



Short Communication

Support for the monophyletic origin of Gnathifera from phylogenomics

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This manuscript is dedicated to Prof. em. Dr. Peter Ax, the first describer of gnathostomulids

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ABSTRACT

The monophyletic origin of Spiralia within the metazoan tree of life is supported by many large-scale phylogenomic data. While there is now substantial molecular evidence for Lophotrochozoa being a monophyletic taxon within Spiralia, the phylogenetic affiliations of many other spiralian phyla remain unclear. Here we focus on the question of a monophyletic taxon Gnathifera, which was originally characterized by jaw morphology as comprising the taxa Rotifera, Acanthocephala and Gnathostomulida. Based on a large-scale molecular sequence dataset of 11,146 amino acid residues, we reconstructed phylogenetic trees of spiralian phyla using maximum-likelihood and Bayesian approaches. We obtain the first phylogenomic evidence for the clade Gnathifera, linking Syndermata (Rotifera + Acanthocephala) with Gnathostomulida. Furthermore, our data support recent findings concerning the paraphyly of Eurotatoria.

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1. Introduction

In the metazoan taxon Protostomia, most recent molecular phylogenetic reconstructions support a monophyletic clade of moulting animals (Ecdysozoa) on the one hand (e.g., Aguinaldo et al., 1997; Dunn et al., 2008; Giribet et al., 2000), and an assembly of very diverse taxa, often now summarized as Spiralia, on the other (Giribet, 2008; Giribet et al., 2000; Hausdorf et al., 2007). However, even the analysis of large phylogenomic datasets has not facilitated complete resolution of relationships within Spiralia (Dunn et al., 2008; Hausdorf et al., 2007; Helmkampf et al., 2008). Thus, support for a spiralian clade 'Platyzoa', comprising Rotifera, Acanthocephala, Gnathostomulida, Gastrotricha and Platyhelminthes (Cavalier-Smith, 1998), is still ambiguous, possibly due to limited taxon sampling (Giribet, 2008).

Crucial for testing the Platyzoa hypothesis is the phylogenetic position of the enigmatic taxon Gnathostomulida, which represents microscopic marine animals with a specialized and eponymous jaw. Originally, Gnathostomulida were regarded as Platyhelminthes (Ax, 1956) or their sistergroup (Ax, 1984). Based on the possibility that certain morphological characters such as

very small body size and the absence of a body cavity and anus could represent the bilaterian ground pattern, a basal placement of the gnathostomulids has also been hypothesized (Ax, 1995). By their monociliated epithelial cells, Cavalier-Smith (1998) grouped gnathostomulids together with gastrotrichs and named this new taxon Monokonta. On the basis of the complex jaw ultrastructure, Ahlrichs (1995, 1997) postulated a sistergroup relationship between Gnathostomulida and the taxon Syndermata, which comprises free-living and epizoid Bdelloidea, Monogononta and Seisonidea ("Rotifera" in the classical sense) and the endoparasitic Acanthocephala (which have lost a digestion tract). Monophyly of Syndermata is now well supported by ultrastructural characters such as a syncytial epidermis and spermatozoa with anteriorly inserting cilia (Ahlrichs, 1995, 1997) and by molecular analyses of single genes (e.g., Garey et al., 1996; Herlyn et al., 2003; Passamanek and Halanych, 2006) and phylogenomic data (e.g., Hausdorf et al., 2007; Witek et al., 2008). In contrast to the univocal support for Syndermata, a monophylum Gnathifera (Gnathostomulida + Syndermata) is still essentially based on ultrastructural evidence (Ahlrichs, 1997; Herlyn and Ehlers, 1997), while molecular analyses yielded conflicting results: an 18S rRNA dataset (Littlewood et al., 1998) placed Gnathostomulida within Ecdysozoa, while evidence for a monophylum Gnathifera, albeit with moderate statistical support, was inferred from 18S rRNA (Giribet et al.,

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2000) and combined 18S/28S rRNA, histone H3, and cytochrome c oxidase data (Giribet et al., 2004). For this reason, we decided to re-examine the monophyly of Gnathifera by using only very recently available EST (expressed sequence tag) data from Syndermata and Gnathostomulida (Table 1) and assembled a large-scale metazoan dataset of aligned ribosomal protein sequences. We examined the effect of including acoelomorphs and myzostomid species, which have proven difficult to place in Metazoa and may be prone to producing phylogenetic artefacts (e.g., Bleidorn et al., 2009; Dunn et al., 2008; Egger et al., 2009; Zrzavy, 2001). We also tested the recently observed paraphyly of Eurotatoria (Garcia-Varela and Nadler, 2006; Witek et al., 2008) under inclusion of the Gnathostomulida.

2. Materials and methods

2.1. Ribosomal protein alignment

Ribosomal protein sequences were extracted from the publicly available EST and trace sequence data for one gnathostomulid (*Gnathostomula peregrina*), one gastrotrich (*Turbanella ambronensis*) and three platyhelminths (*Paraplano-cera* sp., *Dugesia japonica* and *Schmidtea mediterranea*) by their annotation, translated into amino acid sequences and used to enlarge the ribosomal protein alignments compiled by Witek et al. (2008). The myzostomid sequences (*Myzostoma seymourcollegiorum*, *Myzostoma cirriferum*) and the acoelomorph sequences (*Convolvata pulchra*, *Convolvata roscoffensis*, *Neochildia fusca*) were also obtained from publicly available EST and trace sequence data and processed likewise. All ribosomal protein sequences obtained were aligned by the ClustalW algorithm (Thompson et al., 1994) using default parameters. Resulting alignments were inspected and adjusted manually for misaligned positions using GeneDoc (Nicholas et al., 1997). Questionably aligned positions were eliminated with GBLOCKS (Castresana, 2000) using the “less stringent” parameters option.

2.2. Phylogenetic reconstruction

The phylogenetic information content of the alignments was estimated by the likelihood mapping approach as implemented in Tree-Puzzle 5.2 (Schmidt et al., 2002; Strimmer and von Haeseler, 1997), testing all 46,376 possible quartets with exact parameter estimation. For the likelihood mapping we used the WAG+I+G+F, as the RtREV model is not implemented in Tree-Puzzle 5.2, and the WAG+I+G+F model was the next best choice according to the ProtTest (Abascal et al., 2005) analysis. Bayesian inference analyses based on the site-heterogeneous CAT model [which allows the amino acid replacement pattern to vary across a protein alignment; (Lartillot and Philippe, 2004)] were performed using PhyloBayes v2.1c (Blanquart and Lartillot, 2006). Ten independent chains were run simultaneously for 11,000 points each. Chain equilibrium was estimated by plotting the log-likelihood and the alpha parameter as a function of the generation number. The first 1500 points were subsequently discarded as burn-in. According to the divergence of bipartition frequencies, all chains reached conver-

gence (maximal difference <0.34, mean difference <0.006), supported by the fact that all chains produced the same consensus tree topology. Taking every 10th sampled tree, a 50% majority rule consensus tree was computed using both chains. ProtTest was used to assess the appropriate model of sequence evolution for maximum likelihood-based tree reconstruction. As ribosomal proteins are likely to evolve similarly, the model was determined for the full concatenated dataset. Analyses were then conducted using PhyML (Guindon and Gascuel, 2003) with 100 bootstrap replicates and Treefinder (Jobb, 2007; Jobb et al., 2004) with the RtREV+I+G+F substitution model (Dimmic et al., 2002). Confidence values for the edges of the maximum-likelihood tree (Treefinder) were computed by applying expected likelihood weights (ELWs) (Strimmer and Rambaut, 2002) to all local rearrangements of tree topology around an edge (1000 replications) and in a second approach with 100 bootstrap replicates. A partitioned analysis was conducted using Treefinder (Jobb, 2007; Jobb et al., 2004) with the substitution models RtREV (Dimmic et al., 2002), WAG (Whelan and Goldman, 2001) and JTT (Jones et al., 1992) with varying parameters of G, I and F, as determined by ProtTest analyses for each individual protein (see Supplementary Table 1). Trees produced in the course of the analysis were further edited using TreeView (Page, 1996). 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. Thereafter we investigated whether the maximum likelihood trees for these hypotheses are part of the confidence set of trees applying the approximately unbiased (AU) test (Shimodaira, 2002). These analyses were conducted on the dataset without myzostomids and acoelomorphs, as well as on the dataset including both taxa.

3. Results and discussion

3.1. Compilation of the dataset

Ribosomal proteins have proven to be valuable for phylogenomic analyses due to their good representation in EST datasets and easy-to-infer orthology relationships (Hausdorf et al., 2007; Hughes et al., 2006; Landais et al., 2003; Struck and Fisse, 2008). We thus retrieved ribosomal protein coding sequences for *G. peregrina* (Gnathostomulida), *T. ambronensis* (Gastrotricha) and the three Platyhelminthes *D. japonica*, *S. mediterranea* and *Paraplano-cera* sp. from public databases, and used the inferred amino acid sequences to complement a concatenated alignment of 79 ribosomal proteins previously compiled by Witek et al. (2008). After cleaning the raw data from ambiguously aligned positions, the final alignment had a length of 11,146 amino acid positions with sequence coverage per taxon ranging from 29% to 100%, compared to the vertebrate reference sequence (Table 1). The suitability of the present dataset for phylogenetic reconstruction was corroborated by likelihood mapping analysis, in which 99.2% of the quartets were fully resolved and none of the quartet-trees was star-like (Supplementary Fig. 1).

Table 1
Gnathiferan taxa included in this analysis. Indicated are the number of ribosomal proteins (RP) within the concatenated sequence alignment, the overall amount of amino acid residues per species and the percent coverage in the total alignment.

Taxon	Species	# RP	# Amino acids	Coverage [%]
Acanthocephala	<i>Echinorhynchus truttae</i>	28	3194	28.65
Acanthocephala	<i>Pomphorhynchus laevis</i>	65	7346	65.90
Bdelloidea	<i>Philodina roseola</i>	72	9963	89.38
Gnathostomulida	<i>Gnathostomula peregrina</i>	62	7961	71.42
Monogononta	<i>Brachionus plicatilis</i>	28	4231	37.95

3.2. Phylogenetic reconstructions: support for Gnathifera

Independently of the tree reconstruction method used, our phylogenetic analyses (Fig. 1 for the PhyloBayes tree; for the other original trees see Supplementary Figs. 2–5) consistently showed a monophyletic origin of Syndermata and Gnathostomulida when acoelomorphs and myzostomids were not included. As discussed below, we believe the effect of these taxa on tree topology is artefactual, and we conclude that our results provide support for monophyletic Gnathifera, with support values ranging from high to maximum support (PhyloBayes (PB): 0.98, Treefinder-partitioned (TFp): 100, Treefinder (TF): 100, Treefinder-bootstrap (TFb): 89, PhyML (PM): 96). Notably, the partitioned analysis showed the same result as the analysis using a uniform evolution model for the dataset.

Hypothesis testing showed that in both cases, with or without acoelomorphs and myzostomids, the hypothesis (i) Gnathostomulida standing basal in the Bilateria (Ax, 1995), (ii) Gnathostomulida belonging to the Ecdysozoa (Littlewood et al., 1998) and (v) Gnathostomulida being the sistergroup to the platyhelminths (Ax, 1984) could be rejected (Table 2). From the remaining three hypothesis (iii) Gnathifera (Ahlrichs, 1995, 1997), (iv) Gnathostomulida belonging to the platyhelminths (Ax, 1956) and (vi) Monokonta (Cavalier-Smith, 1998) we do find more confidence from the

dataset without myzostomids and acoelomorphs for the Gnathifera hypothesis than for the other ones.

Regarding the phylogenetic position of Gnathifera within the metazoan tree, four out of five analyses placed Gnathifera within Spiralia, mostly with solid support values (PB: 0.99, TFp: 99, TF: 100, PM: <50), but varying positions, whereas the fifth approach (TFb) did not show clades with less than 50% support and therefore did not display the deeper protostome relationships. While PB analysis placed Gnathifera basally within Spiralia, TF and PM reconstructions grouped Gnathifera together with Platyhelminthes and Gastrotricha, producing the taxon *Platyzoa sensu Cavalier-Smith (1998)*, albeit with very weak statistical support. Notably, the phylogenetic relations of Gnathifera, Platyhelminthes and Gastrotricha differ between the reconstruction methods and must be regarded unresolved by this dataset.

Another important aspect of our phylogenetic analysis is the consistent support for the monophyletic origin of Bdelloidea and Acanthocephala (PB: 0.87, TFp: 90, TF: 90, TFb: 88, PM: 63), which strongly suggests a paraphyletic origin of Bdelloidea and Monogononta (“Eurotatoria”). Support values for this observation are higher (PB: 0.87, TF: 90) after the inclusion of Gnathostomulida as a more closely related taxon, than they were in the original analyses (PB: 0.83, TF: 76) without Gnathostomulida (Witek et al., 2008). Finally, the present analyses also support the monophyly

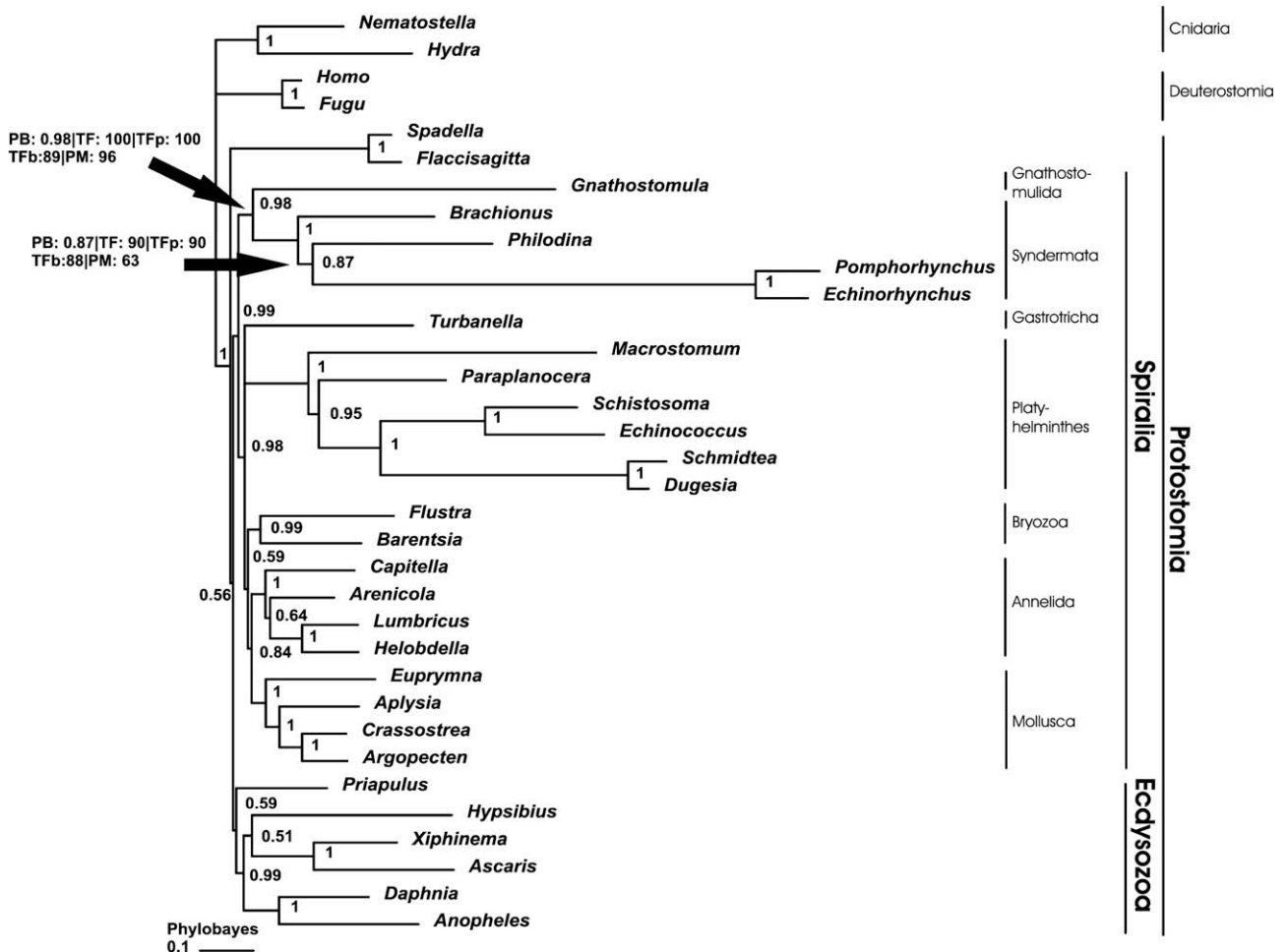


Fig. 1. Phylogenetic reconstruction using PhyloBayes. Monophyletic Gnathifera are shown as a basal spiralian taxon. Within Syndermata, Bdelloidea are more closely related to Acanthocephala than to Monogononta. Lophotrochozoa appear monophyletic, as do Spiralia. Numbers at internal nodes represent posterior probabilities. For monophyletic Gnathifera and Bdelloidea + Acanthocephala the support values from the other reconstruction methods are also shown. For the other original trees see Supplementary Figs. 2–5.

Table 2
Results from hypotheses testing. Results from hypotheses testing based on the competing phylogenetic positions of Gnathostomulida using the approximately unbiased (AU) test.

Hypothesis	AU (dataset without Myzostomida and Acoela)	AU (dataset with Myzostomida and Acoela)
(i) Gnathostomulida basal in Bilateria	0	0
(ii) Gnathostomulida in Ecdysozoa	0.04019085	0
(iii) Gnathifera	0.9684348	0.7971718
(iv) Gnathostomulida in Platyhelminthes	0.09527434	0.3167382
(v) Gnathostomulida sistergroup to Platyhelminthes	0	0
(vi) Monokonta	0.05478665	0.2870257

of Lophotrochozoa, comprising annelids, molluscs and bryozoans (PB: 0.56, TFp: 99, TF: 98, Tfb: 51, PM: 73).

3.3. Influence of unstable taxa

The recent phylogenomic approach by Dunn et al. (2008) suggested a grouping of gnathostomulids with myzostomids and acoelomorph flatworms. However, these latter two taxa were shown to be unstable in the phylogenetic analysis. Interestingly, pruning them from the 77-taxon tree from Dunn et al. (2008) would result in monophyletic Gnathifera as well. However, Dunn et al. (2008) also removed gnathostomulids and rotifers from their final analysis, and their datasets did not include acanthocephalans. Because of the possible deteriorating effect of problematic taxa on tree reconstruction, we systematically re-analyzed our own dataset with and without myzostomids, acoels, and the platyzoan taxa individually and in various combinations (results summarized in Supplementary Table 2).

The addition of myzostomids to our dataset did not affect monophyly of Gnathifera. Myzostomida were variably placed with low support in Ecdysozoa (TF), along with chaetognaths (PM) or basal within Spiralia (PB), indicating some artificial behaviour of the myzostomid data. A recent maximum-likelihood analysis of ribosomal proteins grouped myzostomids at yet another position (as sister of gastrotrichs), although mtDNA and other data strongly suggest that myzostomids are related to annelids (Bleidorn et al., 2009). These authors hypothesize a bias of yet unknown kind in myzostomid ribosomal protein sequence data.

When we added the acoelomorphs to the analysis, they were placed as sister taxon to the gnathostomulids, with strong support by TF, but only weak support by PB and PM reconstructions. As in Dunn et al. (2008), the simultaneous addition of myzostomids and acoelomorphs produced a well-supported sistergroup relationship of these taxa and (with lower support) an affinity to gnathiferans. These groupings persisted when platyzoan taxa were pruned individually or jointly from the extended dataset, suggesting that the myzostomid and acoelomorph data themselves are primarily responsible for this hypothetical clade.

Since recent molecular work convincingly argues for a basal position of acoelomorphs in the metazoan tree (Baguna et al., 2008; Egger et al., 2009; Paps et al., 2009; Sempere et al., 2007), we regard the acoelomorph affinities to myzostomids and also (occasionally) to the gnathiferan clade as artefacts, possibly induced by long-branch attraction. We also note that our PM analysis placed the Priapulida inside the spiralian clade. This must also be regarded artificial, as the ecdysozoan affinity of priapulids is well supported (Bourlat et al., 2008; Philippe et al., 2005; Webster et al., 2006, 2007). Together, these examples demonstrate the necessity to compare multiple tree reconstruction methods and to evaluate molecular trees on the basis of additional morphological evidence.

4. Conclusions

In summary, we provide for the first time support from large-scale molecular data for Gnathifera comprising Syndermata and

Gnathostomulida. The present findings thus complement previous evidence from ultrastructural (Ahlrichs, 1995, 1997; Herlyn and Ehlers, 1997) as well as limited molecular data (Giribet et al., 2000, 2004). The present results moreover confirm earlier findings (e.g., Giribet et al., 2000; Witek et al., 2008) of a phylogenetic position of Gnathifera within Spiralia. However, the exact phylogenetic affiliations of Gnathifera within Spiralia remain to be resolved by future analyses. These will also have to include data from the enigmatic animal taxa Micrognathozoa and Cyclophora, which have been grouped within Gnathifera as possible extensions of the original characterization based on morphological structures of the jaws (Kristensen and Funch, 2000) and molecular data (Giribet et al., 2000). In addition to the monophyly of Gnathifera, our data provide further evidence for the Spiralia/Lophotrochozoa clade within protostomes, thus corroborating results of several recent studies (e.g., Hausdorf et al., 2007; Helmkampf et al., 2008). Finally, we obtained evidence for the paraphyly of Eurotatoria (García-Varela and Nadler, 2006; Witek et al., 2008), even under inclusion of a more closely related taxon, the Gnathostomulida.

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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.2009.07.031.

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