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Testing the new animal phylogeny: A phylum level molecular analysis of the animal kingdom

Sarah J. Bourlat [a,*,1], Claus Nielsen [b], Andrew D. Economou [a], Maximilian J. Telford [a]

[a] Department of Biology, University College London, Darwin Building, Gower Street, London WC1E 6BT, UK
[b] Zoological Museum, Invertebrate Department, Universitetsparken 15, DK-2100 Copenhagen, Denmark

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ABSTRACT

The new animal phylogeny inferred from ribosomal genes some years ago has prompted a number of radical rearrangements of the traditional, morphology based metazoan tree. The two main bilaterian clades, Deuterostomia and Protostomia, find strong support, but the protostomes consist of two sister groups, Ecdysozoa and Lophotrochozoa, not seen in morphology based trees. Although widely accepted, not all recent molecular phylogenetic analyses have supported the tripartite structure of the new animal phylogeny. Furthermore, even if the small ribosomal subunit (SSU) based phylogeny is correct, there is a frustrating lack of resolution of relationships between the phyla that make up the three clades of this tree. To address this issue, we have assembled a dataset including a large number of aligned sequence positions as well as a broad sampling of metazoan phyla.

Our dataset consists of sequence data from ribosomal and mitochondrial genes combined with new data from protein coding genes (5139 amino acid and 3524 nucleotide positions in total) from 37 representative taxa sampled across the Metazoa. Our data show strong support for the basic structure of the new animal phylogeny as well as for the Mandibulata including Myriapoda. We also provide some resolution within the Lophotrochozoa, where we confirm support for a monophyletic clade of Echiura, Sipuncula and Annelida and surprising evidence of a close relationship between Brachiopoda and Nemertea.

1. Introduction

The interrelationships among living phyla of metazoans have been the subject of controversy for a century, and represent a challenge in both morphological and molecular terms. The morphology based view of metazoan phylogeny implicit or explicit in zoology textbooks arranges metazoan phyla into acoelomates, pseudocoelomates and coelomates in accordance with a gradual increase in complexity of body plans. However, an alternative scheme dividing the bilaterian animals into protostomes and deuterostomes was established by Grobben (1908, 1910) and has had many adherents over the years, e.g. Kaestner (1954/55), Remane et al. (1976), Nielsen (1985, 2001), and Storch and Welsch (2004). The first molecular phylogenetic analyses of animal phylogeny date back 20 years (Field et al., 1988) and since this time our understanding of animal relationships has undergone a series of revolutions (Halanych, 2004). Almost all of these changes in our understanding of how the animals evolved derive from analyses of a single gene: the small subunit ribosomal RNA gene (SSU rRNA).

The revolutionary paper of Aguinaldo et al. (1997) recovered monophyletic clades of protostomes and deuterostomes, and divided the protostomes into ecdysozoans and lophotrochozoans. The Ecdysozoa—named for the shared characteristic moulting of the cuticle (ecdysis)—unites the arthropods with the pseudocoelomate nematodes, priapulids and other worms bearing an introvert. The Lophotrochozoa, originally identified by Halanych (Halanych et al., 1995) unites the annelids and molluscs and a number of other protostome phyla, most of which possess one or other of the two characteristics that give the clade its name: a ciliated feeding structure called a lophophore and a trochophore type larva. In traditional phylogenies, a clade called the Articulata united the annelids and arthropods on the basis of shared segmentation, but the molecular analyses separate these two groups and place Arthropoda in the Ecdysozoa and Annelida in the Lophotrochozoa.

Another radical aspect of this so-called "new animal phylogeny" is the relocation of several phyla that had been thought to represent intermediate grades of complexity and early branches (Platyhelminthes, Nemertea and Nematoda) amongst the coelomate groups at the crown of the tree (Aguinaldo and Lake, 1998). Analyses of SSU rRNA also moved phyla that had been linked to the deuterostomes, such as chaetognaths and lophophorates, into the protostomes. Additional support for the

* Corresponding author. Fax: +46 8 5195 4125.
E-mail address: Sarah.Bourlat@nrm.se (S.J. Bourlat).
[1] Present address: Department of Invertebrate Zoology, Swedish Museum of Natural History, Box 50007, SE-104 05 Stockholm, Sweden.

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Ecdysozoa/Lophotrochozoa split has been obtained from the analysis of Hox genes (de Rosa et al., 1999), horseradish peroxidase (HRP) antibody staining (Haase et al., 2001), large subunit ribosomal RNA (LSU) (Mallatt and Winchell, 2002), myosin heavy chain (Ruiz-Trillo et al., 2002), and sodium/potassium ATPase (Anderson et al., 2004). Resolution within the Lophotrochozoa and Ecdysozoa remains limited.

In contrast to the support for the new animal phylogeny, a series of studies focussing on large datasets and few taxa (reflecting the availability of whole genomes from a small number of model organisms (Blair et al., 2002; Wolf et al., 2004)) resulted in support for a monophyletic clade of coelomate animals—'Coelomata': essentially a return to more traditional gradist theories. It seems likely, however, that this result is due to phylogenetic errors stemming from the fast evolving *Caenorhabditis elegans* (Copley et al., 2004; Irimia et al., 2007; Philippe et al., 2005).

Perhaps the most comprehensive study of metazoan relationships to date is of Philippe et al., with 146 genes from 35 species (Philippe et al., 2005), in which the problem of long branch attraction is addressed by removing fast evolving taxa from the analysis, and using a better model of sequence evolution, resulting in the placement of nematodes and platyhelminthes in the Ecdysozoa and Lophotrochozoa, respectively. Some authors claimed that metazoan relationships cannot be fully resolved because they 'represent a closely spaced series of cladogenetic events', reflecting the Cambrian explosion (Rokas et al., 2005). However, using strategies to reduce the non-phylogenetic signal, such as increasing the number of species, replacing fast evolving species by a slowly evolving one, and using a better model of sequence evolution (Baurain et al., 2007) results in improved resolution in the metazoan tree. In addition, small sets of genes can be just as effective given increased attention to taxon sampling (Hedtke et al., 2006).

In the present study, we use molecular data from the large and small ribosomal subunits (3524 reliably aligned nucleotide positions), from whole mitochondrial genomes (2048 reliably aligned amino acids) and from 8 nuclear protein coding genes (3090 reliably aligned amino acids). To cover the broad range of divergences in our tree (deep splits at the level of the Precambrian) as well as more recent divergences (towards the tips of the tree), we have used genes with different levels of conservation across the metazoan phyla, and which overlap in their resolution (see Section 4).

We have sampled 168 species across the Metazoa and combined them into 37 higher order taxa, each representing a phylum or a class of the animal kingdom. Within the Ecdysozoa we include priapulids, nematomorphs, nematodes, pycnogonids, arachnids, chilopods, diplopods, xiphosurans, crustaceans and insects. Within the Lophotrochozoa, we have sampled polychaete and clitellate annelids, sipunculids, echiurans, bivalve, gastropod, polyplacophoran, and cephalopod molluscs, nemerteans, phoronids and brachiopods as well as several deuterostomes. Our aim was first, to test the Lophotrochozoa/Ecdysozoa hypothesis and second, to provide more resolution within these groups. To address the problem of long branch attraction, we use the slowest evolving taxa from our dataset and we root the tree using the closest possible outgroups (Porifera, Hydrozoa and Anthozoa).

Data were analyzed using Bayesian analysis of concatenated translated protein sequences and the rRNA DNA sequences. To make the datasets more complete, we have, where necessary, pooled data from several species into a composite concatenated sequence representative of a given monophyletic group (see Table 1); since we aim to obtain resolution at the level of the 'phyla' we consider this to be acceptable for this type of analysis.

2. Results and discussion

The tree obtained with our dataset of concatenated nuclear, ribosomal and mitochondrial genes is in agreement with the basic structure of the 'new animal phylogeny' and supports the Ecdysozoa/Lophotrochozoa hypothesis (Fig. 1). We find that the Coelomata hypothesis, which groups the animals with a mesodermally lined body cavity to the exclusion of the pseudocoelomate nematodes and the acoelomate flatworms is very strongly rejected using the Bayes factor test ($2\log_e(B_{10}) = 749.56$) (Bayes factors represent the ratio of the model likelihoods of the topologies of the two models under consideration and values of $2\log_e(B_{10})$ (two times the difference between the harmonic means of the two models) > 10 are considered strong evidence to support one model over another).

Our tree (Fig. 1) supports a monophyletic clade of protostomes (Bootstrap Value (BV) 96, Bayesian Posterior Probability (BPP) 1.00), monophyletic deuterostomes (92/1.00) and confirms the Ecdysozoa/Lophotrochozoa split (70/1.00 and 96/1.00, respectively).

2.1. Ecdysozoa

The clade Ecdysozoa, originally described by Aguinaldo et al. on the basis of SSU DNA, groups the animals that share a cuticle shed by moulting (or ecdysis) (Aguinaldo et al., 1997). The ecdysozoan clade is recovered in our tree (Fig. 1, 70/1.00) and consists of the nematodes + nematomorphs at the base (73/1.00) and then the priapulids as a sister group to all arthropods (77/1.00).

2.2. Arthropoda: Mandibulata or Paradoxopoda?

Within the Arthropoda, our tree (Fig. 1) groups Crustacea with Hexapoda (93/1.00) in the Pancrustacea. This relationship had been observed previously and confirms existing molecular phylogenies based on LSU and SSU (Mallatt et al., 2004), and studies based on mitochondrial genomes (Boore et al., 1998; Hwang et al., 2001). In a more recent study, hexapods have been shown to be, in effect, terrestrial crustaceans (Regier et al., 2005). More controversially, our tree supports the Mandibulata (88/1.00), the Pancrustacea + Myriapoda grouping together, with Chelicerata as an outgroup, rather than the Paradoxopoda (Myriapoda with Chelicerata). Mandibulata, the monophyletic grouping of all arthropods with mandibles (insects, crustaceans and myriapods) to the exclusion of the chelicerates has been supported in molecular and morphological studies (Snodgrass, 1938; Giribet et al., 2001, 2005; Edgecombe et al., 2003). However, the Paradoxopoda grouping has been recovered in numerous molecular studies based on SSU and LSU (Mallatt et al., 2004; Mallatt and Giribet, 2006), mitochondrial genes (Hwang et al., 2001; Negrisolo et al., 2004) and protein coding genes (Pisani et al., 2004). There are as yet no derived morphological features that unite the Paradoxopoda (Hwang et al., 2001; Mallatt et al., 2004; Pisani et al., 2004).

The relative support for the Mandibulata versus Paradoxopoda hypothesis was tested using Bayes factors. Bayes factor tests on the dataset reject the Paradoxopoda tree topology ($2\log_e(B_{10}) = 60.64$).

2.3. Arthropoda: Chelicerata

In our analysis, we find that the horseshoe crabs (Xiphosura) cluster with the arachnids (95/1.00), in agreement with other molecular and morphological studies (Regier et al., 2005; Wheeler and Hayashi, 1998; Giribet et al., 2001), and that there is weak support for placing pycnogonids as basal chelicerates (57/0.97).

Table 1
Species used in concatenation

Higher order taxon	Lineage	Species used in data concatenation	% Missing data and gaps
Xenoturbella	Phylum Xenoturbellida	*Xenoturbella bocki*	45.8
Asteroids	Phylum Echinodermata	*Asterias forbesii* *Asterias amurensis* *Asterina miniata* *Asterina pectinifera* *Asterias rubens* *Astropecten brasiliensis*	13.8
Echinoids	Phylum Echinodermata	*Encope michelini* *Dendraster excentricus* *Echinus esculentus* *Strongylocentrotus purpuratus* *Eucidaris tribuloides* *Anthocidaris crassispina* *Lytechinus variegatus* *Arbacia punctulata* *Arbacia lixula*	6.4
Hemichordates	Phylum Hemichordata Class Enteropneusta	*Saccoglossus bromophenolosus* *Saccoglossus* sp. *Harrimania planktophilus* *Balanoglossus carnosus* *Saccoglossus kowalevskii* *Ptychodera flava*	14.8
Homo	Phylum Chordata	*Homo sapiens*	0
Mus	Phylum Chordata	*Mus musculus*	0.2
Teleosts	Phylum Chordata	*Danio rerio* *Oncorhynchus mykiss*	13.9
Arachnids	Phylum Arthropoda Subphylum Chelicerata	*Tetranychus urticae* *Rhipicephalus appendiculatus* *Tegenaria gigantea* *Phormictopus* sp. *Aphonopelma chalcodes* *Loxosceles reclusa* *Neacarus texanus* *Heptathela hangzhouensis* *Habronattus oregonensis* *Aphonopelma* sp. *Mastigoproctus giganteus*	4.2
Xiphosurans	Phylum Arthropoda Subphylum Chelicerata	*Limulus polyphemus* *Carcinoscorpius rotundicauda*	13.2
Pycnogonids	Phylum Arthropoda Subphylum Chelicerata	*Anoplodactylus lentus* *Tanystylum orbiculare* *Endeis laevis* *Colossendeis* sp. *Callipallene* sp. *Nymphon gracile*	21.1
Chilopods	Phylum Arthropoda Subphylum Myriapoda	*Lithobius* sp. *Strigamia maritima* *Scolopendra polymorpha* *Scutigera coleoptrata* *Scolopocryptops sexspinosus* *Tuoba laticeps* *Thereuonema* sp.	8.2
Diplopods	Phylum Arthropoda Subphylum Myriapoda	*Polyxenus fasciculatus* *Diplopoda* sp. *Archispirostreptus gigas* *Oxidus gracilus* *Narceus annularus* *Narceus americanus* *Orthoporus* sp. *Thyropygus* sp.	6.7
Crustaceans	Phylum Arthropoda Subphylum Crustacea	*Artemia* sp. *Triops longicaudatus* *Triops cancriformis* *Daphnia pulex* *Dilocarcinus pagei* *Ostracoda* sp. *Lepas anserifera* *Carcinus maenas* *Argulus sp.* *Acanthocyclops vernalis* *Harbansus paucichelatus* *Skogsbergia lerneri*	6.8
Thysanurans	Phylum Arthropoda Class Insecta	*Thermobia domestica* *Ctenolepisma lineata* *Ctenolepisma longicaudata*	18.3
Anopheles	Phylum Arthropoda Class Insecta	*Anopheles albimanus* *Anopheles gambiae* *Anopheles quadrimaculatus*	14.8
Drosophila	Phylum Arthropoda Class Insecta	*Drosophila melanogaster*	0.5
Apis	Phylum Arthropoda Class Insecta	*Apis mellifera*	16.7
Clade III nematodes	Phylum Nematoda	*Ascaris lumbricoides* *Ascaris suum* *Brugia malayi* *Onchocerca volvulus*	21.2
Caenorhabditis	Phylum Nematoda	*Caenorhabditis elegans*	0.5
Nematomorphs	Phylum Nematomorpha	*Paragordius varius* *Gordius aquaticus* *Chordodes morgani*	35.1
Priapulids	Phylum Priapulida	*Priapulus caudatus* *Halicryptus spinulosus*	13.3
Polychaetes	Phylum Annelida Class Polychaeta	*Phyllodoce* sp. *Arenicola marina* *Nereis macrydi* *Sabella pavonina* *Ophelina* sp. *Marenzelleria viridis* *Nereis succinea* *Nereis limbata* *Platynereis dumerilii*	15.7
Echiurans	Phylum Echiura	*Urechis caupo* *Echiurus echiurus* *Listriolobus pelodes*	22.4
Clitellates	Phylum Annelida Class Clitellata	*Allolobophora* sp. *Lumbricus terrestris* *Hirudo medicinalis* *Eisenia fetida* *Helobdella stagnalis* *Lumbricus terrestris*	17.8
Sipunculids	Phylum Sipuncula	*Phascolion strombus* *Phascolopsis gouldii*	29.2
Bivalves	Phylum Mollusca Class Bivalvia	*Mytilus edulis* *Crassostrea gigas* *Pinctada fucata* *Ostrea edulis* *Mytilus galloprovincialis* *Macoma nasuta* *Lampsilis cardium* *Nucula proxima*	7.1
Gastropods	Phylum Mollusca Class Gastropoda	*Marisa* sp. *Ilyanassa obsoleta* *Tectura testudinalis* *Lottia austrodigitalis* *Batillus cornutus* *Tegula brunnea* *Philine aperta* *Biomphalaria* sp. *Lottia digitalis* *Deroceras reticulatum*	11.2

(*continued on next page*)

Table 1 (*continued*)

Higher order taxon	Lineage	Species used in data concatenation	% Missing data and gaps
Polyplacophorans	Phylum Mollusca Class Polyplacophora	*Leptochiton* sp. *Chaetopleura apiculata* *Lepidochitona hartwegi* *Katharina tunicata*	16.3
Cephalopods	Phylum Mollusca Class Cephalopoda	*Loligo pealei* *Octopus cyanea* *Octopus rubescens* *Loligo bleekeri* *Octopus vulgaris*	27.7
Nemerteans	Phylum Nemertea	*Cerebratulus* sp. *Lineus longissimus* *Lineus ruber* *Amphiporus* sp.	44.2
Brachiopods	Phylum Brachiopoda Order Articulata	*Terebratalia transversa* *Terebratulina retusa*	29.2
Phoronids	Phylum Phoronida	*Phoronis psammophila* *Phoronis vancouverensis* *Phoronopsis viridis*	27.1
Bryozoans	Phylum Bryozoa Class Gymnolaemata	*Tubulipora* sp. *Watersipora subtorquata* *Bugula turrita* *Flustrellidra hispida*	27.6
Urochordates	Phylum Chordata Subphylum Urochordata	*Ciona intestinalis* *Ascidia* sp. *Ciona savignyi*	17.7
Poriferans	Phylum Porifera	*Leucosolenia* sp. *Geodia neptuni* *Tethya actinia* *Axinella corrugata* *Scypha* sp. (*Sycon* sp.) *Leucosolenia* sp.	16.1
Anthozoans	Phylum Cnidaria Class Anthozoa	*Urticina eques* *Anemonia erythraea* *Metridium senile* *Antipathes galapagensis*	22.3
Hydrozoans	Phylum Cnidaria Class Hydrozoa	*Hydra* sp. *Hydra vulgaris* *Hydractinia echinata* *Hydra circumcincta* *Hydra littoralis*	50.7

Early studies, such as those of Snodgrass (Snodgrass, 1938) had already established a morphological relationship between arachnids, xiphosurans and also the sea spiders (pycnogonids). However, some studies based on molecules and morphology combined (Giribet et al., 2001) placed the pycnogonids at the base of all extant arthropods. The phylogenetic position of the pycnogonids leads to debate concerning the origin of arthropod head appendages. The debate centres around the suggested homology of pycnogonid chelifores to the protocerebral 'great appendages' of certain Cambrian stem-group arthropods rather than to the deuteocerebral chelicerae of spiders (Maxmen et al., 2005). If true, this would support the idea of the pycnogonids as a basal branch of the arthropods (Budd and Telford, 2005). We tested the alternative hypothesis that pycnogonids are basal to all other arthropods using Bayes factors, and we find that this tree topology cannot be rejected ($2\log_e(B_{10}) = 6.84$), showing that the placement of pycnogonids is not strongly resolved using our data.

The sistergroup relationship between pycnogonids and chelicerates, while poorly supported, is in agreement with some previous molecular studies (Mallatt et al., 2004; Regier et al., 2005; Mallatt and Giribet, 2006), and with morphological analyses (Dunlop and Arango, 2005; Waloszek and Dunlop, 2002). This result suggests that pycnogonid chelifores may indeed be homologous to spider chelicerae as traditionally thought and subsequent studies of Hox gene expression boundaries seem to confirm this (Jager et al., 2006).

2.4. Nematoida and Priapulida

Our analysis suggests that the nematomorphs (or horsehair worms), nematode-like parasites whose larvae are parasitic in arthropods, are a sister group to the nematodes (73/1.00). This grouping, named the Nematoida, is supported (although weakly) in combined analyses of LSU and SSU (Mallatt et al., 2004; Mallatt and Giribet, 2006), and in morphological studies. Nematodes and nematomorphs share a number of synapomorphies, including the structures of the body wall, the cuticle and the ectodermal nerve cords (Nielsen, 2001).

Our dataset recovers the priapulids as sistergroup of the arthropods (77/1.00). This placement disagrees with other molecular analyses based on SSU and LSU (Mallatt and Giribet, 2006; Mallatt et al., 2004), which position the Nematoida closer to the arthropods than the priapulids.

Morphological studies, on the other hand unite priapulids, nematodes and nematomorphs (together with kinorhychs, loriciferans and gastrotrichs) into a monophyletic Cycloneuralia (Schmidt-Rhaesa, 1998) or Introverta (Nielsen, 2001). The name Cycloneuralia refers to a unique collar-shaped, peripharyngeal brain present in gastrotrichs, nematodes, priapulids, kinorhynchs and loriciferans (Nielsen, 2001). These phyla also share an inversible anterior end (the introvert) and, with the exception of the gastrotrichs, the presence of a moulted cuticle and absence of locomotory cilia.

We tested the alternative 'Introverta' hypothesis, (the monophyletic grouping of priapulids, nematodes and nematomorphs, based on the presence of an introvert), using Bayes factors and find that this tree topology is rejected as significantly less well supported than one grouping the priapulids as sister group to the arthropods ($2\log_e(B_{10}) = 46.18$).

2.5. Lophotrochozoa

The Lophotrochozoa is a clade originally identified by SSU data (Halanych et al., 1995) and includes, amongst several other phyla, the annelids, the molluscs, the phoronids, the brachiopods and the bryozoans. Our tree (Fig. 1) supports the lophotrochozoan assemblage with a BV 96 and BPP 1.00. The relationships within the Lophotrochozoa are still not well understood, possibly due to poor taxon sampling in multigene analyses. Other factors might also have contributed to lack of resolution, such as rapid radiation or multiple substitutions, (as seen within the Mollusca) (Winnepenninckx et al., 1996), rate heterogeneity (Passamaneck et al., 2004) and saturation problems. As an indication of the lack of resolution in SSU analyses, the ribosomal genes alone do not resolve the various classes of molluscs or of the annelids as monophyletic groups (Aguinaldo et al., 1997; Giribet, 2002; Giribet et al., 2000; Halanych, 1998). A recent study of lophotrochozoan phylogeny using LSU and SSU combined recovers the nemerteans, annelids and molluscs as monophyletic groups but suggests that the Lophophorata (brachiopods, phoronids and bryozoans) are polyphyletic (Passamaneck and Halanych, 2006), as previously found in the first study of lophophorates based on complete SSU DNA (Halanych et al., 1995).

2.6. Mollusca: mono- or polyphyletic?

Our tree does not resolve the Mollusca as monophyletic (Fig. 1). We tested the alternative hypothesis that Mollusca are monophyletic but this alternative tree topology is rejected as significantly less well supported using our dataset ($2\log_e(B_{10}) = 188.84$). Monophyletic Mollusca have been recovered from analyses of LSU and SSU combined (Passamaneck and Halanych, 2006).

Running the analyses excluding the mitochondrial data partition results in a monophyletic Mollusca (data not shown), putting into question the choice of mitochondrial genomes used in resolving

the Mollusca in our analysis. The tree constructed using mitochondrial genomes only (Appendix 3c) shows that the bivalves and gastropods have long branches, which might have an effect on the accuracy of tree reconstruction in the combined dataset. In addition, the bivalve *Mytilus galloprovincialis* is known to have an unusual doubly uniparental mode of mitochondrial inheritance (Mizi et al., 2005), which may result in mitochondrial recombination in that species. Ribosomal data alone also do not resolve the Mollusca as monophyletic (Appendix 3b), a result which can be attributed to rate heterogeneity in molluscan SSU and LSU, especially among the cephalopods (Passamaneck et al., 2004). In our tree based on nuclear data only, we find monophyletic Mollusca. The polyplacophorans are basal to the bivalves, gastropods and cephalopods (Appendix 3a), in support of the Conchifera hypothesis, an assemblage which groups the gastropods, cephalopods, bivalves, scaphopods and monoplacophorans based on the presence of a shell with a periostracum, (together secreted by a complex shell gland), to the exclusion of the polyplacophorans, which have eight shells (Nielsen, 2001).

2.7. Annelida includes echiurans and sipunculids

Our data (Fig. 1) support a monophyletic clade of annelids, with high support values (BV 100, BPP 1.00). This clade includes the segmented polychaetes and clitellates (oligochaetes + leeches) as well as the echiurans (spoon worms) and the sipunculids (peanut worms), unsegmented marine worms which have probably secondarily lost their segmentation. The branching order of these phyla is not well resolved in our tree (Fig. 1), and our taxon sampling does not permit us to see if the polychaetes are the paraphyletic stem-group of the other three lineages. Previous molecular studies have already suggested that the echiurans and sipunculids are derived annelids (Halanych et al., 2002; McHugh, 1997; Struck et al., 2007; Bleidorn et al., 2006), and that Clitellata may also be included within the polychaete annelids (Struck et al., 2007; McHugh, 2005). Traditionally, the polychaetes (mainly marine worms) and the clitellates (the terrestrial oligochaetes and leeches) have been regarded as separate monophyletic groups. In agreement with these results, the mitochondrial gene order of the sipunculid *Phascolopsis gouldii* is very similar to that of the oligochaete *Lumbricus terrestris* (Boore and Staton, 2002). According to ribosomal data, the echiurans are related to capitellid polychaetes, and should be regarded as secondarily unsegmented polychaetes (Bleidorn et al., 2003). The observation of a segmented ventral nervous system in juvenile echiurans similarly supports the inclusion of the echiurans in the annelids (Hessling and Westheide, 2002). Embryology of the sipunculids, particularly the existence of a so-called molluscan cross, has been interpreted as indicative of molluscan affinities, but this has now been shown to be a poor character, which has been based on subjective interpretations of blastomere patterns (Jenner, 2003; Maslakova et al., 2004). Our data clearly support the monophyletic origin of polychaete annelids, clitellates, echiurans and sipunculids, suggesting that the former morphological analyses of these groups may have underestimated their morphological diversity, and implies that segmentation is likely to have been lost in the lineages leading to the echiurans and sipunculids.

2.8. Lophophorates

The lophophorates (brachiopods, phoronids and bryozoans) have traditionally been classified together based on similarities of the ciliated tentacles, called the lophophore, and the putative archimery of the body, i.e. the possession of a prosome, a mesosome with the lophophore, and a metasome, each with one or a pair of coelomic sacs (Hyman, 1959). Some authors have regarded the phoronids and brachiopods as basal deuterostomes, based on embryological characters (Nielsen, 2001), but this idea has been contradicted by molecular studies which show that all the lophophorate groups are protostomes (de Rosa et al., 1999; Halanych et al., 1995). In other studies, based on SSU and morphology, brachiopods and phoronids are found to be sister groups within the protostomes (Halanych et al., 1995; Peterson and Eernisse, 2001), or the phoronids an ingroup of the brachiopods (Cohen, 2000; Cohen et al., 1998). A recent study based on SSU and LSU combined suggests that the Lophophorata are polyphyletic, and that the brachiopods themselves are polyphyletic (Halanych et al., 1995; Passamaneck and Halanych, 2006).

Our tree positions the phoronids as a sister group to the polyplacophorans (70/1.00). We find that the brachiopods, nemerteans and bryozoans form a monophyletic clade.

We tested the hypothesis that the phoronids and brachiopods belong within the deuterostomes using Bayes factor tests, and find that this tree topology is very strongly rejected using the standard criterion ($2\log_e(B_{10}) = 1274.8$).

2.9. Nemerteans

Our results surprisingly show the nemerteans as the sister group of the articulate brachiopods (88/1.00) (inarticulates were not included in this study). The nemertean + brachiopod association is an unexpected result, but our analyses lack platyhelminth sequences, and this may have had some influence on this topology. The nemerteans have traditionally been associated with the flatworms, with which they share some morphological and embryological similarities. The strongest nemertean/flatworm synapomorphy was long thought to be the lack of a body cavity, but there is a debate as to whether the nemerteans are really acoelomate (Jenner, 2004; Turbeville, 2002). Similarities in the larval ciliary bands of the pilidium larva of some nemertines and the Müller's larva of some flatworms have been pointed out by Nielsen (2001). SSU data support the position of nemertines within a protostome coelomate clade (Turbeville et al., 1992). Recent LSU data also suggested an association of the nemertines with the brachiopods and phoronids, the nemertean *Tubulanus* branching with the Brachiopoda in the LSU tree of Passamaneck and Halanych (Passamaneck and Halanych, 2006). However, the brachiopods are polyphyletic in this tree, some grouping with the annelids and others as basal to other lophotrochozoan phyla (Passamaneck and Halanych, 2006). There is also some evidence for a brachiopod-nemertean association from their mitochondrial gene orders. The ND2-CO1 gene boundary seems to be shared between nemerteans, brachiopods and spiralian taxa (Turbeville, 2002). However, it is difficult to point out any morphological synapomorphy between brachiopods and spiralian taxa including nemerteans.

2.10. Deuterostomes

The deuterostomes have traditionally been distinguished from the protostomes based on a number of morphological features such as the fate of the blastopore, the origin of the mesoderm and the radial cleavage pattern of the embryo. The deuterostome grouping has remained largely unchanged in molecular studies, comprising echinoderms, hemichordates, urochordates, cephalochordates, vertebrates, and the new phylum Xenoturbellida (Bourlat et al., 2003). We find high support for the deuterostome grouping in our dataset (92/1.00), with the exception of the urochordates (see below).

Within the deuterostomes, our tree supports the Ambulacraria (echinoderms + hemichordates) (75/1.00). The Ambulacraria grouping has been recovered in other molecular studies based on SSU (Halanych, 1995) and on LSU and SSU combined (Furlong and Holland, 2002; Winchell et al., 2002). In terms of synapomorphies,

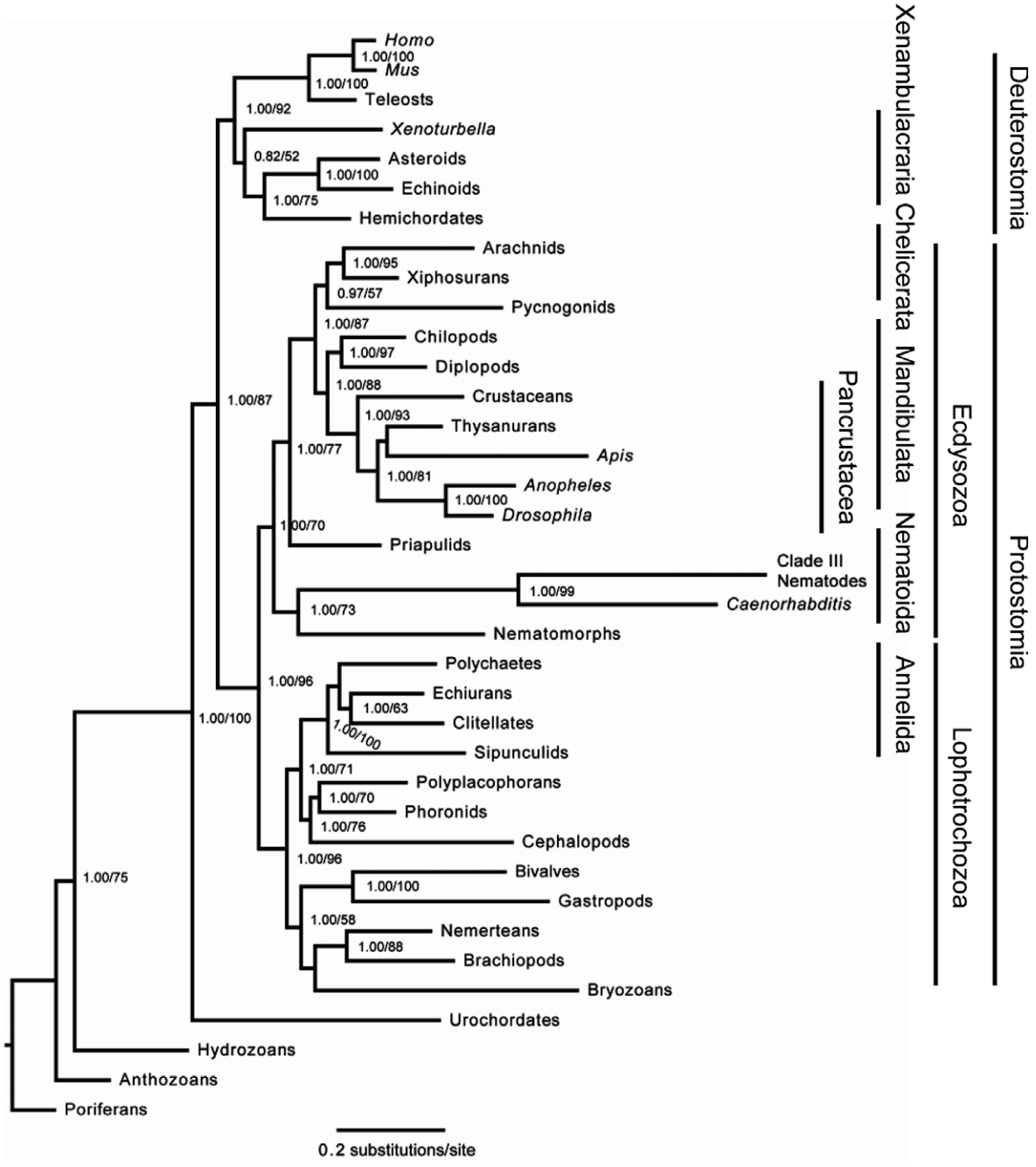

Fig. 1. Bayesian inference protein tree of the animal kingdom based on concatenated nuclear, ribosomal and mitochondrial genes (support values: BI posterior probability/bootstrap).

the echinoderms and hemichordates share a dipleurula larva (Nielsen, 2001).

Our data confirm the phylogenetic position of *Xenoturbella* as a sister group to the Ambulacraria (52/0.82), and the Ambulacraria/*Xenoturbella* clade as a sister group to the vertebrates, as previously shown in studies based on SSU, CO1 and CO2 genes (Bourlat et al., 2003), whole mitochondrial genome and phylogenomic data (Bourlat et al., 2006).

2.11. Urochordates

Our tree shows the urochordates at the base of the Bilateria. This is presumably an artefact of long branch attraction, and can possibly be explained by their fast rate of evolutionary change attracting them to the base of the tree. This result has also been seen in previous studies (Telford et al., 2003). We tested the alternative hypothesis that the urochordates belong to the deuterostomes using Bayes factors, and we find that this topology is rejected as significantly less well supported using the standard criterion ($2\log_e(B_{10}) = 56.39$).

3. Conclusions

To address the problems of both stochastic and systematic errors in reconstructing the phylogeny of the Metazoa, we have assembled a dataset from 22 nuclear and mitochondrial genes.

We have used the traditional PCR approach to generate novel data from 3 nuclear loci to add to the previously available datasets to which we have added new sequences from several taxa. To maximise the completeness of each operational taxonomic unit (OTU), we merged the data from a total of 168 species into 37 composite sequences representing almost all major animal lineages biasing the selection towards the more slowly evolving exemplars. The structure of our tree is in general agreement with previous efforts and, while obviously not entirely independent, gives further support to the tripartite structure of the new animal phylogeny consisting of deuterostomes, lophotrochozoans and ecdysozoans. We also draw attention to a number of relationships that, being controversial, are of particular note, (i) the monophyly of the Mandibulata (i.e. Myriapoda grouping with Crustacea and Hexapoda rather than with Chelicerata), (ii) the monophyletic group of annelids, echiurans and sipunculids, (iii) the basal position of Nematoida within Ecdysozoa and the sistergroup relationship between Priapulida and Euarthropoda, (iv) the relationship between Brachiopoda and Nemertea.

Most of these clades, although not all found in previous molecular phylogenies based on smaller data sets do make sense of the morphological features of the animals in question. The Mandibulata share a common head morphology which includes but is not restricted to the common presence of a mandible (Edgecombe et al., 2003). The Pycnogonida and Chelicerata share chelate first appendages which have been confirmed as positionally homologous by recent studies of Hox gene expression (Jager et al., 2006). The Echiura and Sipuncula have been shown to have a very similar mitochondrial gene order to the Annelida and even to have an annelid-like metameric nervous system in the larvae (Hessling and Westheide, 2002; Wanninger et al., 2005).

We have compared the support for each of these relationships with alternative topologies using Bayes factors and in almost all cases (apart from the position of the pycnogonids as chelicerates rather than basal arthropods, $2\log_e(B_{10}) = 6.84$) we have been able to reject the alternative topology as significantly well supported. While this may seem encouraging, we believe that the current criterion for the use of Bayes factors is not stringent enough.

We do not put great faith in the relative positions of Priapulida and Nematoida on our tree as the reverse pattern has also been described but highlight it in order to emphasise the lack of resolution here. The most surprising of the clades we highlight is the nemertean plus brachiopod clade. While highly supported and seemingly robust, it seems problematic to accept the sistergroup relationship between these two very different clades. The nemerteans are classic spiralians with a trochophore larva apparently homologous to that of annelids and molluscs (Maslakova et al., 2004) while brachiopods are radially cleaving and there has been no suggestion that their larva could be a derived trochophore (Nielsen, 2005). Another concern is that we do not find the Brachiopoda and Phoronida as sistergroups suggesting the phylogenetic signal might not be as reliable as one might hope. This said, it is uncontentious that the non-spiralian brachiopods are relatively close to a number of spiralian groups and so, unless shown to be outside of a spiralian clade, an explanation of their different pattern of embryogenesis will always be required. We note additionally, that the phylogenomic analysis of Dunn et al. based on 150 genes from 77 taxa (Dunn et al., 2008) published during the reviewing process of our manuscript also supports a sister group relationship of nemerteans and brachiopods, referred to as 'Clade A', giving further support for this association.

The problems that remain in our analysis include portions of the tree that are plausible yet weakly supported (Pycnogonida + Chelicerata, Priapulida + Euarthropoda) or implausible (non-monophyletic Mollusca and basal Urochordata). In contrast, the EST analysis of Dunn et al. clearly supports Pycnogonida + Chelicerata, Cycloneuralia, as well as the more plausible monophyletic Mollusca and Urochordata as the sister group to Chordata. These discrepancies highlight the strength of the EST approach and demonstrate that better resolution can be achieved with larger datasets and better gene selection strategies that avoid paralogy (Dunn et al., 2008). There are a number of other ways in which resolution in the Metazoan tree can be improved, the most important of which are the inclusion of data from additional taxa in order to reveal homoplasy, and the development of methods to deal with systematic error such as unequal or uneven rates of evolution and compositional bias (Phillipe and Telford, 2006).

4. Materials and methods

4.1. Degenerate primer design

To find orthologs across the metazoan taxa, protein coding genes from *Saccharomyces cervisiae*, *Caenorrhabditis elegans*, *Drosophila melanogaster* and Human were searched using the Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST/) against each other and in all possible combinations. Genes were chosen according to percentage similarity, alignable length and the availability of conserved regions for primer design. Primers were designed using codehop (http://blocks.fhcrc.org/blocks/codehop.html). The chosen genes were dyskerin (a centromere/microtubule binding protein of 60–70% similarity across the yeast, fly, worm and human genomes), vacuolar ATP synthase subunit (with 70–80% percentage similarity) and carnitine palmytoyltransferase (30–40% similarity). In addition, other genes were chosen according to their prior use in other studies and availability in GenBank, such as enolase, glyceraldehyde 3-phosphate dehydrogenase, elongation factor 1-α, sodium/potassium ATPase and RNA polymerase II. Others such as myosin were avoided deliberately because they have a large number of paralogs. Mitochondrial genomes and SSU and LSU ribosomal DNA sequences were downloaded from GenBank through NCBI (http://www.ncbi.nlm.nih.gov/).

4.2. Specimen collection

Marine invertebrates were collected from Millport Marine Station, UK, Kristineberg Marine Research Station, Sweden or Friday Harbour Laboratories, Washington, USA, and identified to species level by Claus Nielsen. Arthropod specimens were kindly donated by colleagues.

4.3. RT-PCR, cloning, sequencing

Total RNA was prepared using the RNeasy minikit (Qiagen). cDNA for RT-PCR was prepared using Expand Reverse Transcriptase (Roche) and random hexanucleotide primers (Roche). Polymerase chain reaction was carried out using degenerate primers (see Appendix 1) and Taq polymerase (Roche) at the following temperatures: 1 cycle: 94 °C, 2 min; 30 cycles 94 °C, 30 s; 50 °C, 60 s; 72 °C 90 s; 1 cycle 72 °C, 10 min. These non-stringent annealing conditions fit all primer pairs in most species, but meant that multiple bands were obtained on the gel.

The right sized PCR products were gel purified using QIAquick gel extraction kit (Qiagen) and cloned using pGEM-T vector (Promega). Clones were sequenced using T7 (5′-TAATACGACTCACTA TAGGG-3′) and SP6 (5′-GATTTAGGTGACACTATAG-3′) primers and BigDye Terminator v3.1 (Applied Biosystems). Sequences were analyzed and contigs made using Sequencher (Gene Codes Corporation) and Lasergene (DNASTAR).

4.4. Alignment

Protein coding nucleotide sequences were translated according to the universal genetic code for nuclear protein or their taxon specific genetic code (for mitochondrial proteins) using the software translatorX, and the amino acids were aligned using Muscle (Edgar, 2004) as the alignment tool. The amino acid alignment was edited by eye in MacClade (version 4.07 (Maddison and Maddison, 2000)). The original nucleotides were finally aligned according to the amino acid alignment again using translatorX (Telford, unpublished). Unreliably aligned positions were excluded from the analyses using MacClade version 4.06.

4.5. Data concatenation

In order to reduce the proportion of missing data in the concatenated alignment, sequences from several species were combined into a composite higher-level taxon (Class or Phylum) and renamed accordingly (e.g. Asteroids, Echinoids, Arachnids, Gastropods). In cases where more than one species representing the particular higher-level taxon was represented for a given gene, the available sequences (e.g. several different mollusc sequences) were ranked according to their average distance from all other sequences in the alignment (see Table 1 for constituent species for each higher-level taxon). A composite sequence was then built up using as much of the top ranked sequences (shortest average distance) as was present. A Perl script was written to automate this procedure. In the case of *Xenoturbella bocki*, *Homo sapiens*, *Mus musculus*, *Drosophila melanogaster*, *Apis mellifera*, and *Caenorhabditis elegans* we used sequence data from those species only.

4.6. Phylogenetic analyses

For Bayesian analyses of amino acid data (Huelsenbeck and Ronquist, 2001) the data were divided into three partitions: ribosomal RNA, mitochondrial protein and nuclear protein. For nucleotides the GTR + G5 was used. MtREV substitution matrix (mitochondrial sequences) or WAG substitution matrix (nuclear genes) were fixed and likelihood settings were set to rates = gamma, ngammacat = 5. Two independent runs with two chains were run for 5,000,000 generations and sampled every 100th generation. A majority rule consensus tree was constructed from trees sampled after the likelihood estimates had plateaued. MrBayes, version 3.1.2 was used (Huelsenbeck and Ronquist, 2001).

4.7. Non-parametric bootstrapping

As the mixture of evolutionary models we used (separate models for nuclear and mitochondrial amino acids and rRNA nucleotides) are not available in likelihood packages with which we could run a standard non-parametric bootstrap, we constructed 100 boostrapped datasets followed by 100 Bayesian analyses. Each of the three data types was bootstrapped 100 times and then the separate bootstrap datasets concatenated. The same models were used as for the main analysis in Bayesian analyses of each of the 100 concatenated datasets. The MCMC was run for 500,000 generations. The first 80% of trees were discarded before the Bayesian consensus tree was estimated. We used the consensus tree from each MrBayes run to produce the final bootstrap consensus. The PHYLIP program consense (Felsenstein, 1989) was used to construct an overall consensus from the 100 Bayesian consensus trees.

4.8. Bayes factor tests

MrBayes was also used to calculate Bayes factors to compare the optimal topology with that of topologies in which the Bayesian analysis was constrained to contain alternative potential monophyletic groups. The Bayes factor (B_{10}) represents the ratio of the model likelihoods of the two topologies under consideration (Nylander et al., 2004; Wiens et al., 2005). Values of $2\log_e(B_{10})$ were calculated (i.e., two times the difference between the harmonic means of the post burn-in log-likelihoods of the two models) and values > 10 are considered to be very strong evidence favouring one model over the other (Kass and Raftery, 1995).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2008.07.008.

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