermiin 2004) are common problems in this respect. Lartillot and

Philippe (2008) noted that the assumption of a time-homogeneous amino acid replacement process made by conventional protein models is strongly violated in the metazoan phylogenomic data set they examined. As this entails a risk of observing artifacts as a result of compositional bias, we studied the influence of compositional heterogeneity on phylogenetic analyses of metazoan relationships. We show that approaches to reduce compositional heterogeneity like the exclusion of taxa with strongly differing amino acid composition, the employment of distance methods using LogDet distances (Lockhart et al. 1994; Tamura and Kumar 2002) or the recoding of amino acids in groups with similar properties (Embley et al. 2003; Rodríguez-Ezpeleta et al. 2007; Susko and Roger 2007) diminish artifacts and result in higher confidence values. We focused on the relationships of the lophophorate lineages and entoprocts and improved the taxon sampling of these groups by adding two new expressed sequence tag (EST) data sets for so far uncovered clades of ectoprocts and by combining all data sets of lophophorates and entoprocts existing to date. The new data set includes eleven representatives of the lophophorate lineages and entoprocts, which more than doubles the amount of data of these groups compared with previous phylogenomic analyses that comprised four (Helmkampf et al. 2008b) or five (Dunn et al. 2008) of these taxa. Using this data set we investigated especially hypotheses concerning the relationships of ectoprocts and entoprocts, and the relationships of the major clades within ectoprocts.

Materials and Methods

EST generation and processing

Two new EST (expressed sequence tag) data sets of the ectoproct bryozoans *Alcyonidium diaphanum* Lamouroux, 1813 and *Tubulipora* sp. were generated for this study. Both species represent major ectoproct clades that have not been covered by genomic-scale data so far, namely Ctenotomata and Cyclostomata, respectively. Specimens of *Alcyonidium* were from Helgoland, Germany, whereas *Tubulipora* was obtained from a culture kept at the laboratories of the Marine Biological Association in Plymouth, Great Britain, that is descended from a specimen collected off Stoke Point, South Devon, Great Britain. Total RNA was extracted from a lobe of an adult *Alcyonidium* colony by homogenization in liquid

nitrogen and employing the TRIzol Plus purification system (Invitrogen, Karlsruhe, Germany). The subsequent cDNA library construction was carried out at the Max Planck Institute for Molecular Genetics in Berlin as follows. Isolation of mRNA was performed with the Dynabeads mRNA Purification Kit (Invitrogen, Karlsruhe, Germany), and transcription into cDNA achieved by the primer extension method. The products were fractioned according to size and cloned directionally using CloneMiner technology (Invitrogen, Karlsruhe, Germany) and the vector pDONR222. In the case of *Tubulipora,* total RNA and mRNA were isolated from a whole colony as above; the mRNA was transcribed and amplified by long distance PCR. The products were size fractioned and cloned directionally by the Creator SMART cDNA Library Construction Kit (Clontech, Heidelberg, Germany) using the vector pDNR-LIB. By 5' sequencing clones with the automated capillary sequencer system ABI 3730 XL (Applied Biosystems, Darmstadt, Germany), 2331 and 2040 ESTs were generated from the *Alcyonidium* and *Tubulipora* libraries, respectively. These single reads are available at NCBI's dbEST. EST assembly was accomplished by a processing pipeline at the Center for Integrative Bioinformatics in Vienna as previously described (Hausdorf et al. 2007, with the addition of a second clustering step to improve contig assembly), and resulted in a total of 1369 contigs obtained from *Alcyonidium*, and 1375 from *Tubulipora.*

Extraction and alignment of ribosomal protein sequences

Ribosomal protein gene sequences were retrieved from the newly-generated ESTs and 46 additional, publicly available data sets. These sets comprised both non-redundant transcriptome data derived from whole genome projects (e.g., NCBI's RefSeq and JGI's filtered model data sets) as well as EST data processed as described above, and included all lophophorate and ectoproct taxa currently represented by genomic-scale data. Slow-evolving taxa were selected instead of fast-evolving ones whenever possible (e.g., *Paraplanocera*, *Xiphinema*). These data were surveyed by the tblastn algorithm based on a query set of 78 human cytoplasmic ribosomal protein sequences acquired from the Ribosomal Protein Gene Database (http://ribosome.med.miyazaki-u.ac.jp, excluding *rps4y* and *rpl41*, which are redundant or too short, respectively). All hits with an *e*-value lower than 1×10^{-10} were again queried

against the human ribosomal protein sequences by employing the genewisedb algorithm (score cut-off < 50) as implemented in the Wise2 package (Birney et al. 2004). This was done to receive protein translations corrected for frameshift errors due to sequencing inaccuracy. Of each gene and taxon, the longest sequence was taken. The resulting, non-redundant gene sets were individually aligned by the L-INS-i algorithm implemented in MAFFT (Katoh et al. 2002; Katoh and Toh 2008) and edited by Gblocks (Castresana 2000) using low stringency parameters. The final alignment, spanning 11544 amino acid positions, was attained by concatenating all single alignments and has been deposited at TreeBASE (http://www.treebase.org, preliminary accession number SN4402). In this dataset, *Alcyonidium* is represented by 10383 positions (89.9 %) derived from 74 genes, and *Tubulipora* by 7192 positions (62.3 %) gathered from 49 genes. The overall density of the matrix, i.e. the proportion of determined amino acid positions, amounts to 82.6 %. Alignments with reduced taxon sets were attained by removing taxa from the final, complete alignment.

Phylogenetic analyses

Maximum likelihood analyses were conducted with Treefinder, version of October 2008 (Jobb et al. 2004; Jobb 2007). An appropriate model for protein evolution was determined with the 'propose model' option of Treefinder based on the Akaike Information Criterion with a correction term for small sample size. According to this criterion a mixed model that is a linear combination of 14 empirical models of protein evolution considering among-site rate variation with a four-category discrete gamma-distribution for rates was chosen. Confidence values for the edges of the maximum likelihood tree were computed by bootstrapping (Felsenstein 1985) (100 replications).

Some amino acid substitutions are found less frequently in real data than predicted by empirical models, which may lead to biases in phylogenetic reconstruction. Thus, we also used the class frequency mixture model LG + cF + Γ that adjusts for site-specific amino acid frequencies proposed by Wang et al. (2008) and implemented in the maximum likelihood program QmmRAxML. No bootstrap analyses were performed with this model, because the calculations proved as computationally too extensive.

We used the disparity index test (Kumar and Gadagkar 2001a, 2001b) as implemented in MEGA version 4.1 (Tamura et al. 2007) with pairwise deletion of positions with gaps or missing data to identify sequence pairs characterized by dissimilar amino acid compositions, which indicate differences in the substitution process among lineages. We applied three approaches to reduce the potential impact of compositional bias on phylogeny reconstruction. First, we excluded those species which have most significant differences with the remaining species. Secondly, we constructed a neighbor-joining tree (Saitou and Nei 1987) with MEGA based on LogDet distances as modified by Tamura and Kumar (2002) considering among-site rate variation using gamma distributed rates with a shape parameter as determined in the maximum likelihood analysis (called Equal Input model with rate variation and pattern heterogeneity in MEGA). Thirdly, we recoded the data using the six groups of amino acids (AGPST, C, DENQ, FWY, HKR, ILMV) that tend to replace one another (Dayhoff et al. 1978). The recoded sequences were analyzed with Treefinder with a 6-state general time-reversible model treating each of these groups as one state (Embley et al. 2003), modeling among-site rate variation with a four-category discrete gamma-distribution.

To test predefined phylogenetic hypotheses, we used constrained trees and the 'resolve multifurcations' option of Treefinder to obtain the maximum likelihood tree for a specified hypothesis. Then we investigated whether the maximum likelihood trees for these hypotheses are part of the confidence set of trees applying the approximately unbiased test (Shimodaira 2002) and the expected likelihood weights method (Strimmer and Rambaut 2002).

Results and Discussion

Reducing compositional bias improves molecular phylogenetic inference

In the maximum likelihood tree based on 11544 amino acid positions derived from 78 ribosomal protein genes of 48 metazoan taxa and calculated with a mixed protein model (fig. 1A; this tree has been found by constrained searches), Brachiopoda and Phoronida are sister groups (bootstrap value 71 %). The corresponding clade, Brachiozoa, has been recovered in previous phylogenomic analyses with a

more sparse sampling of lophophorate taxa (Helmkampf et al. 2008b) as well as some other molecular (Mackey et al. 1996; Cohen et al. 1998; Cohen 2000; Halanych 2004; Anderson et al. 2004; Cohen and Weydmann 2005; Baguñà et al. 2008; Paps et al. 2009; but see Passamaneck and Halanych 2006) and morphological (Nielsen 2001) analyses. Also congruent with earlier phylogenomic studies (Dunn et al. 2008; Helmkampf et al. 2008b), Brachiozoa constitutes a clade with Nemertea. These relationships were also confirmed by the maximum likelihood analysis with the class frequency mixture model that adjusts for site-specific amino acid frequencies proposed by Wang et al. (2008) (fig. 2A). In contrast to previous phylogenomic (Hausdorf et al. 2007; Helmkampf et al. 2008b; Struck and Fisse 2008; Witek et al. 2008) and rDNA analyses (Baguñà et al. 2008; Paps et al. 2009) Ectoprocta and Entoprocta do not compose a monophylum in the maximum likelihood trees with the complete taxon sampling (fig. 1A, 2A). Instead, ectoprocts are the sister group of Eutrochozoa + Brachiozoa in the analysis with the mixed protein model (fig. 1A) respectively of all Lophotrochozoa including entoprocts and platyhelminths in the analysis with the class frequency mixture model (fig. 2A). Entoprocts appear as sister group of Platyzoa in analyses with either of these models. However, the bootstrap values of these new groupings are below 50 %. These contradictory results indicate that there might be systematic errors as a result of model assumption violations either in previous or in the present analyses.

To evaluate one possible cause of systematic errors we examined the amino acid composition of the used sequences. A total of 53.7% of the sequence pairs of the examined 48 taxa show significantly dissimilar amino acid compositions according to the disparity index test (Kumar and Gadagkar, 2001a,b). This confirms the observation of Lartillot and Philippe (2008) that the assumption of a time-homogeneous amino acid replacement process made by conventional protein models is strongly violated in metazoan phylogenomic data and, thus, there is a risk of observing artifacts related to compositional bias.

We tried to reduce the potential impact of dissimilar amino acid compositions in our sequence data set on phylogenetic analyses using three approaches. First, we reduced the number of sequence pairs with dissimilar amino acid compositions by excluding those species that significantly differ in amino acid composition from more than half of the examined species. Consequently, we had to exclude 21 species,

among others all representatives of Platyhelminthes and Syndermata, because these significantly deviated in amino acid composition from at least 33 of the 48 originally included taxa. Maximum likelihood analyses of the remaining 27 taxa with the mixed protein model (fig. 1B) resulted in monophyletic, but weakly supported Bryozoa (bootstrap value 59 %) and also an increased support for Brachiozoa (bootstrap value 88 %). Another topological change in the tree obtained with the 27 taxa set concerns the position of Brachiozoa, which is no longer the sister group of Nemertea, but instead of Eutrochozoa, i.e., Nemertea + Annelida + Mollusca. Similar topological changes occurred also in the maximum likelihood tree obtained with the class frequency mixture model (fig. 2B).

Secondly, we constructed a neighbor-joining tree based on LogDet + Γ distances as modified by Tamura and Kumar (2002) considering among-site rate variation (fig. 3). In this tree Bryozoa is also monophyletic, but not supported in a bootstrap analysis. The support for Brachiozoa increased (bootstrap value 86 %). In the neighbor-joining tree based on modified LogDet + Γ distances Brachiozoa is the sister group of Nemertea + Annelida. Moreover, Chaetognatha, Platyhelminthes and Syndermata form a group with Nematoda and Tardigrada, which is probably the result of long branch attraction.

Thirdly, we recoded the amino acid sequences using the six groups of chemically similar amino acids that tend to replace one another (Dayhoff et al. 1978) to reduce compositional bias as suggested by Embley et al. (2003). After recoding, 30.8 % of the pairs of sequences still show significantly dissimilar compositions according to the disparity index test. Again, we reduced the number of sequence pairs with dissimilar amino acid compositions by excluding those species that significantly differ in amino acid composition from more than half of the examined species. Fortunately, only six taxa had to be excluded and the reduced 42 taxa data set still includes representatives of Platyhelminthes and Syndermata. Phylogenetic analyses of the recoded 42 taxa data set with a 6-state general time-reversible model (Embley et al. 2003) and considering among-site rate variation confirmed the monophyly of Bryozoa (bootstrap value 86 %) and Brachiozoa (bootstrap value 92 %) (fig. 4). Despite the loss of information resulting from using a reduced amino acid alphabet, the support for these groups was higher in the tree based on the analysis of the recoded data set than in the tree resulting from the maximum likelihood analysis of the unmodified amino acid data set from which the taxa most strongly differing in amino acid

composition had been excluded (fig. 1B) and in the neighbor-joining tree based on modified LogDet + Γ distances (fig. 3). Other aspects of the tree were improved as well. Whereas the monophyly of Deuterostomia was not recovered in the maximum likelihood analyses of the 27 taxa amino acid data set (fig. 1B, 2B) and the neighbor-joining tree using modified LogDet + Γ distances (fig. 3), it was more strongly supported in the maximum likelihood analyses of the recoded data set (fig. 4) than in the analyses of the 48 amino acid data set (fig. 1A). This shows that using reduced amino acid alphabets is a more efficient method for mitigating artifacts in phylogenetic analyses resulting from compositional heterogeneity than is the simple exclusion of taxa with a divergent amino acid composition or the use of distance methods based on LogDet distances.

Testing hypotheses concerning relationships of the lophophorate lineages and the entoprocts

We used the approximately unbiased test (Shimodaira 2002) and the expected likelihood weights method (Strimmer and Rambaut 2002) to test predefined phylogenetic hypotheses. These maximum likelihood based topology tests also depend on the assumption of a time-homogeneous amino acid replacement process. Therefore, we employed both the 27 taxa amino acid data set from which the taxa that differ most strongly in amino acid composition have been deleted, and the 42 taxa recoded data set for these tests (Table 1). For the analysis of ectoproct interrelationships (Table 2) we constructed an additional data set restricted to Bryozoa and Brachiozoa as reciprocal outgroups, because *Cristatella*, the only representative of Phylactolaemata, is not contained in the other reduced data sets. In the recoded data set restricted to Bryozoa and Brachiozoa, the composition of the sequences of *Cristatella* is not significantly different from the majority of the other species. The relationships of the considered groups in the maximum likelihood tree based on this data set are identical with those shown in the maximum likelihood trees based on the complete data set (fig. 1A, 2A).

Hypotheses derived from morphology and embryology suggesting relationships of lophophorates and deuterostomes (Ax 1995; Lüter and Bartolomaeus 1997; Sørensen et al. 2000; Nielsen 2001) have already been rejected in previous phylogenomic analyses (Hausdorf et al. 2007; Helmkampf et al. 2008b).

Here we focused on alternative hypotheses concerning the phylogenetic position of ectoprocts and entoprocts. The hypotheses that ectoprocts are sister to all other Lophotrochozoa including Platyzoa (Halanych et al. 1995; Halanych 2004; Passamaneck and Halanych 2006), and that entoprocts belong to Platyzoa (Halanych 2004) could be rejected by both topology tests (Table 1, hypotheses 1-2). The rejection of the last hypothesis is especially remarkable, because entoprocts cluster with Platyzoa in the maximum likelihood trees based on the complete amino acid data set (fig. 1A, 2A). The hypothesis that entoprocts are sister to molluscs that has been strongly favored by morphological studies (Bartolomaeus 1993; Haszprunar 1996; 2000; Ax 1999; Wanninger 2004; Wanninger et al. 2007; Haszprunar and Wanninger 2008) could be rejected by both topology tests with the 42 taxa recoded data set, and also by the expected likelihood weights method with the 27 taxa amino acid data set (Table 1, hypothesis 3). Haszprunar and Wanninger (2008) provided a list of nine potential synapomorphies of entoprocts and molluscs, but this list is not based on an analysis of a character matrix of these and possibly related phyla like ectoprocts (such a matrix is actually missing), and Haszprunar (1996) conceded that several of these potential synapomorphies are of low phylogenetic significance. However, it could not yet be excluded that entoprocts are sister to Eutrochozoa, together forming Trochozoa (Table 1, hypothesis 4). The in some respect complementary hypothesis that Lophophorata is monophyletic (Emig, 1984) could be rejected only by the expected likelihood weights method with the 42 taxa recoded data set (Table 1, hypothesis 5).

According to the analyses of both the complete and the reduced data sets (fig. 1-4), brachiopods are monophyletic and the sister group of Phoronida. The hypotheses that Brachiopoda is paraphyletic and that phoronids are the sister group of articulate brachiopods (Halanych et al. 1995; Zrzavý et al. 1998) or inarticulate brachiopods (Cohen et al. 1998; Cohen 2000; Cohen and Weydmann 2005) have been significantly rejected by both topology tests based on the 27 taxa amino acid data set (Table 1, hypotheses 6-7). However, none of these hypotheses could be rejected on the basis of the 42 taxa recoded data set (Table 1, hypotheses 6-7), and only a sister group relationship of phoronids and articulate brachiopods could be discarded based on the recoded data set restricted to Bryozoa and Brachiozoa (Table 2, hypotheses 1-2). A denser taxon sampling within brachiopods is necessary to clarify the relationships within Brachiozoa definitively.

In other respects the relationships within Lophotrochozoa remain unclear as well. In the maximum likelihood trees based on the 48 taxa amino acid data set (fig. 1A, 2A) and the recoded data set (fig. 4) Brachiozoa is sister to Nemertea as in the phylogenomic analyses of Dunn et al. (2008) and Helmkampf et al. (2008). In contrast, it is sister to Eutrochozoa including Nemertea, Mollusca and Annelida in the maximum likelihood trees based on the 27 taxa amino acid data set (fig. 1B, 2B), and to Nemertea + Annelida in the neighbour-joining tree using modified LogDet + Γ distances. Neotrochozoa including Mollusca and Annelida (with the annelids comprising Sipuncula and Echiura; see Struck et al. 2007, Hausdorf et al. 2007) has been found only in the maximum likelihood analyses with the mixed protein model (fig. 1). Most of these relationships are not strongly supported by bootstrap tests and none can be rejected by topology tests (Table 1, hypotheses 8-10).

Phylogenetic relationships within Ectoprocta

This is the first analysis in which all four recent major groups of Ectoprocta, i.e., Phylactolaemata, Stenolaemata, Ctenostomata and Cheilostomata, are covered by large protein sequence data sets. The results of our phylogenetic analyses (fig. 1-4) demonstrate the monophyly of Ectoprocta and a sister group relationship between Phylactolaemata, the freshwater bryozoans, and the predominantly marine Gymnolaemata (= Stenolaemata + Ctenostomata + Cheilostomata; as originally defined by Allman 1856 and used, e.g., by Bassler 1953; Jebram 1973; Todd 2000; Ax 2001) as has been suggested by Jebram (1973), Cuffey and Blake (1991), Todd (2000) and Ax (2001). Within Gymnolaemata, Ctenostomata and Cheilostomata form a monophyletic group, Eurystomata (as proposed by Marcus 1938; and equivalent to Gymnolaemata as used by Cuffey 1973 and Fuchs et al. forthcoming). This is corroborated by recent analyses of Fuchs et al. (forthcoming) based on 18S rDNA, 28S rDNA and COI, which have also shown that neither Ctenostomata nor Cheilostomata is monophyletic. However, some ctenostomate bryozoans that have not been included in molecular phylogenetic analyses so far might be the sister group of all other Ctenostomata + Cheilostomata + Stenolaemata (Todd, 2000). Contrary to one of the phylogenetic trees of Todd (1990: fig. 7; this has been questioned by Todd himself), the Alcyonidiidae do not belong to these basal groups, but cluster with Cheilostomata in our tree.

Diphyletic Ectoprocta with phylactolaemates forming a clade with recent phoronids as hypothesized by Mundy et al. (1981) could be significantly rejected by topology tests (Table 2, hypothesis 3). The hypotheses that Stenolaemata constitutes a monophyletic group with Cheilostomata (Cuffey and Blake 1991) and that Phylactolaemata may have been derived from Ctenostomata (Bassler 1953) have also been significantly rejected in the topology tests based on the recoded data set restricted to Bryozoa and Brachiozoa (Table 2, hypotheses 4 and 5). The same is true for the topology proposed by Anstey (1990), (Ctenostomata, (Cheilostomata, (Phylactolaemata, Stenolaemata))) (Table 2, hypothesis 6). However, a sister group relationship between Stenolaemata and all other groups of ectoprocts as proposed by Cuffey (1973) could not be excluded with the available sequence data (Table 2, hypothesis 7), but is unlikely according to morphological characters (Jebram 1973; Cuffey and Blake 1991; Todd 2000; Ax 2001).

Conclusions

As suggested by Baurain et al. (2007), improved taxon sampling, replacing fast-evolving species by more slowly evolving species, and using better models of sequence evolution resulted in a reduction of non-phylogenetic signal and a better resolution of animal phylogeny compared with other analyses (e.g., Rokas et al. 2005). Heterogeneity of the amino acid composition is a widespread among metazoan taxa, with potentially detrimental effects on phylogenetic inference. Despite the loss of information involved, recoding amino acids into groups of functional interchangeability proved to be a more efficient approach to reduce the impact of compositional bias on phylogenetic analyses than simply excluding taxa with strongly deviating amino acid composition or employing distance methods based on LogDet distances. All these methods provided additional evidence for the monophyly of Bryozoa and of Brachiozoa. In addition, by considerably improving the taxon sampling within both groups on the phylogenomic scale, we were able to shed light on their internal relations.

Most relationships between metazoan phyla, however, remained nevertheless and contrary to the expectations raised by Baurain et al. (2007) poorly supported. Even if the support for some of these

relationships might further increase by sampling additional genes and taxa, the generally low support for the relationships between metazoan phyla and the short internodes between the phyla still support the conclusion of Rokas et al. (2005) that many of the phyla originated by a closely spaced series of cladogenetic events. It is possible that the phylogenetic signal of the relatively few amino acid transformations that happened between these events in the Precambrian has mostly eroded and has been obscured by other signals like similarities in amino acid composition in the following 500 million years of evolution to a degree that too little of this signal is left to resolve lophotrochozoan phylogeny robustly. The identification of rare genomic changes shared between phyla will probably be the next major step towards a more robust reconstruction of the relationships of lophotrochozoan phyla.

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FIG. 1.—Maximum likelihood trees based on 11,544 amino acid positions derived from 78 concatenated ribosomal proteins using a mixed protein model. Bootstrap support values larger than 50% are shown to the right of the nodes; 100% bootstrap values are indicated by black circles. (*A*) 48 taxa data set. (*B*) 27 taxa data set (considering only those taxa that differ in amino acid composition from less than half of the other taxa).

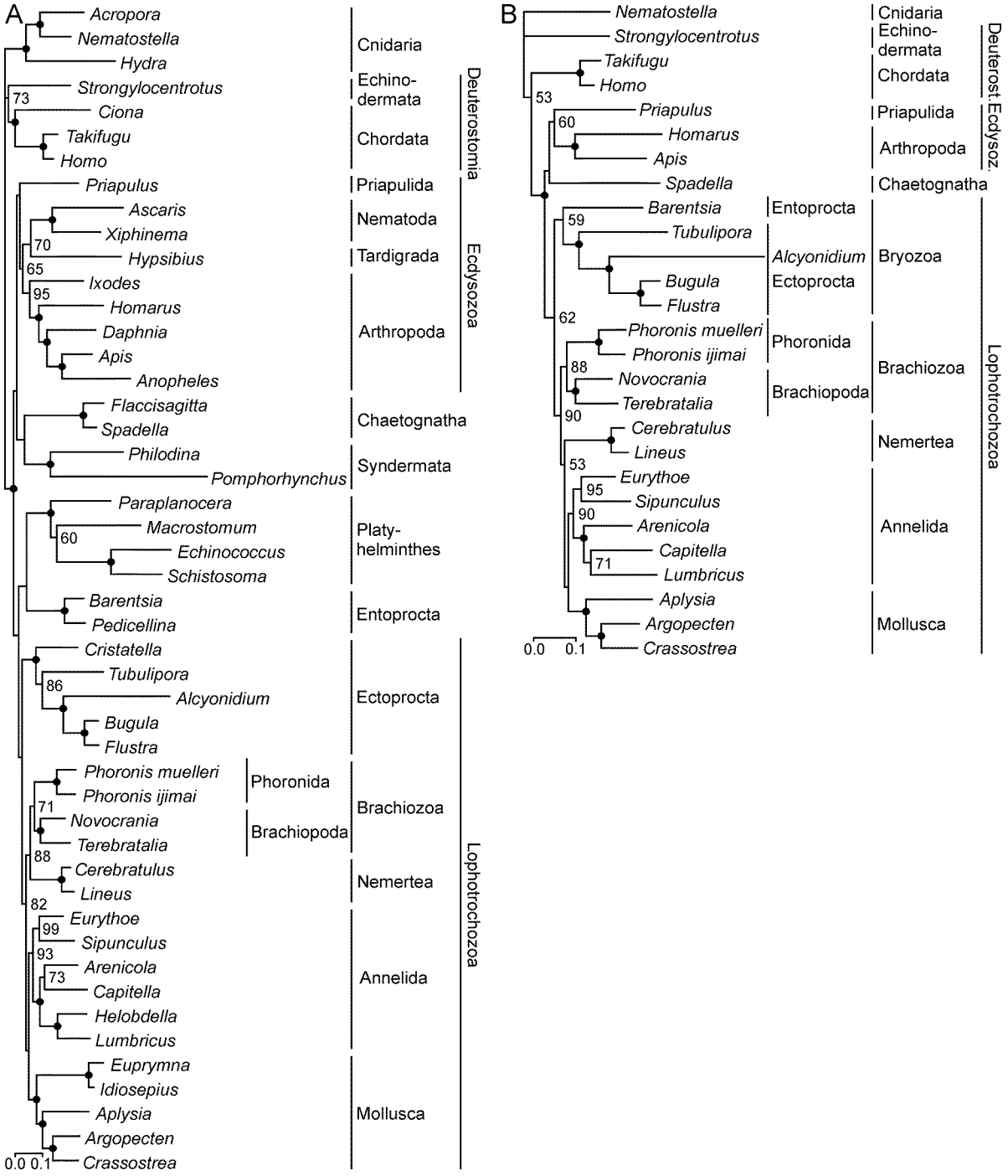

FIG. 2.—Maximum likelihood trees based on 11,544 amino acid positions derived from 78 concatenated ribosomal proteins using a class frequency mixture model that adjusts for site-specific amino acid frequencies. (*A*) 48 taxa data set. (*B*) 27 taxa data set (considering only those taxa that differ in amino acid composition from less than half of the other taxa).

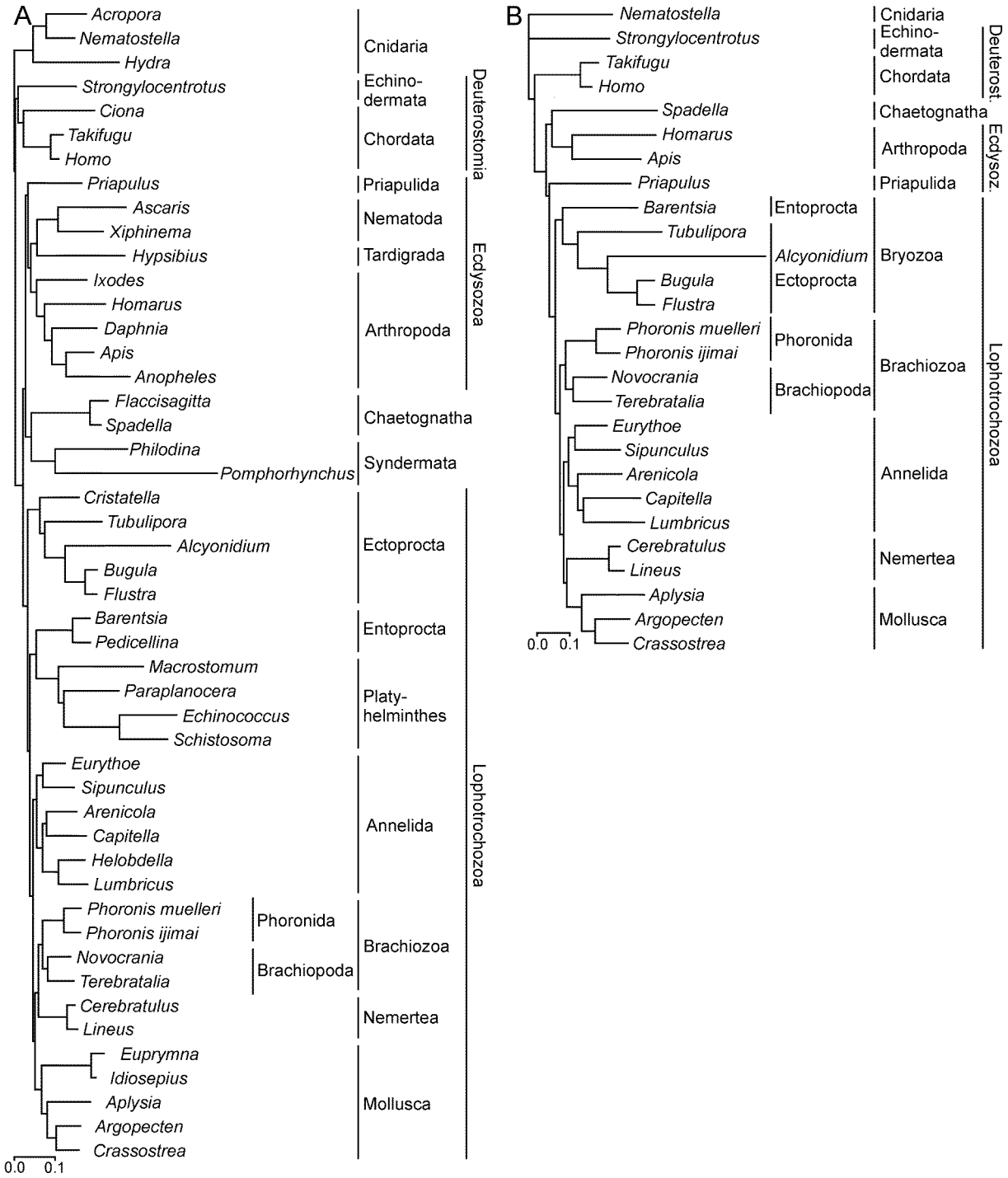

FIG. 3.—Neighbor-joining tree based on modified LogDet + Γ distances. Bootstrap support values larger than 50% are shown to the right of the nodes; 100% bootstrap values are indicated by black circles.

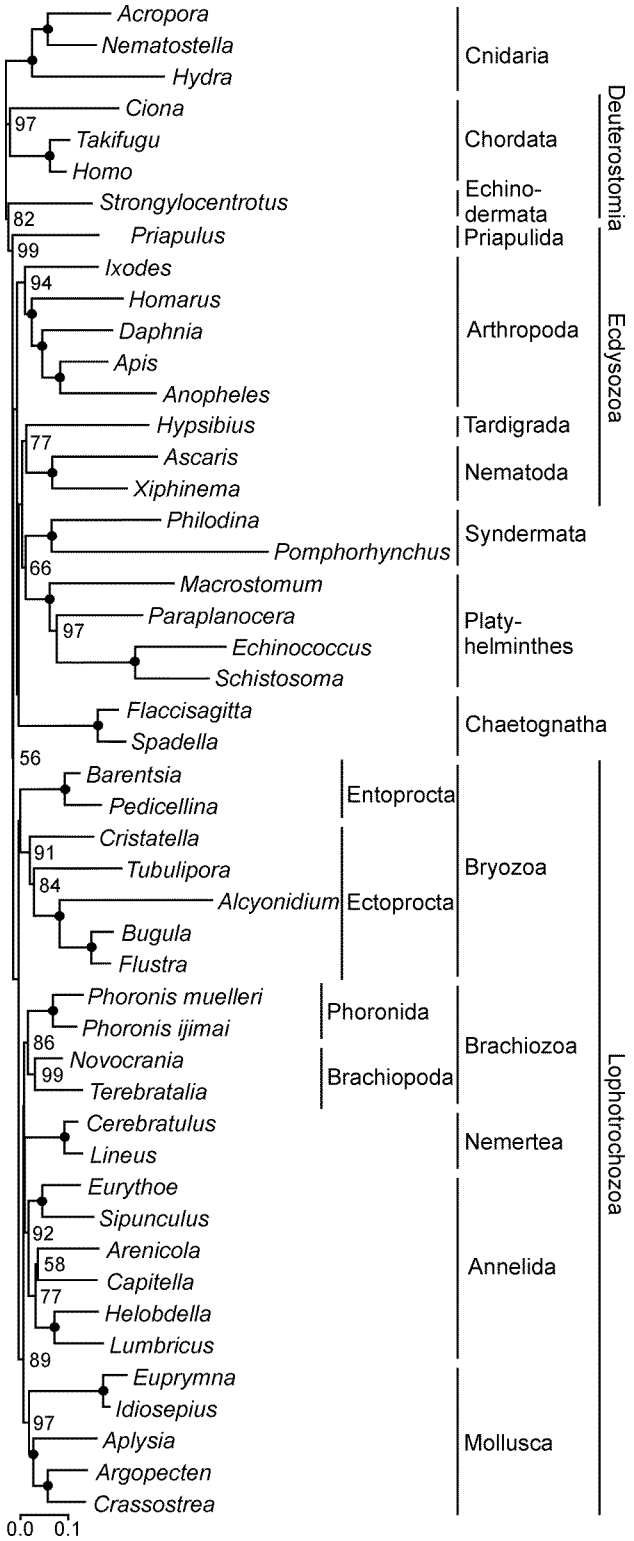

FIG. 4.—Maximum likelihood tree based on the amino acid sequences of 78 concatenated ribosomal proteins recoded using the six Dayhoff-groups with a 6-state general time-reversible model. Only those 42 taxa that differ in amino acid group composition from less than half of the other taxa were considered. Bootstrap support values larger than 50% are shown to the right of the nodes; 100% bootstrap values are indicated by black circles.

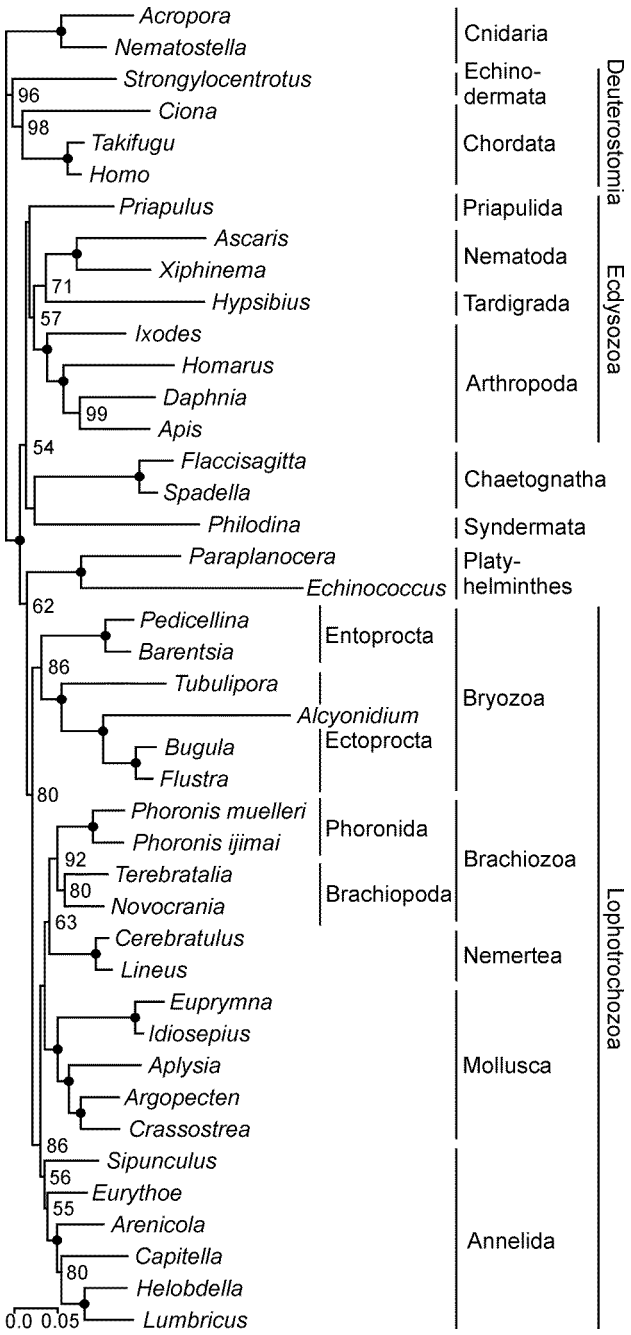

Table 1. Topology test results.

Number[a]	*Phylogenetic hypothesis*	*References claiming the hypothesis*	*Unmodified amino acid data set, 27 taxa*				*Recoded data set, 42 taxa*			
			Likelihood	*Δ Likelihood*[b]	*AU*[c]	*ELW*[d]	*Likelihood*	*Δ Likelihood*[b]	*AU*[c]	*ELW*[d]
	ML tree		-182549	0	0.7083	0.3825	-131467	0	0.7649	0.3977
1	Ectoprocta sister to all other Lophotrochozoa inclusive Platyzoa	Halanych et al. (1995); Halanych (2004); Passamaneck and Halanych (2006)	-182582	33	0.0000*	0.0005*	-131532	65	0.0000*	0.0011*
2	Entoprocta belong to Platyzoa	Halanych (2004)	–				-131537	71	0.0000*	0.0015*
3	Lacunifera (Entoprocta + Mollusca)	Bartolomaeus (1993); Haszprunar (1996, 2000); Ax (1999); Wanninger (2004); Wanninger et al. (2007); Haszprunar & Wanninger (2008)	-182595	46	0.0864	0.0100*	-131522	55	0.0000*	0.0015*
4	Trochozoa (Entoprocta + Eutrochozoa)	Zrzavý et al. (1998); Giribet et al. (2000); Peterson and Eernisse (2001)	-182576	27	0.2295	0.0832	-131499	32	0.1873	0.0732
5	Lophophorata monophyly	Emig (1984)	-182585	36	0.2491	0.1576	-131503	36	0.1445	0.0468*

Number[a]	*Phylogenetic hypothesis*	*References claiming the hypothesis*	*Unmodified amino acid data set, 27 taxa*				*Recoded data set, 42 taxa*			
			Likelihood	*Δ Likelihood*[b]	*AU*[c]	*ELW*[d]	*Likelihood*	*Δ Likelihood*[b]	*AU*[c]	*ELW*[d]
6	Phoronida + Rhynchonelliformea	Halanych et al. (1995); Zrzavý et al. (1998)	-182597	48	0.0000*	0.0000*	-131478	11	0.0654	0.0079
7	Phoronida + Craniformea + Linguliformea	Cohen et al. (1998); Cohen (2000); Cohen and Weydmann (2005)	-182592	42	0.0000*	0.0008*	-131474	7	0.3484	0.1213
8	Brachiozoa + Nemertea	Dunn et al. (2008); Helmkampf et al. (2008)	-182551	1	0.6396	0.3656	ML			
9	Eutrochozoa (Neotrochozoa + Nemertea)	Zrzavý et al. (1998); Giribet et al. (2000); Peterson and Eernisse (2001)	ML				-131480	13	0.3187	0.0847
10	Neotrochozoa (Annelida + Mollusca)	Zrzavý et al. (1998); Giribet et al. (2000); Peterson and Eernisse (2001)	ML				-131469	3	0.7028	0.2643

[a] Numbers refer to the order of appearance in the text.

[b] Δ Likelihood: differences between the likelihood of a constrained tree and the maximum likelihood tree.

[c] AU: approximately unbiased test (*p*-values). Values for topologies significantly rejected at the 0.05 level are indicated by an asterisk.

[d] ELW: expected likelihood weights. Values for topologies not included in the 0.95 confidence set are indicated by an asterisk.

Table 2. Topology test results based on the recoded data set restricted to Bryozoa and Brachiozoa.

Number[a]	*Phylogenetic hypothesis*	*References claiming the hypothesis*	*Likelihood*	*Δ Likelihood*[b]	*AU*[c]	*ELW*[d]
	ML tree		-43445	0	0.8276	0.6721
1	Phoronida + Rhynchonelliformea	Halanych et al. (1995); Zrzavý et al. (1998)	-43458	13	0.0000*	0.0082*
2	Phoronida + Craniformea + Linguliformea	Cohen et al. (1998); Cohen (2000); Cohen and Weydmann (2005)	-43456	11	0.0983	0.0617
3	Phylactolaemata + Phoronida	Mundy et al. (1981)	-43560	115	0.0000*	0.0000*
4	Stenolaemata + Cheilostomata	Cuffey and Blake (1991)	-43573	128	0.0000*	0.0000*
5	Phylactolaemata + Ctenostomata	Bassler (1953)	-43637	192	0.0000*	0.0000*
6	(Ctenostomata, (Cheilostomata, (Phylactolaemata, Stenolaemata)))	Anstey (1990)	-43634	189	0.0000*	0.0000*
7	Phylactolaemata + Eurystomata	Cuffey (1973)	-43450	5	0.2796	0.2580

[a] Numbers refer to the order of appearance in the text.

[b] Δ Likelihood: differences between the likelihood of a constrained tree and the maximum likelihood tree.

[c] AU: approximately unbiased test (*p*-values). Values for topologies significantly rejected at the 0.05 level are indicated by an asterisk.

[d] ELW: expected likelihood weights. Values for topologies not included in the 0.95 confidence set are indicated by an asterisk.

2.5. Tree-based orthology assessment illustrated by the evaluation of ribosomal protein genes

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Tree-based Orthology Assessment Illustrated by the Evaluation of Ribosomal Protein Genes

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Abstract

Despite the availability of unprecedented amounts of molecular data, current efforts to resolve the evolutionary history of animals are still impeded by lack of resolution or even incongruency. One of the sources of systematic error that may mislead phylogenetic inference is the use of paralogs, gene copies arising from gene duplication rather than speciation. The identification of orthologous gene sets is therefore a fundamental requirement of molecular phylogenetic inference.

We propose a novel approach to select genes suitable for a given phylogenetic problem by directly applying the tree-based definitions of paralogy and orthology. Employing an automated pipeline, clusters of homologous gene copies are initially compiled from whole genome and EST data. Subsequently, gene trees are carefully reconstructed for each cluster. Each gene is then assigned to one of three categories representing varying degrees of evidence for orthology or paralogy according to the species overlap criterion, which grants independence from the necessity to match gene trees to a species tree as it is required by alternative phylogenetic orthology assessment methods. In contrast to clustering methods based on reciprocal similarity, this approach allows to decide for which phylogenetic question a gene can be used without problems and for which it should be excluded because gene duplications may affect phylogenetic inference.

As a case study, we evaluated ribosomal protein genes, a functional group of genes used extensively in phylogenomic studies of Metazoa. Most of these genes are characterized by frequent lineage-specific, recent gene duplication events. However, indication for ancient gene duplications is rare and scarcely substantial, making these genes suitable to infer deep metazoan relationships with respect to orthology.

Key words: orthology, orthology assessment, paralogy, phylogenetics, ribosomal proteins

Phylogenomic analyses employing large numbers of genes have become the standard method to infer deep phylogenetic relationships. However, the unprecedented availability of molecular data provides no guarantee to obtain true phylogenies. Despite continuing efforts, lack of resolution or even incongruency still riddle significant parts of the animal tree of life (Rokas et al., 2005; Helmkampf et al., 2008; Lartillot and Philippe, 2008; Dunn et al., 2008). While phylogenomic approaches are effective in overcoming stochastic errors, they are still plagued by a range of systematic error sources. Most of these are related to inadequate modelling of molecular evolution due to diverging evolutionary rates among taxa, heterotachy, compositional bias or selective constraints (Delsuc et al., 2005). Another source of systematic error in phylogenetics are gene duplications that may result in disagreement between a gene's trees and the evolutionary history of the species (Zmasek and Eddy, 2002). The different homology relations arising from speciation and gene duplication are described by the concepts of orthology and paralogy: two genes are defined to be orthologous if they originate by a speciation event, i.e., a single ancestral gene in the last common ancestor of the respective species, while paralogous genes are related via gene duplication (Fitch, 1970; Koonin, 2005). More precise terms like inparalog, outparalog and co-ortholog have been proposed to better reflect the more complex homology relations occurring when both types of event are subsequently involved (Remm et al., 2001; Sonnhammer and Koonin, 2002).

The availability of large amounts of molecular data allows and demands novel ways to address this issue by carefully selecting genes forming orthologous relationships prior to phylogenetic reconstruction. Most methods to infer homology relations can be classified into two broad categories, similarity-based methods and phylogenetic approaches. The former rely on the simple assumption that the sequences of orthologous genes are more similar to each other than to any other genes from the compared genomes. Usually, two genes in two species are considered orthologous if each is the others' best reciprocal hit using the Blast algorithm (Altschul et al., 1997; Wall et al., 2003). Related methods have been developed that extend this principle by comparing more than two taxa (e.g., COG/KOG database: Tatusov et al., 1997, 2003), discriminate between recent, lineage-specific, and ancient gene duplications (e.g., Inparanoid: Remm et al., 2001; Berglund et al., 2007; OrthoMCL: Li et al., 2003) and incorporate information about synteny, i.e., gene order conservation between genes (HomoloGene database: Sayers et al., 2009).

In contrast, phylogenetic approaches (Page and Charleston, 1997; Zmasek and Eddy, 2001; 2002; Storm and Sonnhammer, 2002; Arvestad et al. 2003; Dufayard et al., 2004) present a more direct way to assess homology relations. By reconstructing the evolutionary history of a related group of genes and establishing for each node of the gene tree whether it represents a speciation or duplication event, these methods come closest to the original definition of orthology and paralogy (Fitch, 1970). There are two ways to use phylogenetic information in homology assessment, tree reconciliation and analyses of species overlap between clades in gene trees. In tree reconciliation, homology assessment is done by mapping the presumed species tree onto the gene tree. Gene duplications and gene loss events can then be inferred from incongruence between both trees. However, this approach cannot be used for gene homology assessment prior to phylogenetic reconstruction, because it requires that the true species tree is known. In contrast, rules based on species overlap between clades in a gene tree allow employing tree-based orthology assessment when inferring phylogenetic relationships is intended (van der Heijden et al., 2007).

Although concatenating alignments of many genes combined with thorough orthology assessment is one of the most promising approaches to resolve phylogenetic relationships (Dutilh et al., 2007), tree-based orthology evaluation has rarely been applied systematically for the purpose of analysing metazoan systematics. This is due to the fact that such an approach is computationally intensive and usually requires manual curation. However, in comparison to methods based on sequence similarity, tree-based homology assessment confers several advantages that are valuable in a phylogenetic context, including increased accuracy and scalability to various levels of phylogenetic resolution (van der Heijden et al., 2007; Gabaldón, 2008). We thus present a novel, tree-based method to assess gene homology relations expressly for phylogenetic purposes. It is implemented in a mostly automated pipeline to carefully reconstruct gene trees, which allows evaluating large gene sets with a moderate effort of time and labour. Since EST data are an indispensable resource in molecular phylogenetics as long as many critically important taxa are not yet represented by whole genome data, our approach is also designed to incorporate this type of data. In contrast, many established methods and orthology databases have primarily been designed to predict gene function and rely on (often annotated) whole genome data only (e.g., KOG, Inparanoid, HomoloGene, TreeFam: Li et al., 2006; Ruan et al., 2008, OrthologID: Chiu et

al., 2007, PhylomeDB: Huerta-Cepas et al., 2007; 2008), making them unsuited to assess orthology for the purpose of a comprehensive phylogenetic study of the animal kingdom.

As a case study, we evaluated ribosomal protein genes, which frequently constitute the largest functional group of genes used in phylogenomic studies. While some large-scale analyses rely on all (Helmkampf et al., 2008) or an almost complete selection of these genes (Lartillot and Philippe, 2008), the most extensive study of animal phylogeny to date excludes almost half of the about 80 eukaryotic riboprotein genes due to putative evidence for paralogy. This stands in stark contrast to the assumption that — at least in mammals — each ribosomal protein is encoded by only one functional gene (Wool et al., 1995). By testing our approach on riboprotein genes, the present study also provides a more in-depth look at the homology relations of these genes in order to resolve the uncertainties concerning their phylogenetic suitability.

Material and methods

We developed a semi-automatic procedure for tree-based gene homology assessment, which was realized by Perl programming and a combination of several established software packages used in data mining and phylogenetic reconstruction (Fig. 1). The process is organized in three main stages, namely the compilation of homologous gene clusters (1), the reconstruction of gene trees for each cluster (2), and the visual evaluation of these gene trees to assess orthology on the basis of the species overlap criterion (3).

1. Compilation of Homologous Gene Clusters

Transcriptomic datasets derived from 10 genome and 13 EST projects representing all major eumetazoan clades were obtained from public archives (supplementary material, Table S1). This included three datasets from lophotrochozoan taxa that constitute the first whole genome datasets available of this critically important animal group. Data from genome projects was restricted to non-redundant datasets of transcribed sequences, e.g., NCBI's RefSeq and JGI's filtered model datasets. This approach allowed the inclusion of transcript variants and transcribed pseudogenes, but excluded processed pseudogenes that are common for ribosomal proteins (Zhang et al., 2002). EST data were assembled to contigs as described previously (Hausdorf et al., 2007, with the addition of a second

clustering step to improve contig assembly). For our case study, putative ribosomal protein gene homologs were extracted from this data set using the tblastn algorithm (Altschul et al., 1997) with an *e*-value cut-off < 1×10^{-10} based on a query set of 78 human cytoplasmic ribosomal protein sequences acquired from the Ribosomal Protein Gene Database (http://ribosome.med.miyazaki-u.ac.jp, excluding the extremely short *rpl41,* and arbitrarily favouring isoform *rps4x* over *rps4y* as representative of *rps4*). To obtain a protein translation corrected for frameshift errors due to sequencing inaccuracy, these homologs were again queried against the human ribosomal protein sequences employing the genewisedb algorithm (score cut-off < 50) as implemented in the Wise2 package (Birney et al., 2004).

2. Gene Tree Reconstruction

The resulting protein clusters were aligned using the iterative MAFFT L-INS-i algorithm as of version 6 (Katoh et al., 2002; Katoh and Toh, 2008). Sequences of less than 25 % length in relation to the longest sequence in the cluster were eliminated to mitigate the influence of incomplete sequences on the alignment editing. Then, positions of ambiguous alignment were removed using Gblocks set to low stringency parameters (Castresana, 2000). In a further removal step, sequences covering less than 33 % of the edited alignment length were excluded, as were all but one of several identical sequences from the same taxon (e.g., protein sequences resulting from transcript variants differing only in untranslated regions or synonymous substitutions). This was done to disburden further analyses from redundant sequences or short fragments most likely caused by technical errors. Arithmetic means and medians of the number of sequences per taxon were calculated for each cluster both before and after these removal steps. Each cluster was then phylogenetically analysed by first identifying the best-fitting model of protein evolution according to the Akaike Information Criterion corrected for alignment length by ProtTest 1.4 (Abascal et al., 2005). Based on this model, gene trees were computed by maximum likelihood reconstruction with RAxML 7.0.4 (Stamatakis, 2006) using the batch processing Perl script batchRAxML.pl written by Olaf Bininda-Emonds (pers. comm.). Support values were calculated from 100 rapid bootstrap calculations (Stamatakis et al., 2008).

3. Categorization

Each gene was assigned to one of three categories that represent varying degrees of evidence for gene duplications that might affect phylogenetic analyses of a given phylogenetic question. As criterion, we considered the incidence and extent of species overlap observable between clades in a gene tree. If the branches of a node have overlapping sets of species, it is assumed to represent a duplication event, while if the species sets are mutually exclusive, the node is considered to reflect a speciation event (van der Heijden et al., 2007). Consequently, if homologous sequences derived from the same species occur in multiple regions of the gene tree, the involved gene duplication event must predate the diversification of the taxa concerned. If all homologous sequences derived from the same taxon constitute a monophylum, the involved gene duplication event must have happened more recently, after the diversification of the taxa in question. The categories in detail are:

A. No species overlap, i.e., all homologous sequence copies from the same taxon appear monophyletic (Fig. 1A). If all of these monophyla are supported by bootstrap values ≥ 70 %, the gene is assigned to category A_1; if at least one such monophylum is supported by less, the gene is assigned to A_2. Both cases are interpreted as evidence that gene duplication occurred only after the splitting of the lineages represented, if at all.

B. Single species overlap, i.e., homologous sequence copies of a single taxon do not appear monophyletic (Fig. 1B). If the corresponding non-monophyletic relationships are supported by bootstrap values below 70 %, the gene is assigned to category B_1; if at least one of these is supported by a bootstrap value ≥ 70 %, the gene is assigned to category B_2. Both cases are interpreted as weak evidence that gene duplication occurred before the splitting of the lineages represented.

C. Multiple species overlap, i.e., homologous sequence copies of more than one taxon do not appear monophyletic (Fig. 1C). If all corresponding non-monophyletic relationships are supported by bootstrap values below 70 %, the gene is assigned to category C_1; if at least one of these is supported by a bootstrap values ≥ 70 %, the gene is assigned to category C_2. Both cases are interpreted as strong evidence that gene duplication occurred before the splitting of the lineages represented.

Sensitivity Test to Ancient Gene Duplications

Several non-ribosomal proteins were selected to assess the sensitivity of the presented approach to ancient gene duplications. These proteins, which are common to all metazoan organisms, namely beta-actin, elongation factor 1–alpha, elongation factor 1–beta, and the histones H1 and H2A, are encoded by genes belonging to prominent gene families in the human genome. Since the history of these genes has been shaped by extensive and presumably ancient gene duplications events, we considered them as potential models for highly paralogy-afflicted genes. In order to test whether the species overlap criterion is suitable to reliably detect patterns caused by ancient gene duplications, six human genes representing these gene families — *actb*, *ef1α1*, *ef1β1*, *h1f0*, *h1f1* and *h2a1* — were therefore used as query sequences and passed through the same orthology assignment process as described above.

Application to Phylogenetic Analyses

The overall influence exerted by ribosomal protein genes of different categories on the reconstruction of metazoan phylogeny was evaluated by assembling three datasets comprising all genes assigned to category A only, A and B, and A, B and C, respectively. To mitigate the effects of poor taxon sampling, we extended these datasets to 62 metazoan taxa. Of the 23 taxa used for the orthology assessment, all but one sequence from each taxon was eliminated according to the following criteria: 1. Branch length: the sequence displaying the shortest branch in relation to *Nematostella* serving as an outgroup was chosen (if there was more than one *Nematostella* sequence, the tree was re-rooted at *Homo*). 2. Completeness: if this sequence possessed less than 67 % of the amino acid positions of the complete alignment, the sequence with the next-shortest branch was selected. If there was a tie in branch length, the longer sequence was taken. In most cases, both criteria were correlated, i.e., shorter sequences were often characterized by long branches as the relative weight of substitutions grows inversely with sequence length.

EST data of another 39 taxa, after being processed as described earlier, were screened for ribosomal protein sequences by taking the longest coding sequence available per taxon and gene. The non-redundant gene sets of all 62 taxa were individually aligned and edited by MAFFT (Katoh et al., 2002; Katoh and Toh, 2008) and Gblocks (Castresana, 2000), respectively, before being concatenated to super-alignments comprising all genes of category A, A and B, and A, B and C, called datasets *A*, *AB* and

ABC. The latter has been deposited at TreeBASE (http://www.treebase.org, preliminary study accession no. SN4364). Each of the aforementioned datasets was phylogenetically analysed under a maximum likelihood framework by Treefinder (version of October 2008, Jobb et al., 2004) on the basis of the $LG+G_4$ model (Le and Gascuel, 2008), which proved to be the best-fitting individual model according to the Akaike information criterion. Support values were computed by bootstrapping (100 replicates, Felsenstein, 1985).

Results

Categorization of Ribosomal and Non-Ribosomal Protein Genes

A summary of the number of ribosomal protein genes assigned to each category is given in Table 1 (more detailed, gene-specific information can be found in the supplementary material, Table S2). Almost two thirds of the 78 investigated genes were classified as category A, suggesting that gene duplications occurred not at all or only within lineages represented in our study (Fig. 1A). This makes these genes unequivocally suitable for deep phylogenetic analyses of bilaterian animals. Another third of the genes was characterized by species overlap in a single taxon (category B, Fig. 1B). An ancient gene duplication occurring before the splitting of the studied lineages, followed by massive gene loss in all but one taxon, could provide an explanation for this kind of topology. However, given the branch lengths involved, it seems more likely that a single highly divergent sequence derived from a recent, lineage-specific duplication followed by a change of function and resulting divergent evolution of one of the copies is responsible for this situation. Finally, approximately a tenth of the genes manifested multiple species overlap and thus more conclusive evidence for a possible deep-level gene duplication (category C, Fig. 1C), which indicates an increased probability to encounter paralogy-related problems when using these genes. In all cases though, multiple independent events of gene loss have to be assumed to explain the observed pattern, as the majority of taxa displayed only single or multiple monophyletic sequence copies. In contrast, all non-ribosomal protein genes serving as potential models for highly paralogy-afflicted genes were assigned to category C. Gene trees derived from analysing *actb, ef1a* and *h2a1* additionally displayed a topological pattern expected to occur in gene families generated by ancient gene duplication events (Fig. 2).

Observed Gene Copy Numbers

In 92 % of the cases where a riboprotein gene was represented by more than one sequence per taxon, these formed — almost exclusively well supported — monophyletic groups. In fact, many taxa possessed several copies of a gene: on average, each gene in our dataset comprised 1.5 (genome data) and 1.6 (EST data) sequences per taxon before short or identical sequences were removed. Removal steps decreased the total number of sequences across all gene clusters by 12 %, resulting in a decline of the species-specific average number of sequences per gene to 1.2 and 1.4, respectively. While ESTs were thus confined to adequately informative sequences by discarding technical fragments (identical sequences had been merged during contig assembly), genome data was predominantly affected by eliminating copies that differed only in untranslated regions (transcript variants) or at the level of nucleotides (e.g., copies with synonymous substitutions).

Only a small number of genes were represented by more than 2.0 sequences per taxon on average. All of these were strongly influenced by one or two taxa exhibiting ten or more sequences. Except in three cases, this always concerned ESTs of the platyhelminth *Macrostomum lignano.* A closer look at these sequences revealed sequencing errors as one, but not the only source of this profusion, especially as the affected genes are among the longest ribosomal protein genes (making them prone to sequencing inaccuracies at the 3' end). Because the *Macrostomum* ESTs were generated from thousands of sexually reproducing individuals (Morris et al., 2006), allelic variation offers another explanation. Species-specific median values larger than 1.0 regarding the number of sequences per gene were likewise the exception, and generally not accompanied by mean values above 2.0. Also, assignment to category C did not — except in one case — coincide with mean or median values concerning the number of sequences per taxon and gene larger than 2.0 and 1.0, respectively.

Most cases of non-monophyly regarding sequences derived from the same taxon were caused by EST taxa, above all *Philodina* and *Crassostrea*. The overall number of ribosomal protein gene sequences found per taxon and the number of times a taxon violated the requirement of sequence monophyly was significantly correlated at the 5 % level (Spearman rank correlation test, $N = 23$, $P = 0.02$), although the number of contigs in the case of EST taxa was not (Spearman rank correlation test, $N = 13$, $P = 0.08$).

Phylogenetic Analyses of Differently Categorized Genes

The restriction of ribosomal protein genes to categorical ranges reflecting various levels of stringency produced three concatenated alignments of different length: dataset *A*, composed of 7328 amino acid positions of all category A genes, dataset *AB*, comprising 10264 positions of all genes assigned to category A or B, and dataset *ABC* consisting of 11544 positions derived from all genes of any category. The maximum likelihood trees based on the datasets *AB* (Fig. 4) and *ABC* differ only slightly in topology (even though rotifers, acanthocephalans and gnathostomulids are artefactually attracted to nematodes in dataset *AB*), recover what is known as the modern view of animal phylogeny and are congruent with the most extensive phylogenomic analysis to date (Dunn et al., 2008, stable taxon analysis). In contrast, the maximum likelihood tree obtained from dataset *A* displays more noticeable evidence of probable tree reconstruction artefacts like the attraction of platyhelminths to Ecdysozoa, or deuterostome paraphyly. Average support values calculated from bootstrapping datasets *A, AB* and *ABC* amount to 78, 82 and 84 %, respectively.

Discussion

Overcoming Limitations of Established Methods

Inferring phylogenetic relations between organisms based on sequence data fundamentally requires the establishment of gene orthology. Many phylogenetic studies rely on similarity-based strategies to predict orthology, which represent only rough approximations (e.g., Rokas et al., 2005; Regier et al., 2007) that cannot accurately reflect the complexity of gene homology relations at different levels of resolution. In addition, these strategies usually depend on (often annotated) whole genome data (e.g., COG/KOG, Inparanoid, HomoloGene), restricting their predictive power to certain branches of the tree of life. The present approach has been designed to overcome these limitations, and to provide a homology assessment tool specifically tailored to application in phylogenetics.

The limitations of accuracy and resolution displayed by similarity-based methods were addressed by adopting a tree-based strategy, which offers the possibility to identify orthologs at high levels of resolution, allowing to pinpoint duplication events to specific lineages and taking the complex, hierarchical nature of gene homology relations into account. This permits the selection of genes qualified

to address a given phylogenetic problem. Partitions of a phylogenetic tree in which a gene is suitable to reconstruct phylogenetic relationships may be identified, even if the same gene is afflicted by paralogy in other parts. For instance, many vertebrate genes exist in multiple copies (e.g., aldolase, enolase) due to genome duplication events in this lineage, making these genes prone to paralogy and requiring particular caution when investigating vertebrate phylogeny. However, non-vertebrate relationships may be safely inferred from the same genes — for instance, no episodes of gene duplication could be identified within protostome animals (data not shown). In other cases, it may be possible to break gene families down into smaller, orthologous groups (e.g., *ef1α1* clades, Fig. 3).

These kinds of relationships are directly observable only by the reconstruction of a gene's phylogenetic tree, and in an amount of detail that is not achieved by similarity-based methods. The latter do not preserve the non-transitivity and hierarchical nature of homology relations (but see Jothi et al., 2006), and have often been criticized as too inclusive (e.g., KOG, see Berglund et al., 2008). Even more balanced methods (e.g., OrthoMCL, see Chen et al., 2007) are vulnerable to varying evolutionary rates that lead to orthology misevaluation (van der Heijden et al., 2007). In contrast, tree-based methods are more robust and provide higher specificity, which is of particular interest in phylogenetics. While sharing the benefits of high resolution and specificity with other phylogenetic orthology assessment methods (Page and Charleston, 1997; Zmasek and Eddy, 2001; 2002; Storm and Sonnhammer, 2002; Arvestad et al. 2003; Dufayard et al., 2004), using the species overlap criterion avoids the most severe limitation of tree reconciliation: the dependency on a reliable, fully resolved species tree (but see Dufayard et al., 2004), which precludes this strategy from use in phylogenetics. Although the price of doing without a species tree is the loss of ability to detect hidden paralogy, i.e., gene duplication followed by reciprocal gene loss, similarity-based methods are equally misled by this kind of situation (van der Heijden et al., 2007). On the plus side, species-overlap is less sensitive to gene tree inaccuracies than tree reconciliation, which may lead to the erroneous exclusion of genes due to putative gene duplication followed by reciprocal loss because of gene tree reconstruction artefacts. Using gene order information from fully annotated genomes, van der Heijden et al. (2007) estimated the proportion of correctly assigned orthology relations based on the species overlap criterion to be 95 %, which is higher than what can be obtained by similarity-base methods and at least on par with the results attainable by tree reconciliation.

EST Incorporation, and Gene Tree Reliability

Because several lineages that are of critical importance for analysing the tree of life are not yet represented by annotated genome data, we designed the present approach to include draft genome and EST data. Some cases of paralogy may be missed in taxa represented by EST data, because the absence of a gene in an EST dataset may arise from the fact that it is not represented in the data rather than missing in the organism itself. The taxon set used here was selected to be representative for bilaterians, allowing conclusions about a gene's general susceptibility to paralogy at the level of bilaterian phyla. Especially the variate yet neglected Lophotrochozoa are represented for the first time by several genome data sets in an orthology assessment study. When focussing on other phylogenetic questions, the taxon set may be tailored to the problem at hand by increasing the density of taxon sampling in the tree partition of interest.

Only a few other orthology assessment strategies that are suitable to evaluate EST data have been proposed for phylogenetic inference so far. Dunn et al. (2008) developed an explicit method for selecting orthologous genes from EST data sets by combining similarity-based Markov clustering, gene tree reconstruction, and a threshold concerning the mean number of sequences observed per taxon and gene. However, of the 38 ribosomal protein genes excluded due to these criteria, all but three displayed no or only weak (category A and B, respectively) evidence for gene duplication preceding the radiation of Bilateria according to the present study (conversely, five genes of category C were included by Dunn et al., 2008). Eight of these were rejected due to above-threshold values regarding the mean number of sequences observed per taxon and gene, although, as we were able to show, this does not coincide with phylogenetic evidence for ancient gene duplication. This strategy therefore leads to the exclusion of genes that are merely characterized by high rates of lineage-specific duplication, which pose no threat of generating paralogy-related tree reconstruction artefacts in the context of deep phylogeny. Another eleven of category A or B genes were eliminated due to phylogenetic indication by Dunn et al. (2008). We suppose gene tree reconstruction artefacts are responsible for this incongruency. As a precaution against this problem, we took efforts to increase gene tree reliability as much as possible, e.g., by careful taxon sampling, alignment editing, calculating best-fit models of protein evolution for each gene, and using maximum likelihood methods including bootstrap analysis. In contrast, alternative phylogenetic

orthology assignment approaches currently rely on less powerful gene tree reconstruction methods like parsimony (Chiu et al., 2006; Dunn et al., 2008) or neighbour-joining (van der Heijden et al., 2007; Li et al., 2006; Dufayard et al, 2005). Working explicitly with non-redundant genome data sets (e.g., RefSeq sequences) and removing sequence fragments probably caused by sequencing or assembly errors early in the homology assessment process also proved to be vital in this context. Due to the shortness of single gene alignments — some ribosomal protein genes contain less than 100 amino acid positions — most gene trees were still badly resolved at the level of phyla, displaying low support values at deeper-level nodes. However, support values within the sampled lineages were almost exclusively high, frequently near 100 % (Fig. 2): of all clades formed by sequences of the same species that were observable in the best maximum likelihood gene trees of category A_1 and A_2, 97 % displayed rapid bootstrap values above 70 % (Table 1). To identify and to distinguish gene duplications that occurred before the diversification of the bilaterian phyla from more recent events, this accuracy, this accuracy at the species level is of primary importance when applying the species overlap criterion. But the approach also proved capable to detect duplication events predating the diversification of the phyla in extensive gene families, as is illustrated by Figure 3. Here, the clades formed by these events are generally well supported. We are therefore confident that the approach presented here can be used reliably to tell gene duplications that might affect phylogenetic inference of relationships between bilaterian phyla from duplications that do not have an impact on phylogenetic reconstruction at this level.

Adaptability, and a Note on Computational Applicability

The approach presented herein is not only adaptable with respect to the level of resolution, but to stringency as well. For instance, to maximize stringency, we evaluated genes according to overlap within species. However, this criterion can be applied to taxonomic units of any level to take effects of taxon sampling into account by effectively collapsing species to higher-level units (e.g., employing phylum overlap when a phylum is represented by several taxa, of which some are possibly undersampled). Further possibilities to adapt stringency include changing the prerequisites for categorization, which were also aimed at high stringency in this study, e.g., by increasing tolerance concerning the number of overlapping species, requiring support values above a predefined threshold to diagnose monophyly more

reliably (e.g., 70 % bootstrap), or considering only topological patterns that unambiguously indicate ancient gene duplications (Fig. 3).

Orthology assessment methods based on sequence similarity are often used due to their speed and ease of implementation in bioinformatic work processes. Tree-based approaches are in contrast perceived as too time-consuming and labour-intensive (Gabaldón, 2008). By automating all work steps from data compilation to gene tree reconstruction, and implementing efficient algorithms in the process (e.g., RAxML), we tried to keep the costs of time and labour at a minimum. The method is fast enough to handle datasets like the one used here in a few days on a single desktop computer. Use of advanced computer infrastructure or refraining from the computation of individual evolutionary models for each gene would cut the cost of time to a fraction thereof. At the moment, gene trees have to be inspected visually, which requires a moderate investment of labour. However, this is compensated by the accuracy and versatility granted by the method. Software like LOFT (van der Heijden et al., 2007) that is capable to visualize species overlap may be used to facilitate tree inspection, especially when larger taxon sets are analysed.

Suitability of Ribosomal Protein Genes for Metazoan Phylogenetics

Using a tree-based approach relying on the species-overlap criterion, we demonstrate that ribosomal proteins are generally not afflicted by ancient gene duplications, and are thus suitable for phylogenetic analyses of Bilateria in this respect. Although on average every second taxon was represented by two or more sequences per gene, the vast majority of these sequences could be located in well-supported monophyletic relationships. Possible explanations for this proliferation of highly similar sequences include technical artefacts (e.g., sequencing or assembly errors), transcript variants, allelic variation (obviously, this plays chiefly a role when a library is generated from more than one individual, like in the case of *Macrostomum*) and recent, lineage-specific gene duplications. None of these phenomena are of any consequence when investigating deep phylogenetic nodes. Evidence for gene duplication events predating lineage diversification as would be indicated by massive species overlap (Fig. 3) could not be found among ribosomal protein genes. Even riboprotein genes of category B and C owe their classification presumably rather to highly divergent sequences than to ancient gene duplication as is indicated by the

observed branch lengths (Fig. 2B). Divergence may be caused by the rapid evolution of a recently duplicated gene, or the occasional contamination of a library with sequences from an unrelated taxon (e.g., one of the sequences from the *Crassostrea* dataset clustering with human sequences in gene *rps2* and thus accounting for this gene's assignment to category C, suspiciously exhibits 100 % similarity to a GenBank entry of the halibut *Hippoglossus*).

Closer inspection of human sequences (which should be unaffected by most technical issues) revealed that many cases of sequence redundancy can be attributed to the existence of splice variants, although an origin from different chromosomal regions or chromosomes does occur as well. The monophyly of such latter sequences suggests a prevalence of recent, lineage-specific duplication events. A detailed study on ribosomal protein genes in the human genome uncovered thousands of processed pseudogenes, but the majority of these proved to be disabled by frameshifts and stop codons (Zhang et al., 2003). Since processed pseudogenes result from retrotransposition events (i.e., reverse transcription of mRNA followed by integration into the genome, Vanin, 1985), the remainder can be assumed to be 'dead on arrival', because in the process, retrotransposed genes are removed from the genetic context governing their transcription. This is also congruent with the observation that each (mammalian) ribosomal protein is presumably encoded by only one functional gene (Wool et al., 1995). We assume that these interpretations apply to all investigated taxa, because the average number of sequences per gene, and their phylogenetic relations observed in this study were very similar.

Impact on Phylogenetics

Based on the evaluation of ribosomal protein gene trees, we conclude that most, if not all, of these genes are unafflicted by paralogy at deep metazoan nodes. As discussed earlier, cases of single species overlap are most likely caused by rapid divergence of individual gene copies. Care must be taken to avoid these copies when compiling phylogenetic datasets, but there is little danger of falling victim to paralogy-related tree reconstruction artefacts when using these genes. The exclusion of genes of categories B and C is accompanied by a 37 % loss of positions in comparison to our full riboprotein gene alignment, leading to a reduction of the overall support of the resulting tree. Also, noticeable long-branch attraction artefacts (Felsenstein, 1978) start strongly affecting the tree topology at this point. In comparison, the exclusion of

category C genes only is of less consequence: the decrease of the number of alignment positions by 11 % is followed by a slight decline in overall tree support, but little topological change. This difference is caused by notoriously unstable taxa (rotifers, acantocephalans and gnathostomulids) presumably subject to a long-branch attraction effect. Systematic artefacts that can be traced to paralogy are consequently not involved. We therefore recommend to make use of all ribosomal protein genes of category A and B for the purpose of studying deep metazoan evolution.

While most ribosomal protein genes are thus suitable with respect to orthology, they alone are insufficient to completely clarify the evolutionary history of animals in terms of resolution. Instability of certain taxa — platyzoans in particular — and low or only moderate support of many inter-phylum relationships (e.g., within Lophotrochozoa) are noticeable even when they are analysed in their entirety. However, the lack of resolution and partial inconsistency experienced by and between phylogenetic studies employing various amounts and combinations of ribosomal protein genes (Rokas et al., 2005; Helmkampf et al., 2008; Lartillot and Philippe, 2008; Dunn et al., 2008) are obviously not founded in unrecognized ancient gene duplication events in these genes. Rather, other causes of systematic error that are yet unaccounted for must lie at the heart of this phenomenon, and be dealt with.

In contrast, the overly conservative selection of riboprotein genes (Dunn et al., 2008) would result in a substantial decrease of the length and density of phylogenomic data matrices, especially because these genes are readily available in EST data. Particularly affected were small taxa of great phylogenetic significance, which are represented by disproportionately little data. As long as there is still a limitation concerning the amount of sequence data available from many taxonomic groups, genes should be evaluated carefully so that as little information as possible is discarded due to excessively stringent criteria during orthology assessment. The tree-based strategy presented herein proved to be a highly informative yet practical alternative to do so. It offers the high level of resolution and accuracy distinguishing phylogenetic methods to derive gene homology relations, is customisable to specific phylogenetic questions — among others by the possibility to incorporate preliminary genome and EST data — and is implemented in a mainly automated pipeline that mitigates the investment of time and labour long associated with phylogenetic orthology assessment methods. Increasing processing speed and the

development of more efficient algorithms to infer phylogenetic trees will likely contribute to popularise tree-based orthology assessment strategies in future phylogenetic studies.

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Table 1. Results of assigning 78 ribosomal protein genes to categories A, B and C reflecting varying levels of evidence for gene duplication. A majority of 60 % was classified as category A, characterized by no or recent, lineage-specific gene duplication events. Another third of the genes displayed only weak evidence for gene duplication predating lineage diversification, and was consequently assigned to category B. The remaining 10 % — category C — manifested stronger evidence for gene duplication preceding lineage diversification. $N_{1,2}$ refers to the number of genes conforming to a 70 % support value threshold level (see 'Categorization', above).

Category	**N**	**$N_{1,2}$**	**% (of 78)**
A	47	36, 11	60
B	23	19, 4	30
C	8	2, 6	10

Figure captions

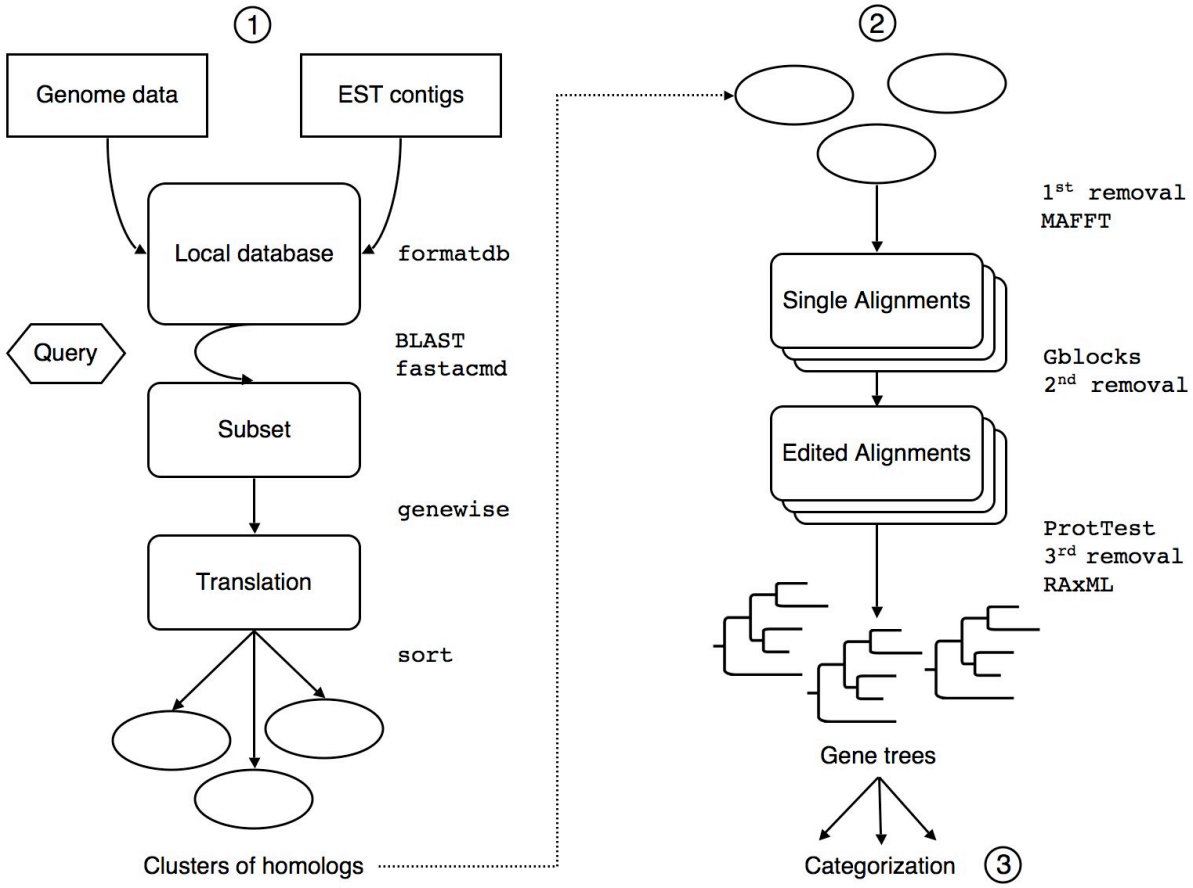
Figure 1. Flow chart depicting the process used to evaluate gene homology relations. Initially, homologs of predefined query sequences are retrieved from a database and sorted into gene clusters (1). A gene tree is subsequently reconstructed for each gene cluster employing advanced inference techniques (2). Finally, each gene tree is inspected visually and assigned to one of three categories representing varying degrees of evidence for gene duplication, depending on the incidence and extent of species overlap (3). Embedded software and other essential work steps are listed at the right of each column.

Figure 2. Gene trees illustrating the three categories established to classify genes according to varying levels of evidence for gene duplication preceding lineage diversification. Category A genes like *rpl6* are characterized by the absence of species overlap, indicating that gene duplications did not occur at all or only recently within lineages represented in this study. In contrast, single or multiple species overlap is observable in genes assigned to categories B (*rpl10a*) and C (*rpl24*), respectively. Overlapping species, i.e., species represented by several sequences not forming monophyletic groups, are indicated by an arrow. Species overlap may be due to recently duplicated yet highly diverging sequences — which appears most likely in singular cases exhibiting long branches — or to gene duplications ancestral to some or all represented lineages, thus causing paralogy that interferes with phylogenetic reconstruction. Vertical bars at the edges of the best maximum likelihood trees shown here denote rapid bootstrap support values above 70 % (one bar) or 95 % (two bars) obtained from RAxML.

Figure 3. Part of the gene tree of *ef1α1* highlighting a topological pattern likely caused by a gene duplication predating lineage diversification. Sequences of all major eumetazoan groups including Cnidaria are sorted into clades that display nearly complete species overlap. This indicates that multiple rounds of gene duplication occurred before the splitting of these groups, and demonstrates that the tree-based orthology assessment approach presented here is capable of detecting this problematic type of gene history. In ribosomal protein genes, a similar pattern was never found. Vertical bars at the edges of the best maximum likelihood tree shown here denote rapid bootstrap support values above 70 % (one bar) or 95 % (two bars) obtained from RAxML.

Figure 4. Maximum likelihood tree of 62 metazoan taxa based on all 70 ribosomal protein genes assigned to category A or B. Topology and support values differ only slightly from the tree obtained from all ribosomal protein genes of all categories, further demonstrating that these genes are generally suitable to phylogenetic reconstruction of Bilateria with respect to paralogy. Numbers at the nodes denote bootstrap support values calculated by Treefinder (100 replications), corresponding to analyses based on all genes (first number), category A and B genes (second number), and category A genes only (third number).

Figure 1


1
Genome data
EST contigs
Local database
formatdb
Query
BLAST
fastacmd
Subset
genewise
Translation
sort
Clusters of homologs
2
1st removal
MAFFT
Single Alignments
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2nd removal
Edited Alignments
ProtTest
3rd removal
RAxML
Gene trees
Categorization
3

Figure 2

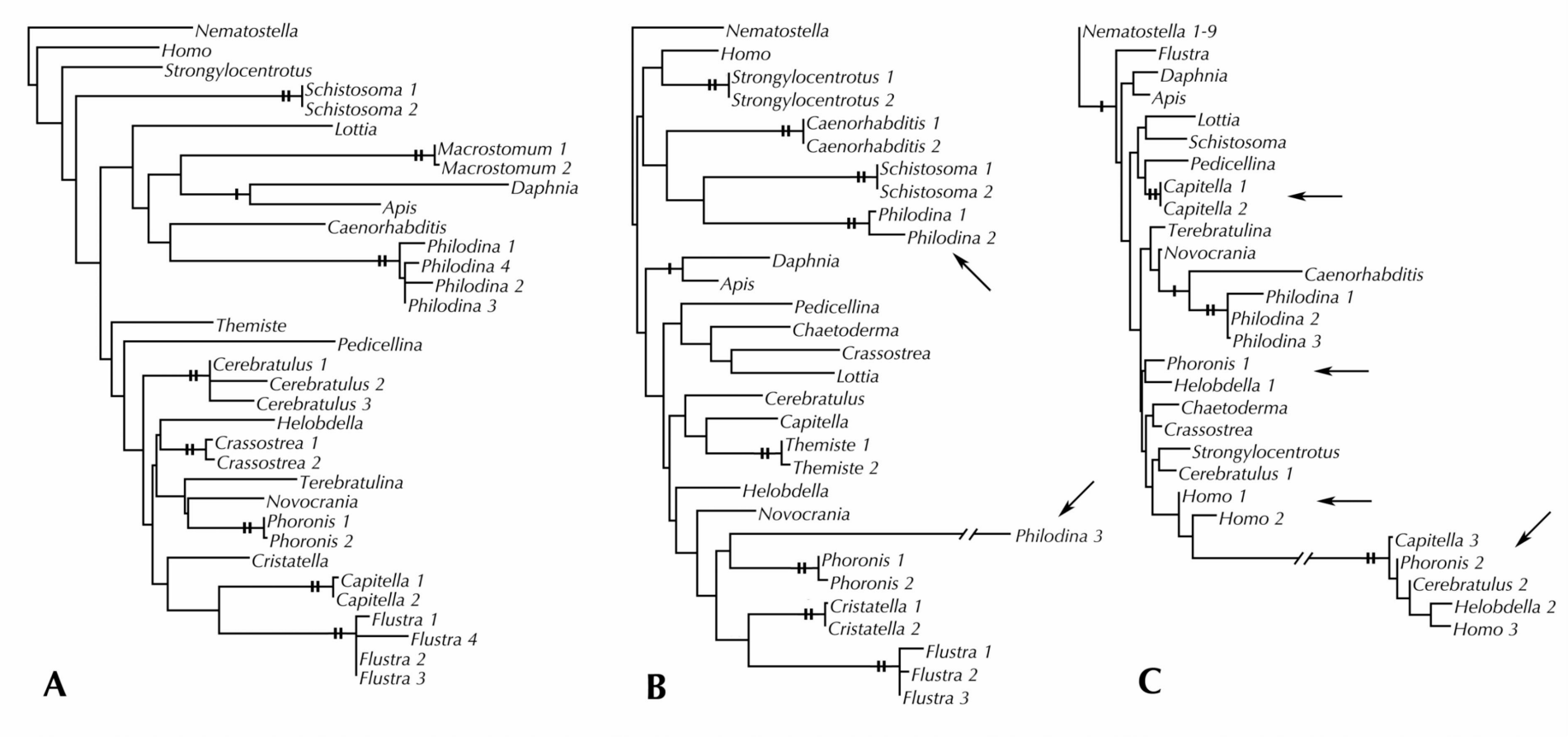

Nematostella
Homo
Strongylocentrotus
Schistosoma 1
Schistosoma 2
Lottia
Macrostomum 1
Macrostomum 2
Daphnia
Apis
Caenorhabditis
Philodina 1
Philodina 4
Philodina 2
Philodina 3
Themiste
Pedicellina
Cerebratulus 1
Cerebratulus 2
Cerebratulus 3
Helobdella
Crassostrea 1
Crassostrea 2
Terebratulina
Novocrania
Phoronis 1
Phoronis 2
Cristatella
Capitella 1
Capitella 2
Flustra 1
Flustra 4
Flustra 2
Flustra 3
A
Nematostella
Homo
Strongylocentrotus 1
Strongylocentrotus 2
Caenorhabditis 1
Caenorhabditis 2
Schistosoma 1
Schistosoma 2
Philodina 1
Philodina 2
Daphnia
Apis
Pedicellina
Chaetoderma
Crassostrea
Lottia
Cerebratulus
Capitella
Themiste 1
Themiste 2
Helobdella
Novocrania
Philodina 3
Phoronis 1
Phoronis 2
Cristatella 1
Cristatella 2
Flustra 1
Flustra 2
Flustra 3
B
Nematostella 1-9
Flustra
Daphnia
Apis
Lottia
Schistosoma
Pedicellina
Capitella 1
Capitella 2
Terebratulina
Novocrania
Caenorhabditis
Philodina 1
Philodina 2
Philodina 3
Phoronis 1
Helobdella 1
Chaetoderma
Crassostrea
Strongylocentrotus
Cerebratulus 1
Homo 1
Homo 2
Capitella 3
Phoronis 2
Cerebratulus 2
Helobdella 2
Homo 3
C

Figure 3

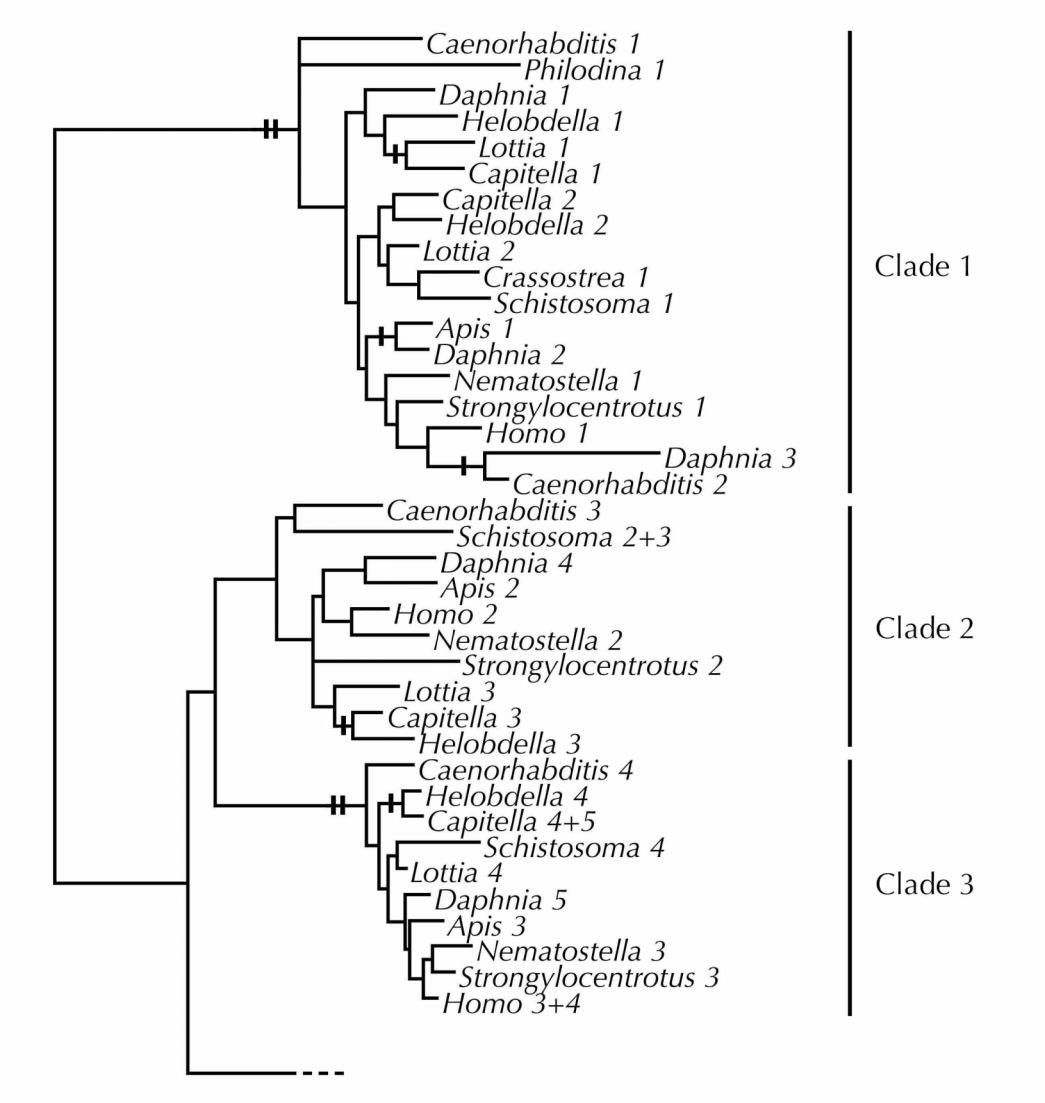
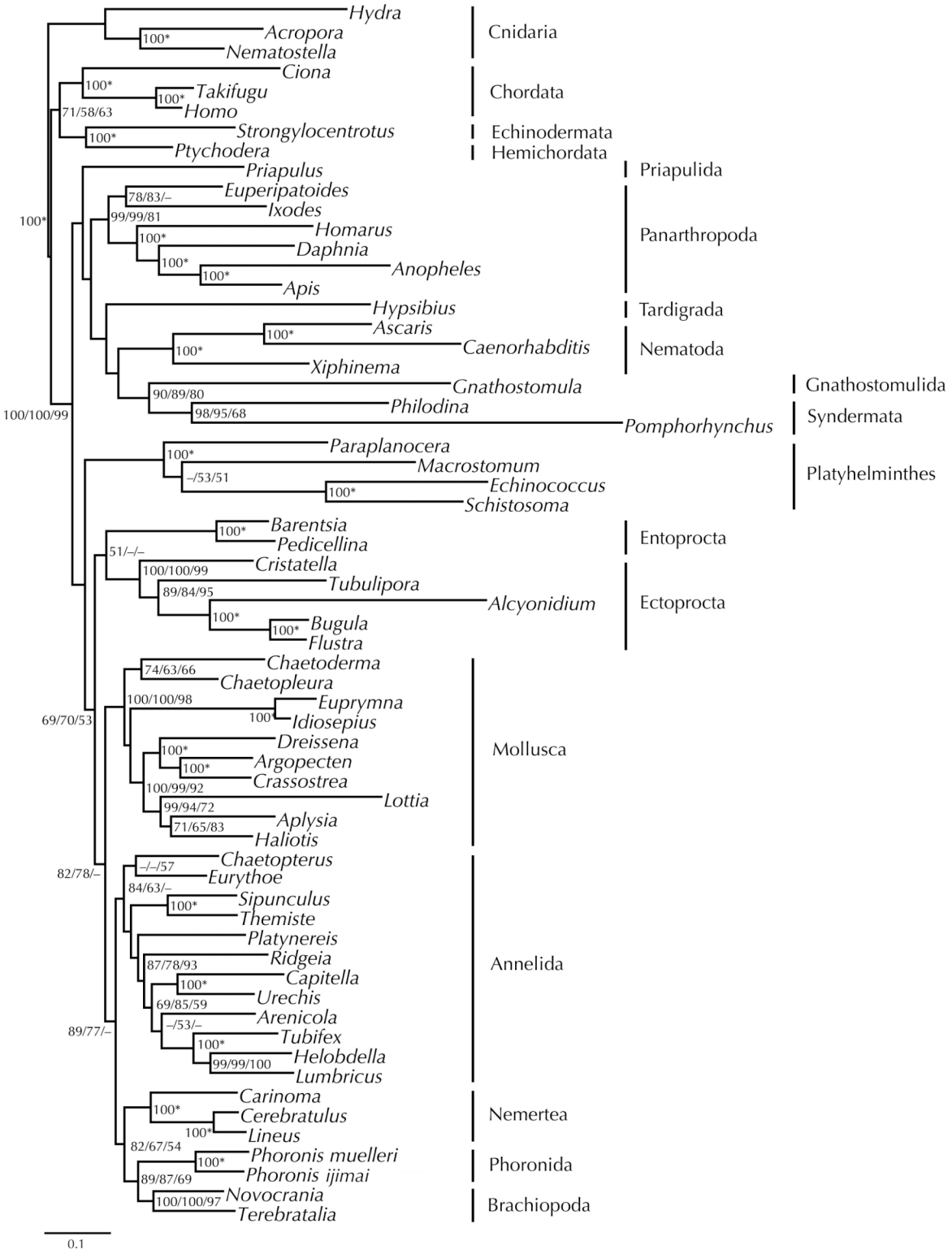

Caenorhabditis 1
Philodina 1
Daphnia 1
Helobdella 1
Lottia 1
Capitella 1
Capitella 2
Helobdella 2
Lottia 2
Crassostrea 1
Schistosoma 1
Apis 1
Daphnia 2
Nematostella 1
Strongylocentrotus 1
Homo 1
Daphnia 3
Caenorhabditis 2
Clade 1
Caenorhabditis 3
Schistosoma 2+3
Daphnia 4
Apis 2
Homo 2
Nematostella 2
Strongylocentrotus 2
Lottia 3
Capitella 3
Helobdella 3
Clade 2
Caenorhabditis 4
Helobdella 4
Capitella 4+5
Schistosoma 4
Lottia 4
Daphnia 5
Apis 3
Nematostella 3
Strongylocentrotus 3
Homo 3+4
Clade 3

Figure 4


Hydra
Acropora
Nematostella
Ciona
Takifugu
Homo
Strongylocentrotus
Ptychodera
Priapulus
Euperipatoides
Ixodes
Homarus
Daphnia
Anopheles
Apis
Hypsibius
Ascaris
Caenorhabditis
Xiphinema
Gnathostomula
Philodina
Pomphorhynchus
Paraplanocera
Macrostomum
Echinococcus
Schistosoma
Barentsia
Pedicellina
Cristatella
Tubulipora
Alcyonidium
Bugula
Flustra
Chaetoderma
Chaetopleura
Euprymna
Idiosepius
Dreissena
Argopecten
Crassostrea
Lottia
Aplysia
Haliotis
Chaetopterus
Eurythoe
Sipunculus
Themiste
Platynereis
Ridgeia
Capitella
Urechis
Arenicola
Tubifex
Helobdella
Lumbricus
Carinoma
Cerebratulus
Lineus
Phoronis muelleri
Phoronis ijimai
Novocrania
Terebratalia
Cnidaria
Chordata
Echinodermata
Hemichordata
Priapulida
Panarthropoda
Tardigrada
Nematoda
Gnathostomulida
Syndermata
Platyhelminthes
Entoprocta
Ectoprocta
Mollusca
Annelida
Nemertea
Phoronida
Brachiopoda
100*
71/58/63
78/83/–
99/99/81
90/89/80
98/95/68
100/100/99
–/53/51
51/–/–
89/84/95
74/63/66
100/100/98
69/70/53
100/99/92
99/94/72
71/65/83
–/–/57
82/78/–
84/63/–
87/78/93
69/85/59
89/77/–
99/99/100
82/67/54
89/87/69
100/100/97
0.1

2.6. Summary of achieved results

The following chapter summarises the results achieved by the phylogenetic studies that comprise this work. It focuses on the central conclusions of each study regarding the position of the lophophorate lineages. For a more detailed presentation, please refer to the complete manuscripts reproduced in the previous chapters. Conclusions pertaining to this work in general are outlined in the next section.

2.6.1. PCR-based multigene analysis (Manuscript 2.1)

This first study represents an early attempt to procure larger amounts of protein-coding sequence data than were available of lophophorate taxa at the beginning of this study. Seven nuclear gene fragments of *ATP synthase* β*, catalase, elongation factor 1–*α*, fructose-bisphosphate aldolase, methionine adenosyltransferase, phosphofructokinase* and *triosephosphate isomerase* were successfully sequenced from seven representatives of ectoproct bryozoans, brachiopods, phoronids, and chaetognaths. The concatenated dataset comprised 2033 amino acid positions, and was complemented by homologous sequences of 31 additional taxa obtained from GenBank. Likelihood-mapping proved that the resulting alignment is characterized a high phylogenetic information content (96.0% of the quartets are fully resolved).

Both maximum likelihood and Bayesian inference analyses of this dataset recovered the main bilaterian clades, i.e., Deuterostomia, Ecdysozoa and Spiralia including Lophotrochozoa and Platyhelminthes (Fig. 2). According to these analyses, ectoproct bryozoans, brachiopods and phoronids are more closely related to nemerteans, molluscs and annelids than to deuterostomes or ecdysozoans. Thus, Lophotrochozoa is monophyletic, although the support for this clade is not strong. Within Lophotrochozoa, the lophophorates lineages do not constitute a monophyletic group: rather, ectoproct bryozoans and phoronids share a more recent common ancestor with annelids and molluscs than with brachiopods (Fig. 2). While articulate and inarticulate brachiopods are sister to each other, bryozoans also do not appear as a monophyletic taxon. Instead, phylactolaemate bryozoans are placed closer to Phoronida than to gymnolaemate bryozoans. The only inconsistency between the trees reconstructed with maximum likelihood and Bayesian inference is that Annelida is monophyletic in the former tree,

whereas it is paraphyletic with respect to Phoronida and phylactolaemate Bryozoa in the latter reconstruction.

Several disputed hypotheses, among others that lophophorates are more closely related to Deuterostomia than to Protostomia, that it is monophyletic, and that Bryozoa is sister to Spiralia plus Ecdysozoa could be significantly rejected with both the approximately unbiased test and the expected likelihood weights method. The monophyly of the segmented phyla, Annelida plus Arthropoda, i.e., the Articulata hypothesis, and the monophyly of Neotrochozoa including Annelida and Mollusca, have also been significantly excluded with both tests. All other tested phylogenetic hypotheses could not be ruled out with the approximately unbiased test. However, the expected likelihood weights method did reject hypotheses stating the monophyly of Phoronozoa (Brachiopoda plus Phoronida, also known as Brachiozoa), and a sister group relationship between Phoronozoa and Mollusca.

Thus, the results based on the present multigene dataset disagree with the traditional perspective based on embryological and morphological characters that the lophophorate lineages, i.e., Bryozoa, Brachiopoda, and Phoronida, form the sister group or the paraphyletic stem group of Deuterostomia (Ax, 1995; Lüter and Bartolomaeus, 1997; Brusca and Brusca, 2003). Instead, the analyses support the Lophotrochozoa concept. Even though the support for this clade is not strong, this corroborates studies based on rDNA (Halanych et al., 1995; Peterson and Eernisse, 2001; Mallatt and Winchell, 2002; Passamaneck and Halanych, 2006), *hox* genes (de Rosa et al., 1999; Passamaneck and Halanych, 2004), *myosin* (Ruiz-Trillo et al., 2002), *ATPase* (Anderson et al., 2004) and mitochondrial protein sequences (Stechmann and Schlegel, 1999; Helfenbein and Boore, 2004; Waeschenbach et al., 2006). As most of these studies also have shown before, the multigene analyses presented here further indicate that Lophophorata is not a valid monophyletic taxon, suggesting that lophophore structures originated several times independently during animal evolution. In contrast to many previous investigations (e.g., Cohen, 2000; Giribet et al., 2000; Peterson and Eernisse, 2001; Anderson et al., 2004; but see Ruiz-Trillo et al., 2002; Passamaneck and Halanych, 2006), Phoronozoa (=Brachiozoa) was not recovered, and neither was the monophyly of ectoproct bryozoans. However, the support for the responsible clade that includes phylactolaemate bryozoans and phoronids is not strong and the monophyly of bryozoans could not be

rejected by topological tests. The phylogenetic position of the lophophorate lineages within Lophotrochozoa proved to be difficult to assess more reliably, reflected by weak support values within the trees, and lack of statistical significance of the topology tests. Although, for instance, the hypothesis that Bryozoa is the sister group of Spiralia and Ecdysozoa (Giribet et al., 2000) has been rejected with the expected likelihood weights method, a sister group relation of Bryozoa and all other Lophotrochozoa (or Spiralia, according to our trees) as advocated by Halanych et al. (1995) and Halanych (2004) can not be ruled out according to both topology test methods.

Both maximum likelihood and Bayesian inference analyses indicate that Platyhelminthes do not belong to Lophotrochozoa, which is defined as the last common ancestor of the three traditional lophophorate taxa, the molluscs, and the annelids, and all descendants of that ancestor (Halanych et al., 1995). Assuming that the spiral-quartet cleavage of plathyhelminths is homologous to that of nemerteans, annelids, and molluscs, the name Spiralia for the clade including platyhelminths (and possibly other platyzoans) and lophotrochozoans suggests itself (Giribet et al., 2000). However, it cannot be ruled out that Platyhelminthes indeed belong to Lophotrochozoa, because the topology tests did not reject a position of the bryozoan lineages as sister to Spiralia. Since platyhelminths are the only representatives of Platyzoa in this dataset, a denser sampling of Platyzoa is required for more robust conclusions.

Although the data presented here constituted one of the largest molecular datasets with respect to deep metazoan phylogeny at the date of publication, drawing further conclusions with confidence proved to be difficult. The lack of resolution provided by this amount of data is reflected by weak support values in the trees, particularly within Lophotrochozoa, and the statistical insignificance of many tests concerning alternative topologies. This situation may be remedied by increasing the number of taxa and genes analysed. Since the most efficient method to do so at the moment includes the sequencing of expressed sequence tags (ESTs), the following studies rely exclusively on this technique.

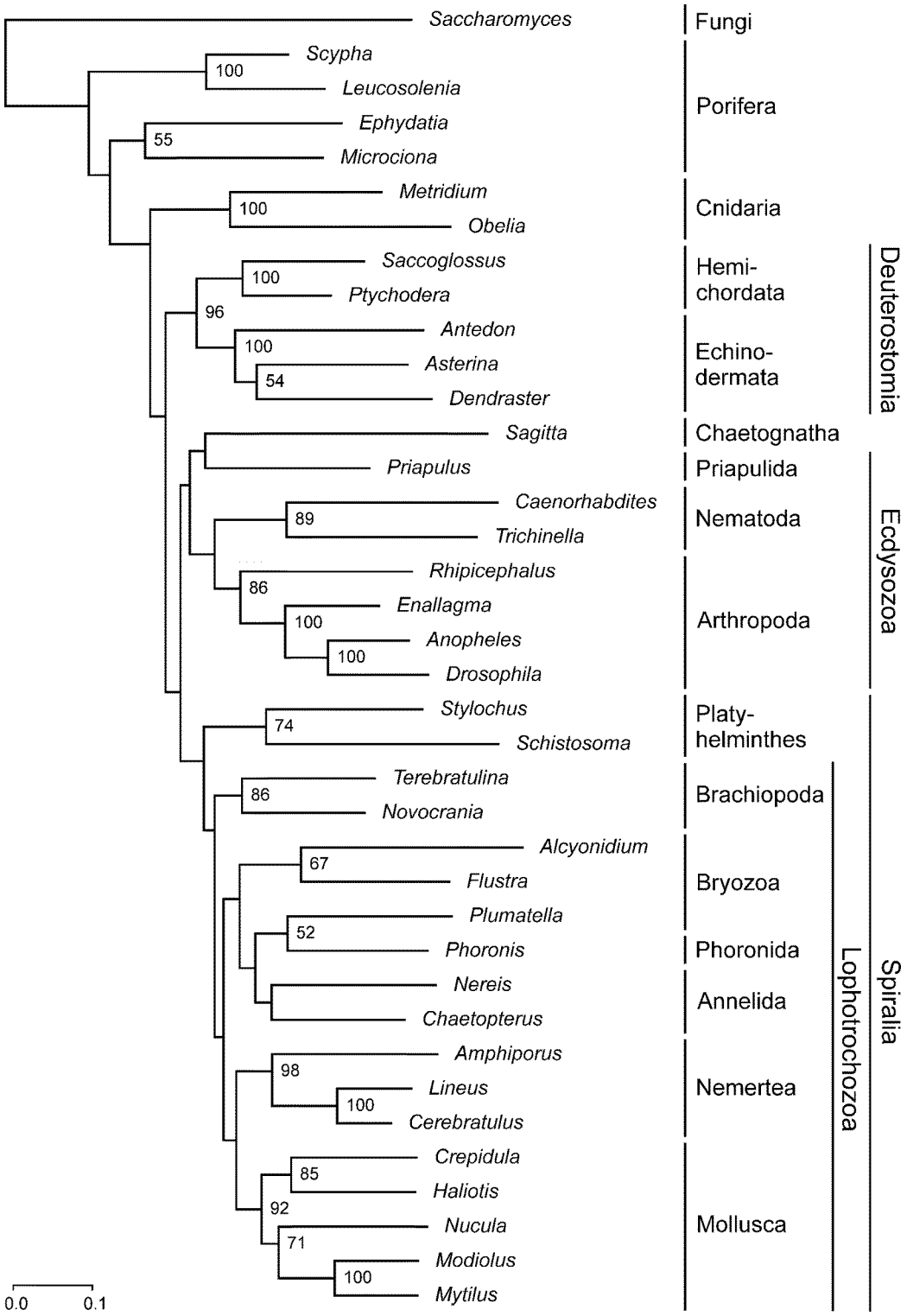


Figure 2. Maximum likelihood tree based on the analysis of seven protein-coding gene fragments obtained by PCR-amplification and sequencing, demonstrating the lophotrochozoan affinities of ectoproct bryozoans, brachiopods, and phoronids. Numbers denote bootstrap support values larger than 50 %.

2.6.2. EST-based phylogenetic analysis of Bryozoa (Manuscript 2.2)

The first study based on genomic-scale data presented in this work includes ESTs of the ectoproct *Flustra foliacea* and the entoproct *Barentsia elongata.* Both phyla were represented for the first time in a phylogenomic investigation. The analyses were based on 79 ribosomal protein sequences derived from 38 metazoan taxa, and demonstrate for the first time in a molecular study that Bryozoa comprises both Entoprocta and Ectoprocta in a monophyletic group positioned at the base of Lophotrochozoa (Fig. 3). This arrangement is supported by strong nodal support values and topology tests. Most alternative hypotheses concerning the phylogenetic position of ectoprocts and entoprocts can be rejected by topology tests, including the hypotheses that ectoprocts are related to Deuterostomia, that they are sister to all remaining Spiralia (Halanych et al., 1995; Halanych, 2004; Passamaneck and Halanych, 2006), and that they are sister to all other protostomes except chaetognaths (Giribet et al., 2000). With regard to entoprocts, a sister group relationship between these and Mollusca (Bartolomaeus, 1993; Haszprunar, 1996; Ax, 1999), a neotenic origin of entoprocts from annelids (Emschermann, 1982) and its placement within Platyzoa (Halanych, 2004; Passamaneck and Halanych, 2006) can be ruled out according to the expected-likelihood weights test based on this data. However, a sister group relationship between Entoprocta and Neotrochozoa, which comprises Mollusca, Sipuncula and Annelida (Zrzavý et al., 1998; Giribet et al., 2000; Peterson and Eernisse, 2001), cannot be significantly rejected. Nonetheless, these results strongly support the monophyly of Bryozoa in the broader sense, suggesting that this controversial, century-old hypothesis has to be resurrected.

When first discovered, entoprocts (Kamptozoa) were treated together with the ectoproct bryozoans because of their sessile life style and ciliated tentacles. Nitsche (1869) pointed to the differences between the position of the anus and the retractability of the tentacle crowns and proposed the names Entoprocta and Ectoprocta for the two main groups of bryozoans. Subsequently, the two groups have almost unanimously been treated as separate higher taxa, mainly based on the differences in cleavage patterns and body cavities (Hatschek, 1891; Hennig, 1979; Emschermann, 1982; Zrzavý et al., 1998; Ax, 1999; Giribet et al., 2000; Sørensen et al., 2000; Brusca and Brusca, 2003). Only Nielsen (1971, 2001) and Cavalier-Smith (1998) maintained the monophyly of Bryozoa in the broader sense.

Ectoprocts have been included in Lophophorata based on similarities of the tentacular apparatus and the radial cleavage they share with phoronids and brachiopods. As discussed earlier, this taxon was traditionally considered the sister or paraphyletic stem group of Deuterostomia (Hennig, 1979; Ax, 1995; Brusca and Brusca, 2003). In contrast, studies based on various molecular markers, including rDNA, *hox* genes, *myosin*, *atpase*, and mitochondrial protein sequences unequivocally showed that Ectoprocta as well as Phoronida and Brachiopoda are more closely related to Annelida, Mollusca and allies than to Deuterostomia or Ecdysozoa (Halanych et al., 1995; Peterson and Eernisse, 2001; Mallatt and Winchell, 2002; Ruiz-Trillo et al., 2002; Anderson et al., 2004; Passamaneck and Halanych, 2004; Passamaneck and Halanych, 2006; Waeschenbach et al., 2006), leading to the establishment of Lophotrochozoa (Halanych et al., 1995). Some of these studies further demonstrated that Lophophorata is polyphyletic (Halanych et al. 1995; Mackey et al. 1996; Giribet et al. 2000; Passamaneck and Halanych 2006).

On the other hand, since entoprocts exhibit spiral cleavage and trochophora-type larvae, a closer connection to taxa also displaying these features was assumed (Ax, 1995, 1999; Zrzavý et al., 1998; Giribet et al., 2000; Peterson and Eernisse, 2001). Molecular phylogenetic analyses of 18S rDNA generally confirmed the affiliation of entoprocts with taxa possessing trochophora larvae, but their exact relationships remained contentious (e.g., Mackey et al., 1996; Giribet et al., 2000; Peterson and Eernisse, 2001). However, so far all analyses of rDNA sequences did support the assumption that ectoprocts and entoprocts do not constitute sister taxa (Mackey et al., 1996; Zrzavý et al., 1998; Giribet et al., 2000; Peterson and Eernisse, 2001; Passamaneck and Halanych, 2006).

These results are now clearly called into question by the reestablishment of Bryozoa in the broader sense proposed by the present study, the first employing large amounts of protein-coding genes that include both taxa. Because morphological data (Funch and Kristensen, 1995; Zrzavý et al., 1998; Sørensen et al., 2000) and rDNA sequences (Passamaneck and Halanych, 2006) indicate that Entoprocta and Cycliophora are sister groups, the latter should be included in Bryozoa as well (as has been done by Cavalier-Smith, 1998), even though genomic data for Cycliophora is unfortunately still missing.

Further, the analyses strongly support the clade Syndermata, formed by Rotifera and Acanthocephala (Fig. 3). This taxon has been established on the basis of morphological evidence (Ahlrichs, 1995a, b,

1997) and has been further supported by analyses of 18S rDNA sequences (Garey et al., 1996; Garey and Schmidt-Rhaesa, 1998; Zrzavý et al., 1998; Giribet et al., 2000). The position of Platyhelminthes remains ambiguous: while the Bayesian inference analysis favours a sister-group position to Neotrochozoa, maximum likelihood methodology places Platyhelminthes next to Syndermata. The latter confirms the Platyzoa hypothesis. Platyzoa comprise Platyhelminthes, Syndermata, Gastrotricha and Gnathostomulida (Garey & Schmidt-Rhaesa, 1998; Cavalier-Smith, 1998; Giribet et al., 2000) and has first been hypothesized by Ahlrichs (1995a) based on sperm morphology. Previously, Platyzoa was either corroborated (Giribet et al., 2000; Passamaneck and Halanych, 2006) or contradicted (Zrzavý et al., 1998; Peterson and Eernisse, 2001) by rDNA and total evidence analyses. The lack of a robust resolution of the phylogenetic relationships of Platyhelminthes within Spiralia despite the large available dataset is probably due to increased substitution rates in Platyhelminthes and Syndermata causing long-branch attraction artefacts. However, the Eubilateria hypothesis (Hennig, 1979) can clearly be rejected by topology testing. According to this hypothesis, Platyhelminthes, which do not have an anus, are considered to be the sister group of all other Bilateria possessing a one-way gut and an anus. Because Bryozoa is more closely related to Neotrochozoa than to Syndermata in the present analyses, syndermatans (and platyhelminths, depending on the inference method) are not lophotrochozoans according to the node-based definition of this clade (Halanych et al., 1995), even though to further substantiate this conclusion genomic data of Phoronida and Brachiopoda are necessary. For the clade including Lophotrochozoa, Platyhelminthes and Syndermata, some authors have used the name Spiralia (Garey & Schmidt-Rhaesa, 1998; Giribet et al., 2000). This usage seems adequate, because spiral quartet cleavage might be an autapomorphy of that taxon (see below).

Cleavage pattern was often considered a key character for the reconstruction of metazoan phylogeny. Typical spiral quartet cleavage with mesoderm formation by the 4d mesoteloblast or one of its daughter cells (Sørensen et al., 2000; Nielsen, 2001) is known from several lophotrochozoan groups (Mollusca, Annelida, Nemertea, Entoprocta), Platyhelminthes, and Gnathostomulida. If this character state is mapped on the tree (Fig. 3), considering the close relationship of Syndermata to Gnathostomulida discussed above, it turns out to be a possible autapomorphy of the clade including Syndermata,

Platyhelminthes and Lophotrochozoa. For this clade, the name Spiralia lends itself, even though spiral cleavage must have been secondarily modified several times within this group (e.g., in Syndermata, Neoophora, Ectoprocta, Brachiopoda, Cephalopoda). The sister group relation of ectoprocts and entoprocts demonstrates that the transition from spiral to radial cleavage can happen within a clade without any transitional stages being preserved. After all, the different cleavage types were one of the main reasons that the two taxa were classified in different major groups for more than a century. Often coelomic cavities were considered an autapomorphy of a clade Coelomata (Hennig, 1979; Philip et al., 2005). If the coelomic cavities of lophotrochozoans are considered homologous to those of deuterostomes and to the small coelomic cavities present in some ecdysozoans, the tree would indicate a frequent reduction of coelomic cavities in several bilaterian lineages (e.g., in chaetognaths, priapulids, nematodes, platyzoans, entoprocts). However, the varying developmental origin of coelomic cavities in the different bilaterian lineages cast doubts on the homology of the coelom across bilaterians (Nielsen, 2001). The results presented herein therefore indicate that several of the supposed key characters of animal phylogeny such as cleavage pattern and coelomic cavities are subject to greater evolutionary plasticity previously thought.

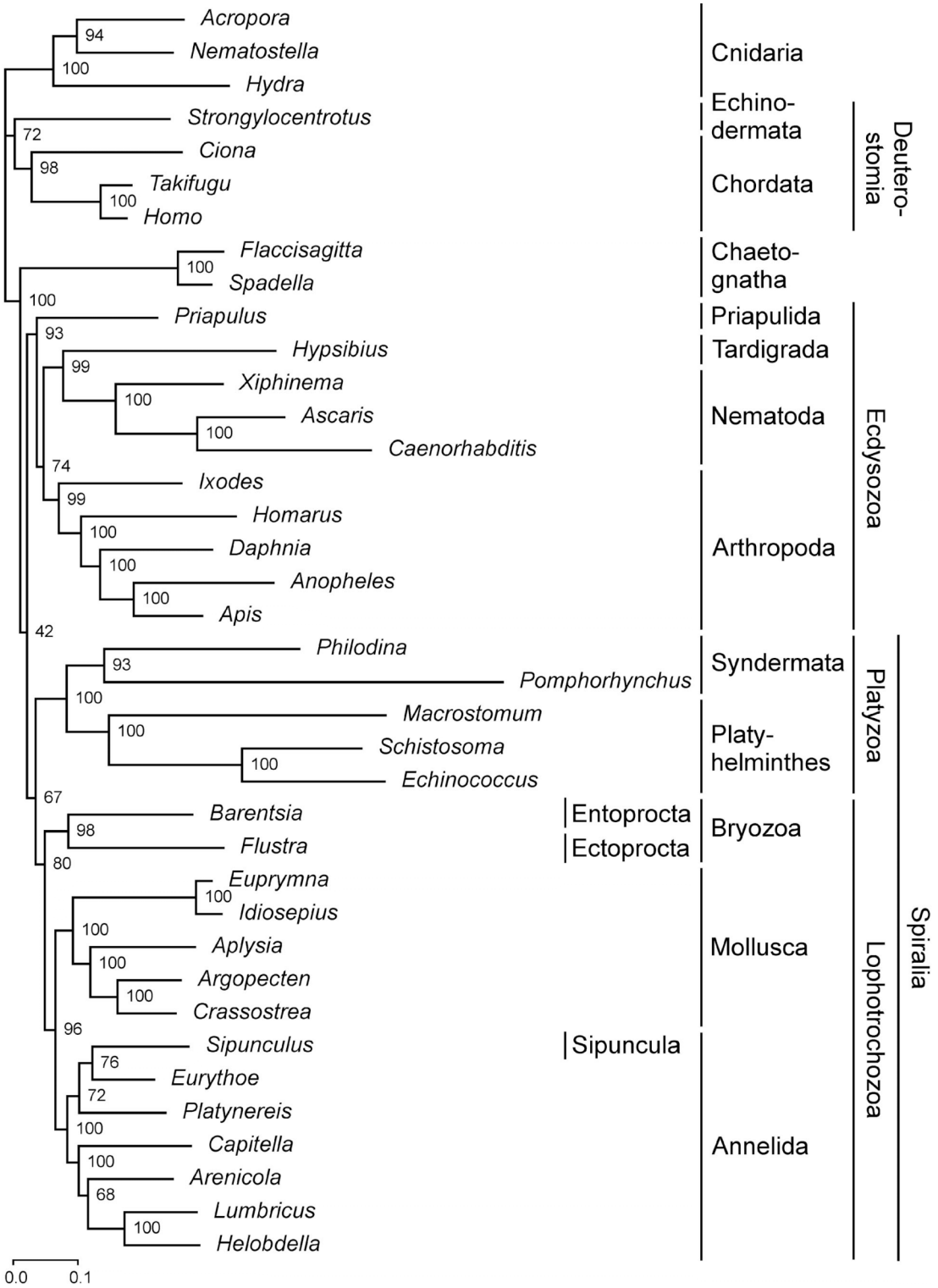


Figure 3. Maximum likelihood tree based on the analysis of 79 ribosomal protein genes, providing evidence for the monophyly of Bryozoa in the broader sense, including Ectoprocta and Entoprocta. Approximate bootstrap support values (LR-ELW) are shown to the right of the nodes.

2.6.3. EST-based phylogenetic analysis of Brachiopoda and Phoronida (Manuscript 2.3)

By including EST data of a brachiopod (*Novocrania anomala*) and a phoronid (*Phoronis muelleri*), the validity of the Lophotrochozoa concept and the monophyly of the lophophorate lineages could be investigated for the first time by genomic-scale data. The results of maximum-likelihood as well as Bayesian analyses, based on concatenated sequences of 79 ribosomal proteins encompassing 11,445 amino acid positions from 39 taxa, demonstrate that the three lophophorate lineages Ectoprocta, Brachiopoda and Phoronida are more closely related to trochozoan phyla than to deuterostomes. In addition, they do not form a monophyletic group (Fig. 4). Thus, the analyses confirm the results previously obtained by the smaller multigene dataset (see above).

Characters that were traditionally used to unite Lophophorata with deuterostomes include a body organization with three distinct coelomic cavities, namely protocoel, mesocoel and metacoel (archimery, Siewing, 1980), a mesosomal tentacular apparatus, entomesoderm derived from the archenteron by enterocoely, larvae with upstream-collecting ciliary bands and heterogeneously assembled metanephridia (Hennig, 1979; Ax, 1995; Lüter and Bartolomaeus, 1997; Sørensen et al., 2000; Brusca and Brusca, 2003). However, the hypothesis that all lophophorate lineages are more closely allied to Deuterostomia than to Protostomia can be rejected by topology tests based on the present ribosomal protein data. Nielsen (2001) argued that ectoprocts show no trace of archimery and that only Brachiopoda and Phoronida form a monophyletic group with Deuterostomia *sensu stricto*. Lüter (2000) suggested that the origin of the coelomic anlage from differentiated archenteral epithelium, which he defined as enterocoely, is a synapomorphy of Brachiopoda and Deuterostomia; he therefore considered these two taxa as sister-groups. Consequently, the hypotheses that Brachiozoa or Brachiopoda alone are the sister-group of Deuterostomia were tested, and both possibilities were rejected.

The conflicting results concerning the phylogenetic relationships of the lophophorates is a major incongruity between morphological and molecular phylogenetic approaches. However, in the last decade the morphological evidence for a close relationship between the lophophorate groups and the deuterostomes has become weaker by careful re-examinations of the characters. It has been shown that neither brachiopods nor phoronids possess three coelomic cavities, because a protocoel is lacking in all

lophophorate groups (Lüter, 2000; Bartolomaeus, 2001). Thus, the archicoelomate concept (Siewing, 1980) uniting Lophophorata and Deuterostomia, founded on the similarities of three distinct coelomic cavities, lost its basis. Additionally, the finding that Pterobranchia may nest within the enteropneusts (e.g., Peterson and Eernisse, 2001) suggests that the ancestral deuterostome more closely resembled a mobile worm-like enteropneust than a sessile colonial pterobranch. This means that the similar tentacular feeding structures of lophophorates and pterobranchs are not a synapomorphy of lophophorates and deuterostomes as supposed previously (Hennig, 1979; Ax, 1995; Lüter and Bartolomaeus, 1997), but evolved independently as convergent adaptations to the sessile lifestyle (Halanych, 1996). Moreover, Lüter (2000) argued that the mesoderm does not originate by enterocoely in Ectoprocta and Phoronida, but that this is the case in Brachiopoda and Deuterostomia only. What is more, whether the mesoderm of brachiopods originates by enterocoely is also in dispute. Jenner (2004) tentatively concluded that reports of true enterocoely, i.e., mesoderm origin by epithelial folding, in brachiopods appear unsupported and that no fundamental difference in the source of mesoderm and mode of coelomogenesis exists between brachiopods and various protostomes. Thus, there are fewer morphological characters arguing against protostome affiliations of brachiopods and phoronids than traditionally assumed.

The phylogenetic analyses discussed here further indicate that Brachiopoda and Phoronida constitute a monophyletic group, Brachiozoa (= Phoronozoa) (Fig. 4). This corroborates previous results based on rDNA (Cohen, 2000; Mallatt and Winchell, 2002; but see Passamaneck and Halanych, 2006), *atpase* (Anderson et al., 2004), morphology (Nielsen, 2001), and a combination of morphological and 18S rDNA datasets (Zrzavý et al., 1998; Giribet et al., 2000; Peterson and Eernisse, 2001). Brachiopods plus phoronids appear as the sister-group of nemerteans in the maximum-likelihood tree (Fig. 4). In contrast, the Bayesian inference analysis shows a sister-group relation of Brachiozoa and Eutrochozoa. The relationships of Brachiozoa within Lophotrochozoa thus remain uncertain. Merely the Conchozoa hypothesis (Cavalier-Smith, 1998; Mallatt and Winchell, 2002), according to which Brachiozoa is the sister-group of Mollusca, can be clearly dismissed on the basis of topology tests.

The central findings of the previous study — considered tentative until a phoronid and a brachiopod could be added to the analysis — were recovered. In particular, Ectoprocta and Entoprocta

remain strongly united (Fig. 4). This agrees with the hypothesis that Bryozoa in the broader sense is monophyletic (Nielsen, 1971, 2001; Cavalier-Smith, 1998). Alternative hypotheses concerning the phylogenetic position of ectoprocts, namely that they are sister to all other Lophotrochozoa including Platyzoa, i.e., Platyhelminthes, Syndermata and allies (Halanych et al., 1995; Halanych, 2004; Passamaneck and Halanych, 2006), or that they are sister to all other protostomes except chaetognaths (Giribet et al., 2000) were again rejected.

From this follows that the three traditional lophophorate lineages, Ectoprocta, Phoronida, and Brachiopoda, do not join into a monophyletic clade in this study (Fig. 4). The monophyly of Lophophorata was rejected with the expected likelihood weights method (but not with the approximately unbiased test). If the monophyly of Lophophorata is constrained, it becomes the sister-group of Eutrochozoa (Peterson and Eernisse, 2001) in the resulting maximum-likelihood tree (not shown). In this tree, Entoprocta is the sister-group of Lophophorata plus Eutrochozoa. Even if this topology should prove correct, the radial cleavage of Lophophorata would be a secondary modification derived from spiral cleavage given that the spiral cleavage of Entoprocta is homologous to that of Annelida and Mollusca. When in contrast the monophyly of Eutrochozoa is enforced, then Brachiozoa and Bryozoa (including Ecto- and Entoprocta) form a monophyletic group in the resulting maximum-likelihood tree. This extended version of "Lophophorata" including Entoprocta is part of the confidence set of trees given the present ribosomal protein dataset, a possibility that is especially interesting, because it is in better agreement with morphological data than topologies that suggest independent origins of Ectoprocta and Brachiozoa within Lophotrochozoa. Potential synapomorphies of Brachiozoa and Bryozoa are the transition to a sessile lifestyle accompanied by the evolution of a horseshoe-shaped, tentacular feeding apparatus and a hydrostatic skeleton consisting of a lophophore coelom and a trunk coelom. In this view, both coelomic cavities were connected in the common ancestor of the two bryozoan subgroups and then were lost in Entoprocta. Most potential synapomorphies of Brachiozoa and Bryozoa are characters that were once thought to support a sister-group relationship between Lophophorata and Deuterostomia, but in light of current evidence that these two groups are unrelated, must have originated by convergence (see above). Hypotheses that suppose that Ectoprocta and Brachiozoa originated independently of each

other from different lophotrochozoan ancestors would require additional convergences of these characters.

Despite the progress presented here, the resolution achieved is still insufficient to fully reconstruct the evolutionary history of Lophotrochozoa. This lack of resolution could neither be avoided by the inclusion of many riboprotein genes and all major lophotrochozoan taxa, nor by the use of the CAT model, which has been shown often to overcome long branch attraction artefacts when other models fail (Baurain et al., 2007; Lartillot et al., 2007). The grouping of taxa with the longest branches in the maximum-likelihood tree (Fig. 4), namely Syndermata and Platyhelminthes, is indeed dissolved in the Bayesian inference reconstruction calculated with the CAT model. Further systematic errors unaccounted for by current tree reconstruction methods, aggravated by the presumably rapid radiation of the lophotrochozoan taxa in the late Precambrian and the limited taxon sampling within many phyla, might be responsible for the lack of resolution within Lophotrochozoa that has been observed both here and in other studies (Halanych et al., 1995; Giribet et al., 2000; Peterson and Eernisse, 2001; Mallatt and Winchell, 2002; Ruiz-Trillo et al., 2002; Anderson et al., 2004, Passamaneck and Halanych, 2006). Promising strategies to combat these issues include improved taxonomic sampling within lophophorates and other lophotrochozoans, and taking measures to reduce the impact of systematic error sources. Two of these, compositional heterogeneity and paralogy, are specifically addressed in the following studies.

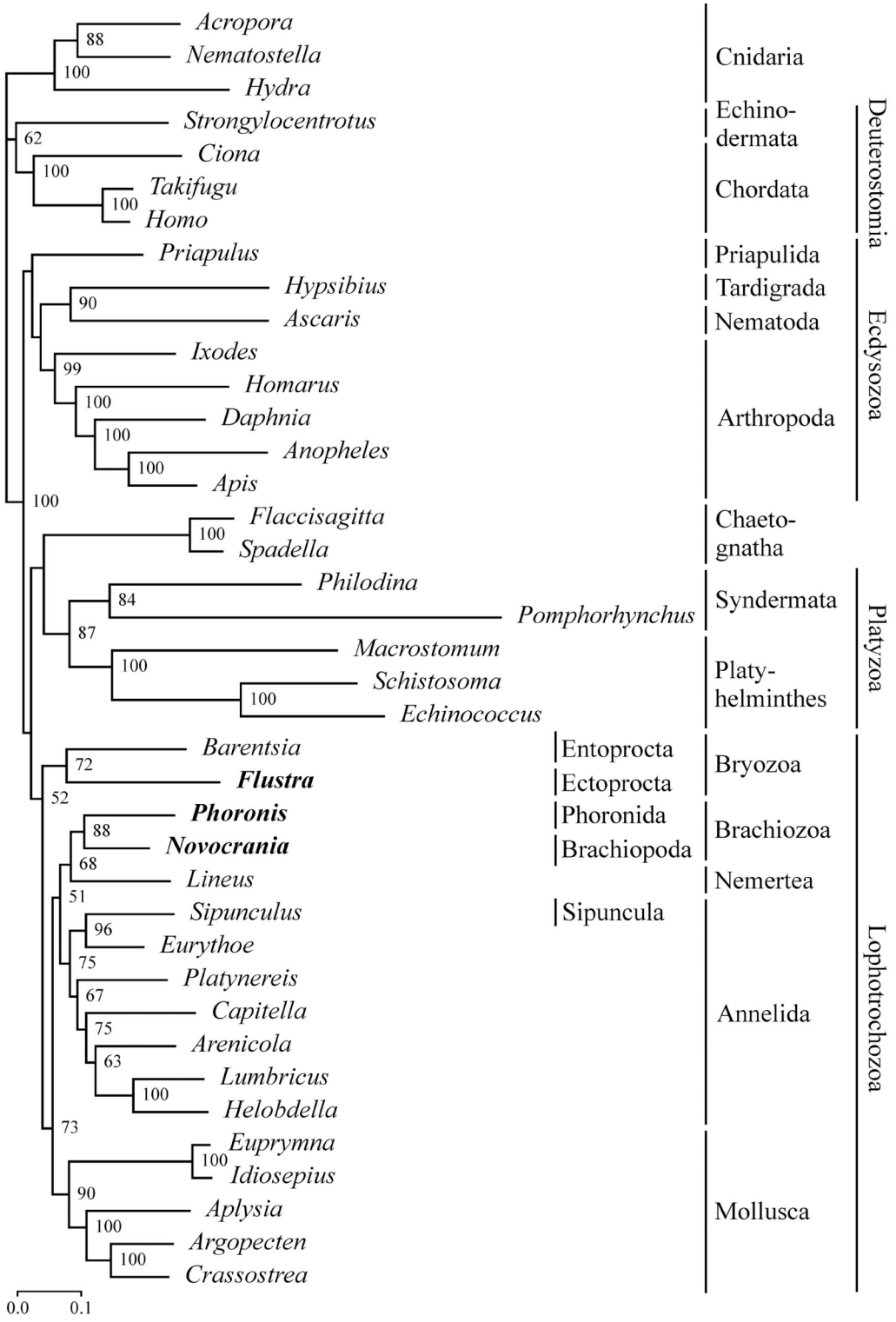


Figure 4. Maximum likelihood tree based on the analysis of 79 ribosomal protein genes, with bryozoans, brachiopods, and phoronids (in bold) represented for the first time by genomic-scale data. Bryozoans and brachiopods plus phoronids (= Brachiozoa) form well supported monophyla, while Lophophorata is polyphyletic according to this analysis. Numbers at the nodes indicate bootstrap support values larger than 50 %.

2.6.4. Reduction of compositional heterogeneity (Manuscript 2.4)

For studying the impact of heterogeneity of the amino acid composition across lineages, the previously used dataset was updated. New taxa were added to account for all lophophorate and entoproct taxa currently represented by EST data, including two EST datasets conducted for this study (*Alcyonidium diaphanum* and *Tubulipora sp.*). In addition, selected taxa employed previously were substituted by more slowly evolving species. Maximum likelihood analysis of the resulting dataset derived from 78 ribosomal protein genes of 48 metazoan taxa recover Brachiopoda and Phoronida are sister groups using an improved taxon sampling. Also congruent with earlier studies and the most extensive phylogenomic analysis to date (Dunn et al., 2008), Brachiozoa constitutes a clade with Nemertea. These relationships were also confirmed by the maximum likelihood analysis with the class frequency mixture model that adjusts for site-specific amino acid frequencies proposed by Wang et al. (2008). In contrast to previous results (and Paps et al., 2009), however, Ectoprocta and Entoprocta do not compose a monophylum in either analysis. Instead, entoprocts appear as sister group of Platyzoa, even though the bootstrap values of these new groupings are below 50 %. These contradictory results indicate that there might be systematic errors as a result of model assumption violations either in previous or in the present analyses.

To evaluate one possible cause of systematic error that may have plagued previous analyses, the amino acid composition of the new dataset was examined: a total of 53.7% of the sequence pairs of the 48 taxa represented showed indeed significantly dissimilar amino acid compositions according to the disparity index test (Kumar and Gadagkar, 2001a,b). This confirms the observation of Lartillot and Philippe (2008) that the assumption of a time-homogeneous amino acid replacement process made by conventional protein models is strongly violated in metazoan phylogenomic data and, thus, there is a risk of observing artefacts related to compositional bias. Three approaches to reduce the potential impact of dissimilar amino acid compositions on the phylogenetic investigation of the present dataset were subsequently attempted:

First, the number of sequence pairs with dissimilar amino acid compositions was reduced by excluding those species that significantly differ in amino acid composition from more than half of all examined species. Accordingly, 21 species had to be excluded, among others all representatives of

Platyhelminthes and Syndermata. Maximum likelihood analyses of the remaining 27 taxa with a mixed protein model recovered monophyletic, but weakly supported Bryozoa and also an increased support for Brachiozoa in comparison to the analysis of the complete dataset.

Secondly, a neighbour-joining tree based on LogDet + Γ distances as modified by Tamura and Kumar (2002) considering among-site rate variation was constructed. In this tree Bryozoa is also monophyletic, but not supported in a bootstrap analysis. The support for Brachiozoa increased, though. However, chaetognaths, platyhelminths, syndermatans and nematodes are clearly subjected to long branch attraction.

Thirdly, the amino acid sequences were recoded using the six groups of chemically similar amino acids that tend to replace one another (Dayhoff et al., 1978) to mitigate compositional bias as suggested by Embley et al. (2003). After recoding, 30.8 % of the pairs of sequences still showed significantly dissimilar compositions according to the disparity index test. The number of sequence pairs with dissimilar amino acid compositions was thus reduced again by excluding those species that significantly differ in amino acid composition from more than half of all examined species. This time, only six taxa had to be excluded and the reduced 42 taxa dataset still includes representatives of Platyhelminthes and Syndermata. Phylogenetic analyses of the recoded 42 taxa dataset with a six-state general time-reversible model (Embley et al., 2003) and considering among-site rate variation confirmed the monophyly of Bryozoa and Brachiozoa with strong support values (Fig. 5). Despite the loss of information resulting from using a reduced amino acid alphabet, the support for these groups was higher in this tree than in those resulting from the first two approaches. Other aspects of the tree were improved as well, including the recovery of Deuterostomia. This shows that using reduced amino acid alphabets is a more efficient method for mitigating artefacts in phylogenetic analyses resulting from compositional heterogeneity than is the simple exclusion of taxa with a divergent amino acid composition or the use of distance methods based on LogDet distances.

Topology tests of alternative hypotheses focused on the phylogenetic position of ectoprocts and entoprocts. The hypotheses that ectoprocts are sister to all other Lophotrochozoa including Platyzoa

(Halanych et al., 1995; Halanych, 2004; Passamaneck and Halanych, 2006), and that entoprocts belong to Platyzoa (Halanych, 2004) could be thus rejected. The latter is especially remarkable, because entoprocts cluster with Platyzoa in the maximum likelihood trees based on the complete amino acid dataset. The hypothesis that entoprocts are sister to molluscs that has been strongly favoured by morphological studies (Bartolomaeus, 1993; Haszprunar, 1996; Wanninger et al., 2007; Haszprunar and Wanninger, 2008) could be rejected by both topology tests with the 42 taxa recoded dataset. Haszprunar and Wanninger (2008) provided a list of nine potential synapomorphies of entoprocts and molluscs, but this list is not based on an analysis of a character matrix of these and possibly related phyla like ectoprocts (such a matrix is actually missing). Haszprunar (1996) conceded that several of these potential synapomorphies are of low phylogenetic significance. However, it could not yet be excluded that entoprocts are sister to Eutrochozoa, together forming Trochozoa. The in some respect complementary hypothesis that Lophophorata is monophyletic (Emig, 1984) could be rejected only by the expected likelihood weights method with the 42 taxa recoded dataset, and should therefore be considered unlikely but conceivable. The hypotheses that Brachiopoda is paraphyletic and that phoronids are the sister group of articulate brachiopods (Halanych et al., 1995; Zrzavý et al., 1998) or inarticulate brachiopods (Cohen et al., 1998; Cohen, 2000; Cohen and Weydmann, 2005) have been significantly rejected by both topology tests based on the 27 taxa amino acid dataset. However, none of these hypotheses could be rejected on the basis of the 42 taxa recoded dataset, and only a sister group relationship of phoronids and articulate brachiopods could be discarded based on the recoded dataset restricted to Bryozoa and Brachiozoa. A denser taxon sampling within brachiopods is necessary to clarify the relationships within Brachiozoa definitively. In other respects the relationships within Lophotrochozoa remain unclear as well. In the maximum likelihood trees based on the 48 taxa amino acid dataset and the recoded dataset (Fig. 5) Brachiozoa is sister to Nemertea as in previous analyses and those of Dunn et al. (2008). In contrast, it is sister to Eutrochozoa including Nemertea, Mollusca and Annelida in the maximum likelihood trees based on the 27 taxa amino acid dataset, and to Nemertea + Annelida in the neighbour-joining tree using modified LogDet + Γ distances. Neotrochozoa including Mollusca and Annelida (with the annelids comprising Sipuncula and Echiura) has been found only in the maximum likelihood analysis

with the mixed protein model. Most of these relationships are not strongly supported by bootstrap tests and none can be rejected by topology tests.

The present study also provided the unprecedented possibility to investigate the internal relationships of ectoprocts on a phylogenomic scale, as all four recent major groups of Ectoprocta, i.e., Phylactolaemata, Stenolaemata, Ctenostomata and Cheilostomata were represented by EST data for the first time. The results presented here (Fig. 5) demonstrate the monophyly of Ectoprocta and a sister group relationship between Phylactolaemata, the freshwater bryozoans, and the predominantly marine Gymnolaemata (= Stenolaemata + Ctenostomata + Cheilostomata; as originally defined by Allman, 1856 and used, e.g., by Ax, 2001). Within Gymnolaemata, Ctenostomata and Cheilostomata form a monophyletic group, Eurystomata (as proposed by Marcus, 1938; and equivalent to Gymnolaemata as used by Cuffey, 1973 and Fuchs et al., in press). This is corroborated by recent analyses of Fuchs et al. (in press) based on 18S rDNA, 28S rDNA and COI, which have also shown that neither Ctenostomata nor Cheilostomata is monophyletic. However, some ctenostomate bryozoans that have not been included in molecular phylogenetic analyses so far might be the sister group of all other Ctenostomata + Cheilostomata + Stenolaemata (Alcyonidiidae do obviously not belong to these basal groups, as *Alcyonidium* clusters here with the cheilostomatans). Diphyletic Ectoprocta with phylactolaemates forming a clade with recent phoronids as hypothesized by Mundy et al. (1981) could be significantly rejected by topology tests, as were most alternative hypotheses concerning ectoproct phylogeny discussed in the literature.

To conclude, the present study explicitly addresses the impact of compositional heterogeneity on phylogenetic inference, and provides additional support for the central results of the previous studies, i.e., the lophotrochozoan affinity of the lophophorate lineages, their polyphyly, the monophyly of brachiopods plus phoronids, and of Bryozoa in the broader sense, including both ectoprocts and entoprocts. It also elucidates the internal relationships of ectoprocts. As suggested by Baurain et al. (2007), improved taxon sampling, replacing fast-evolving species by more slowly evolving species, and using better models of sequence evolution resulted in a reduction of non-phylogenetic signal and a better

resolution of animal phylogeny compared with other analyses (e.g., Rokas et al., 2005). Heterogeneity of the amino acid composition is a widespread among metazoan taxa, with potentially detrimental effects on phylogenetic inference. Despite the loss of information involved, recoding amino acids into groups of functional interchangeability proved to be a more efficient approach to reduce the impact of compositional bias on phylogenetic analyses than simply excluding taxa with strongly deviating amino acid composition or employing distance methods based on LogDet distances. Most relationships between metazoan phyla, however, remained nevertheless and contrary to the expectations raised by Baurain et al. (2007) poorly supported. Even if the support for some of these relationships might further increase by sampling additional genes and taxa, the generally low support for the relationships between metazoan phyla and the short internodes between the phyla still support the conclusion of Rokas et al. (2005) that many of the phyla originated by a closely spaced series of cladogenetic events. It is conceivable that the phylogenetic signal of the relatively few amino acid transformations that occurred between these events in the Precambrian has eroded and been obscured since then by other signals like similarities in amino acid composition to a degree that too little of this signal is left to resolve lophotrochozoan phylogeny robustly.

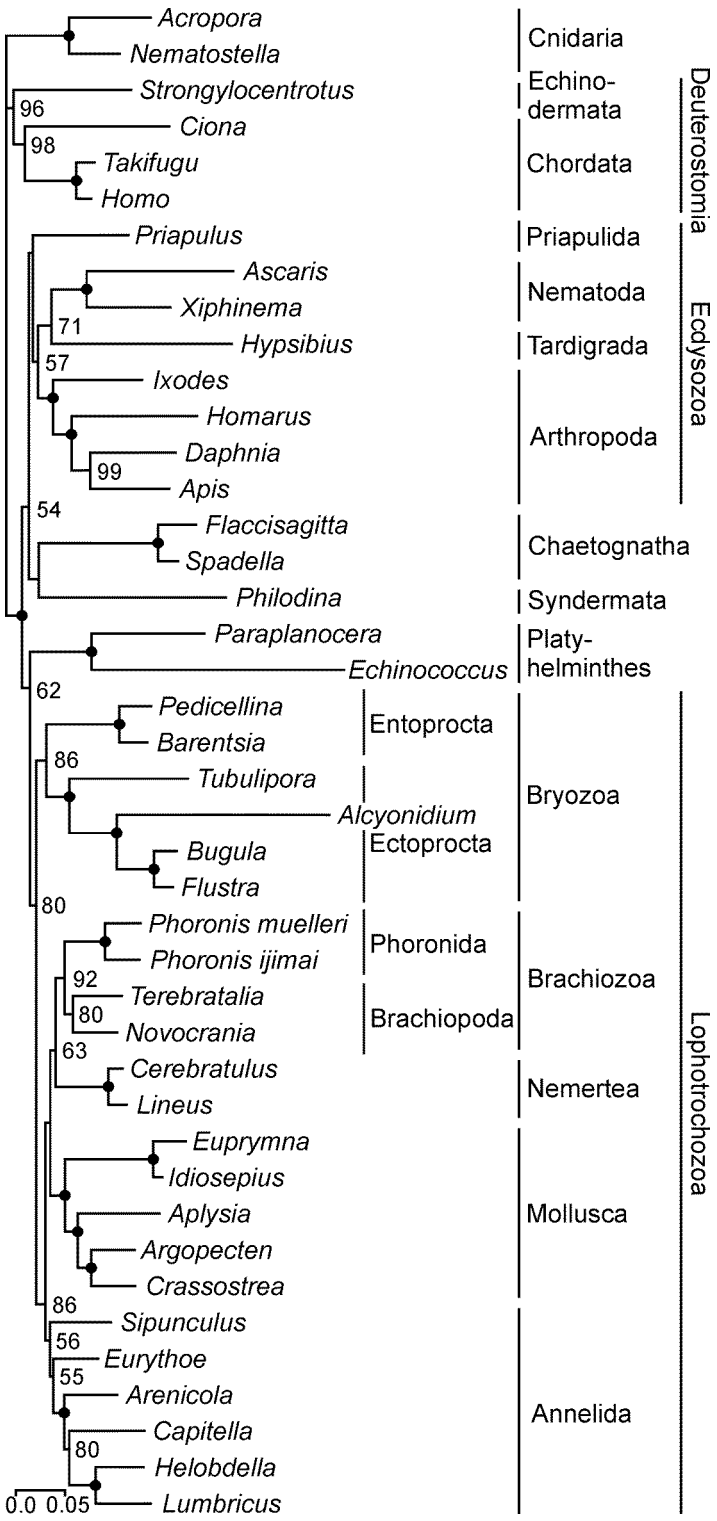


Figure 5. Maximum likelihood tree based on the amino acid sequences of 78 concatenated ribosomal proteins recoded using the six Dayhoff-groups with a six-state general time-reversible model. Only those 42 taxa that differ in amino acid group composition from less than half of all other taxa were considered. Bootstrap support values larger than 50% are shown to the right of the nodes; 100% bootstrap values are indicated by black circles.

2.6.5. Tree-based gene orthology assessment (Manuscript 2.5)

Another source of systematic error in phylogenetics are gene duplications that may result in disagreement between a gene's trees and the evolutionary history of the species. Only orthologous genes, i.e., those arising from speciation rather than gene duplication events (the latter leading to paralogous genes, Fitch, 1970) can be used reliably to infer phylogenetic relationships. Since the availability of large amounts of molecular data allows and demands novel ways to carefully select genes forming orthologous relationships prior to phylogenetic reconstruction, the final study of this work presents a novel, tree-based approach to assess gene homology relations specifically for phylogenetic purposes. To this end, a semi-automatic procedure was realized by Perl programming and a combination of several established software packages used in data mining and phylogenetic reconstruction. The process is organized in three main stages, namely the compilation of homologous gene clusters, the careful reconstruction of gene trees for each cluster, and the visual evaluation of these gene trees to assess homology relations. Each gene was assigned to one of three categories representing varying degrees of evidence for gene duplications, provided by the incidence and extent of species overlap observable between clades in a gene tree. As a case study, ribosomal protein genes were evaluated, because the previous studies relied on this group of genes, and because they are also used extensively in other phylogenomic studies (i.e., Lartillot and Philippe, 2008; Dunn et al., 2008). This allowed to investigate whether artefacts related to paralogy are involved in the incongruencies afflicting these studies.

As a result, ribosomal protein genes were frequently found to be represented by more than one copy per taxon (1.4 on average after fragmentary or identical homologs were removed). However, 92 % of these redundant sequences formed almost exclusively well supported monophyletic groups. Only a small number of genes were represented by more than 2.0 sequences per taxon on average (median values larger than 1.0 were likewise the exception).

Almost two thirds of the 78 investigated genes were classified as category A (defined as lack of species overlap), suggesting that gene duplications occurred not at all or only within lineages represented in this study. This makes these genes unequivocally suitable for deep phylogenetic analyses of bilaterian

animals. Another third of the genes was characterized by species overlap in a single taxon (category B). An ancient gene duplication occurring before the splitting of the studied lineages, followed by massive gene loss in all but one taxon, could provide an explanation for this kind of topology. However, given the branch lengths involved, it seems more likely that a single highly divergent sequence derived from a recent, lineage-specific duplication followed by a change of function and resulting divergent evolution of one of the copies is responsible for this situation. Finally, approximately a tenth of the genes manifested multiple species overlap and thus more conclusive evidence for a possible deep-level gene duplication (category C), which indicates an increased probability to encounter paralogy-related problems when using these genes. In all cases though, multiple independent events of gene loss have to be assumed to explain the observed pattern, as the majority of taxa displayed only single or multiple monophyletic sequence copies.

In contrast, non-ribosomal protein genes representing extensive gene families strongly affected by paralogy and evaluated as a control, were assigned to category C (i.e., *actb, ef1a* and *h2a1*). The approach presented here can thus be used reliably to tell gene duplications that might affect phylogenetic inference of relationships between bilaterian phyla from duplications that do not have an impact on phylogenetic reconstruction at this level.

The present approach has been designed to overcome limitations common to established strategies to predict orthology, including the inability of similarity-based methods to accurately reflect the complexity of gene homology relations at different levels of resolution, and the restriction to (often annotated) whole genome data (e.g., COG/KOG: Tatusov et al., 1997, 2003, Inparanoid: Remm et al., 2001; Berglund et al., 2007). The limitations of accuracy and resolution displayed by similarity-based methods were addressed by adopting a tree-based strategy, which offers the possibility to identify orthologs at high levels of resolution, allowing to pinpoint duplication events to specific lineages and taking the complex, hierarchical nature of gene homology relations into account. This permits the selection of genes qualified to address a given phylogenetic problem. For instance, partitions of a phylogenetic tree in which a gene is suitable to reconstruct phylogenetic relationships may be identified, even if the same gene is afflicted by paralogy in other parts. In other cases, it may be possible to break gene families down into smaller,

orthologous groups. These kinds of relationships are directly observable only by the reconstruction of a gene's phylogenetic tree, and in an amount of detail that is not achieved by similarity-based methods (van der Heijden et al., 2007). In addition, tree-based methods are also more robust and provide higher specificity, which is of particular interest in phylogenetics. While sharing the benefits of high resolution and specificity with other phylogenetic orthology assessment methods (e.g., Zmasek and Eddy, 2002; Storm and Sonnhammer, 2002), using the species overlap criterion avoids the most severe limitation of tree reconciliation: the dependency on a reliable, fully resolved species tree, which precludes this strategy from use in phylogenetics.

Because several lineages that are of critical importance for analysing the tree of life are not yet represented by annotated genome data, the present approach was designed to include draft genome and EST data. The taxon set used here was selected to be representative for bilaterians, allowing conclusions about a gene's general susceptibility to paralogy at the level of bilaterian phyla. Especially the variate yet neglected Lophotrochozoa are represented for the first time by several genome datasets in an orthology assessment study. When focussing on other phylogenetic questions, the taxon set may be tailored to the problem at hand by increasing the density of taxon sampling in the tree partition of interest.

Only a few other orthology assessment strategies that are suitable to evaluate EST data have been proposed for phylogenetic inference so far. For instance, Dunn et al. (2008) developed an explicit method for selecting orthologous genes from EST datasets by combining similarity-based Markov clustering, gene tree reconstruction, and a threshold concerning the mean number of sequences observed per taxon and gene. However, of the 38 ribosomal protein genes excluded due to these criteria, all but three displayed no or only weak (category A and B, respectively) evidence for gene duplication preceding the radiation of Bilateria according to the present study (conversely, five genes of category C were included by Dunn et al., 2008). Eight of these were rejected due to above-threshold values regarding the mean number of sequences observed per taxon and gene, although, as shown above, this does not coincide with phylogenetic evidence for ancient gene duplication. This strategy therefore leads to the exclusion of genes that are merely characterized by high rates of lineage-specific duplication, which pose no threat of generating paralogy-related tree reconstruction artefacts in the context of deep phylogeny. Other sources of the proliferation of highly similar sequences that were observed here, and which are of no consequence

for deep phylogeny, include technical artefacts (e.g., sequencing or assembly errors), transcript variants and allelic variation. Working explicitly with non-redundant genome datasets (e.g., RefSeq sequences) and removing sequence fragments probably caused by sequencing or assembly errors early in the homology assessment process proved to be effective to eliminate some of these sources.

The overly conservative selection of riboprotein genes (Dunn et al., 2008) would result in a substantial decrease of the length and density of phylogenomic data matrices, especially because these genes are readily available in EST data. Particularly affected were small taxa of great phylogenetic significance, which are represented by disproportionately little data. As long as there is still a limitation concerning the amount of sequence data available from many taxonomic groups, genes should be evaluated carefully so that as little information as possible is discarded due to excessively stringent criteria during orthology assessment. The tree-based strategy presented herein proved to be a highly informative yet practical alternative to do so. It offers the high level of resolution and accuracy distinguishing phylogenetic methods to derive gene homology relations, is customisable to specific phylogenetic questions — among others by the possibility to incorporate preliminary genome and EST data — and is implemented in a mainly automated pipeline that mitigates the investment of time and labour long associated with phylogenetic orthology assessment methods.

Based on the evaluation of ribosomal protein gene trees, it can be concluded that most, if not all, of these genes are unafflicted by paralogy at deep metazoan nodes. As discussed earlier, cases of single species overlap are most likely caused by rapid divergence of individual gene copies. Care must be taken to avoid these copies when compiling phylogenetic datasets, but there is little danger of falling victim to paralogy-related tree reconstruction artefacts when using these genes. The exclusion of genes of categories B and C is accompanied by a 37 % loss of positions in comparison to a full riboprotein gene alignment, leading to a reduction of the overall support of the resulting tree. Also, noticeable long-branch attraction artefacts (Felsenstein, 1978) start strongly affecting the tree topology at this point. In comparison, the exclusion of category C genes only is of less consequence: the decrease of the number of alignment positions by 11 % is followed by a slight decline in overall tree support, but little topological change. This difference is caused by notoriously unstable taxa (rotifers, acantocephalans and gnathostomulids) presumably subject

to a long-branch attraction effect. Systematic artefacts that can be traced to paralogy are consequently not involved.

While most ribosomal protein genes are thus suitable with respect to orthology, they alone are insufficient to completely clarify the evolutionary history of animals in terms of resolution. Instability of certain taxa — platyzoans in particular — and low or only moderate support of many inter-phylum relationships (e.g., within Lophotrochozoa) are noticeable even when they are analysed in their entirety. However, the lack of resolution and partial inconsistency experienced by and between phylogenetic studies employing various amounts and combinations of ribosomal protein genes (e.g., previous studies of this work; Rokas et al., 2005; Lartillot and Philippe, 2008; Dunn et al., 2008) are obviously not founded in unrecognized ancient gene duplication events in these genes. Rather, other causes of systematic error that are yet unaccounted for must lie at the heart of this phenomenon, and be dealt with.

3. Conclusion and perspectives

The phylogenetic analyses underlying the present work unambiguously demonstrate that bryozoans, brachiopods, and phoronids are closely related to molluscs, annelids, and relatives, and thus support the Lophotrochozoa instead of the Radialia concept. This could be shown for the first time using large multigene datasets, and convincingly confirms previous single-gene or small multigene analyses (e.g., Mallatt and Winchell, 2002; Ruiz-Trillo et al., 2002; Anderson et al., 2004; Passamaneck and Halanych, 2004; Passamaneck and Halanych, 2006, Waeschenbach et al., 2006). The most comprehensive phylogenomic study on metazoan relationships to date arrives at the same conclusion (Dunn et al., 2008). Considering that many morphological synapomorphies of lophophorates and deuterostomes, including archimery and the mode of mesoderm formation, do not withstand closer scrutiny (see chapter 2.3, "*Phylogenomic analyses of lophophorates (brachiopods, phoronids and bryozoans) confirm the Lophotrochozoa concept*" for a more detailed discussion), evidence for the Lophotrochozoa concept is compelling.

Also consistent with earlier investigations (e.g., Mackey et al., 1996; Giribet et al., 2000; Ruiz-Trillo et al., 2002; Anderson et al., 2004; Passamaneck and Halanych, 2006; Waeschenbach et al., 2006), the analyses presented here strongly indicate lophophorate polyphyly. This conclusion recently received additional support by the phylogenomic study of Dunn et al. (2008) and an up-to-date analysis of rDNA data (Paps et al., 2009). The most likely topology according to the present results is characterized by a basal position of bryozoans within Lophotrochozoa, while brachiopods and phoronids form a more derived lophotrochozoan clade together with molluscs, annelids, and nemertines.

The sister group of ectoproct bryozoans has been identified as Entoprocta, an unexpected grouping that is corroborated by a recent study based on rDNA data (Paps et al., 2009), although morphological evidence is scarce (Nielsen, 2001). However, both ectoprocts and entoprocts also experience — presumably artefactual — attraction by platyzoan taxa (see chapter 2.4, "*Reducing compositional heterogeneity improves phylogenomic inference of lophotrochozoan relationships*", and Passamaneck and Halanych, 2006; Dunn et al., 2008), which makes the validation of bryozoan monophyly difficult, and obscures the branching order of these three groups. Within Bryozoa the present work confirms the

monophyly of Ectoprocta and a sister group relationship between phylactolaemates, the freshwater forms, and the predominantly marine gymnolaemates.

Evidence for Brachiozoa in the form of a sister group relationship between brachiopods and phoronids is likewise strong, and consistent with many other molecular analyses (Giribet et al., 2000; Anderson et al., 2004; Dunn et al., 2008; Paps et al., 2009) and morphology (Nielsen, 2001). No indication was observed that phoronids constitute derived brachiopods (Cohen, 2000). There is uncertainty, however, concerning the sister group of Brachiozoa, with the most probable aspirant according to the phylogenomic analyses included in this work being Nemertea. This grouping also finds support by Dunn et al. (2008), although morphological synapomorphies are unknown at the moment.

Despite these achievements, the resolution of the animal tree of life that is obtained by the analyses presented here is still incomplete, and — as implied above — many questions remain unsolved. This circumstance, however, is shared with comparable studies (e.g., Rokas et al., 2005; Dunn et al., 2008; Lartillot and Philippe, 2008). Some parts of the metazoan tree are even characterized by contradictory results between analyses, e.g. concerning the monophyly of Bryozoa (Dunn et al., 2008) or the position of Platyhelminthes (Lartillot and Philippe, 2008). In contrast to the beginning of this study, most animal phyla are now represented by genomic-scale data, which allows phylogenomic analyses not constrained by insufficient taxon sampling. Yet while approaches employing large numbers of genes from many taxa are effective in overcoming stochastic errors that plagued earlier, single-gene studies, they are prone to a range of systematic error sources. Most of these are related to inadequate modelling of molecular evolution due to diverging evolutionary rates across taxa, heterotachy, compositional bias or selective constraints (Delsuc et al., 2005). During this study, efforts were undertaken not only to improve taxon sampling and replace fast-evolving species by more slowly evolving ones, but also to account for compositional heterogeneity observable in metazoan protein sequences, and to provide evidence for the suitability of the genes used with respect to orthology. While sampling additional genes and taxa will continue to be useful, e.g., by permitting to break long branches or select slow-evolving taxa and genes (Regier et al., 2008), improved models of molecular evolution directly accounting for rate differences

among lineages and across time, compositional heterogeneity, and selective constraints are required to more reliably infer interphyletic relationships.

However, another explanation for the lack of resolution related to the evolutionary process itself is conceivable. The generally low support values and short internodes consistently observed between many phyla indicate that the early evolutionary history of animals was characterized by rapid episodes of radiation. In this case, the phylogenetic signal that has been accumulated between these closely spaced cladogenetic events might be too weak and obscured by non-phylogenetic signal to elucidate metazoan — and particularly lophotrochozoan — phylogeny (Rokas et al., 2005). Simply increasing the amount of sequence data, as is promised by the advent of next-generation sequencing techniques, will then not be sufficient to confidently resolve the branching order within animals. There is hope, though, that rare genomic events that have occurred between these rapid splits, e.g., intron and transposon integration, microRNA innovation or large-scale genome modifications, may serve as phylogenetic signatures shared between phyla. The identification and analysis of these novel markers will probably present the next step in phylogenetics, and might finally grant a more precise understanding of the course of animal evolution.

4. Author's Contributions

The following section describes in detail my own contributions to the studies that comprise this work.

Manuscript 2.1: Multigene analysis of lophophorate and chaetognath phylogenetic relationships

For this study, I collected specimens of *Plumatella repens,* and performed all laboratory work, including RNA preparation, target gene amplification and sequencing. I was also responsible for most computational work including sequence and alignment assembly, Bayesian inference analysis and data submission to public archives. I revised the manuscript in close cooperation with Bernhard Hausdorf, who composed the initial draft.

Manuscript 2.2: Spiralian phylogenomics supports the resurrection of Bryozoa comprising Ectoprocta and Entoprocta

My contributions to this study included the preparation of RNA from *Flustra foliacea,* and the compilation and editing of the alignment in cooperation with Alexander Witek (University of Mainz). I coordinated the protein model evaluation for each gene, conducted the Bayesian inference analysis, and submitted the majority of data to public archives. Finally, I contributed parts to and edited the manuscript written by Bernhard Hausdorf.

Manuscript 2.3: Phylogenomic analyses of lophophorates (brachiopods, phoronids and bryozoans) confirm the Lophotrochozoa concept

For this study, I collected samples of *Novocrania anomala* and *Phoronis muelleri* in Sweden, and carried out the RNA preparation. Computational work performed by me included the retrieval of ribosomal protein sequences from EST data, alignment building and editing, and the submission of the data to

public archives. I conducted all Bayesian as well as individual maximum-likelihood analyses, and wrote and revised the manuscript together with Bernhard Hausdorf.

Manuscript 2.4: Reducing compositional heterogeneity improves phylogenomic inference of lophotrochozoan relationships

Experimental work conducted by me for this study involved RNA preparation from *Alcyonidium diaphanum* and *Tubulipora sp.* I also compiled the dataset, and participated in the computation of the phylogenetic trees. I contributed parts to and edited the manuscript written by Bernhard Hausdorf.

Manuscript 2.5: Tree-based orthology assessment illustrated by the evaluation of ribosomal protein genes

The approach underlying this manuscript was developed, programmed and implemented by me. I further performed all phylogenetic computations, and wrote the manuscript, which was edited by Bernhard Hausdorf, who also conceived the initial idea.

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Declaration

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