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# Interrelationships of the Gastrotricha and their place among the Metazoa inferred from 18S rRNA genes

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The phylum Gastrotricha includes about 700 species. They are small worm-like organisms abundant among marine and freshwater meiobenthos. In spite of their ubiquity, diversity and relative abundance, phylogenetic relationships of these animals remain enigmatic due to the conflicting results of morphological and molecular cladistic analyses. Also unclear are the alliances within the phylum. In order to best estimate the position of Gastrotricha among the Metazoa and to shed some light on the ingroup phylogenetic relationships, small subunit (SSU) ribosomal DNA (rDNA) from 15 species of Chaetonotida (eight genera) and 28 species of Macrotrichida (26 genera) were included in an alignment of 50 metazoan taxa representing 26 phyla. Of the gastrotrich SSU rDNA sequences, eight are new and, along with published sequences represent eight families, including the five marine most speciose. Gastrotricha were resolved within a monophyletic Lophotrochozoa as part of a clade including Micrognathozoa, Rotifera and Cycliophora. The Gnathostomulida were sister to this clade. Nodal support was low for all of these relationships except the grouping of the Micrognathozoa, Rotifera and Cycliophora. Bayesian inference resolved the Gastrotricha as monophyletic with weak nodal support; the Macrotrichida were resolved as paraphyletic with many basal nodes poorly supported. Within the Chaetonotida, the monotypic Multitubulata *Neodasy* was found in alliance with the macrotrichid *Urodasy* while all the Paucitubulata were found to form a single, well-supported clade, with *Musellifer* as the most basal member. Among the more densely sampled Macrotrichida the Lepidodasyidae and Macrotrichidae were each found to be polyphyletic while monophyly was well supported for the Turbanellidae and Thaumastodermatidae. The congruence of our results with those of the cladistic analysis based on morphological traits provides confidence about the value of each dataset, and calls for widening of the research to include additional taxa of particular phylogenetic significance such as the Dactylopodolidae, *Diuronotus*, *Heteroxenotrichula* and *Draculiciteria*. The study highlights the problems in working with small species, the need for voucher specimens and the confused taxonomic status and membership of various gastrotrich families.

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## Introduction

The phylum Gastrotricha includes about 700 species of small, triploblastic, acoelomate, transparent, aquatic organisms ranging from 80 µm to 3 mm in length. The body is dorso-ventrally flattened, covered by a bi-stratified cuticle often sculptured into small scales and spines and bears ventral locomotory cilia and adhesive tubules enwrapped by the cuticle (Ruppert 1991).

Gastrotrichs are an ever-present component of the meiobenthic community of marine sandy sediments and ubiquitous elements of periphyton and benthos in freshwater systems. In marine interstitial habitats, numerically they usually constitute 1–8% of the total meiobenthos, ranking third in abundance among the meiofauna taxa behind the Nematoda and the harpacticoid Copepoda. Indeed, some studies have even found gastrotrichs

to be the first or second most abundant taxon (Coull 1985; Hochberg 1999). In freshwater ecosystems population density may reach 158 individuals/10 cm<sup>2</sup>, ranking the taxon among the top five most abundant groups (Balsamo & Todaro 2002). We can therefore hypothesize that gastrotrichs play an important role in the ecological interactions of the microvorous-detritivorous taxa of the benthos. Nevertheless, in spite of their ubiquity and relative abundance, knowledge of the basic biology and ecology of the phylum is scant when compared with other groups of microinvertebrates. Systematically, gastrotrichs are also poorly known both from morphological and molecular points of view, yet as a group their importance in better understanding protostome evolution cannot be overlooked.

Phylogenetic relationships of these small worms remain enigmatic due to the conflicting results of morphological and molecular cladistic analyses. Traditionally regarded as a class within the Aschelminthes (Hyman 1951), recent morphological analyses highlighting features such as a bilayered cuticle, anterior mouth, cylindrical pharynx and a collar-shaped brain suggest that gastrotrichs are allies of the Introverta, forming with them the clade of the Cycloneuralia (Sørensen *et al.* 2000; Nielsen 2001). Other studies focusing on protonephridial ultrastructure and the monociliated epidermal cells link gastrotrichs with the Gnathostomulida (Rieger 1976; Sterrer *et al.* 1985; Zrzavý *et al.* 1998). In contrast, 18S ribosomal RNA (rRNA) gene sequences indicated a close relationship with the Platyhelminthes (Winnepenninckx *et al.* 1995; Giribet *et al.* 2000) or with the Gnathostomulida (Zrzavý *et al.* 1998, 2001). Combined analyses of morphology and molecules have also failed to find consensus with Gastrotricha resolved as lophotrochozoans within the Platyzoa (Giribet *et al.* 2000; Garey 2001; Zrzavý *et al.* 2001), or as sister taxon of the Ecdysozoa (e.g. Zrzavý 2003). Additional molecular studies have even suggested nonmonophyly of the phylum (Giribet *et al.* 2004; Manylov *et al.* 2004).

Thus, in order to determine accurately the interrelationships of the Metazoa and understand the radiation of major body plans among the animals, the resolution of the inter- and intraphyletic relationships of the gastrotrichs remains an important goal in systematics (Manylov *et al.* 2004). Their relatively basal positions among major clades of Metazoa, according to both morphological and molecular studies, suggest that Gastrotricha may represent a very early group of Bilateria and therefore a key to understanding the evolution of lower worms, in particular at the splitting of Ecdysozoa and Lophotrochozoa (Zrzavý *et al.* 2001).

The phylogenetic relationships within the phylum are also unclear. Gastrotricha is comprised of two orders, Macrotrichida and Chaetonotida, easily distinguished by a number of morphological, ecological and reproductive features. Members of these groups can be so distinct that some authors have doubted the monophyly of the Gastrotricha (e.g. see Rieger

& Rieger 1977; Ruppert 1982). Although anatomical studies, at gross and microscopic levels, have established a coherent phylogenetic framework for the Gastrotricha, with morphology providing the dominant source of characters for the resolution of ingroup evolutionary trends, it appears that different approaches to in- and outgroup evolutionary reconstruction are needed to test independently the current morphological evidence and help to determine plesiomorphic character states. Evolutionary interpretation of the morphological characters is currently problematic because of confusion concerning ancestral character states within the phylum. The first cladistic analysis, involving more than 80 morphological traits from almost all the known genera, corroborated the monophyly of the phylum and of the two orders but cast doubt on the monophyly of some major families, e.g. Lepidodasyidae, Planodasyidae, Chaetonotidae (Hochberg & Litvaitis 2000).

Since the pioneering work of Winnepenninckx *et al.* (1995), only a few authors have employed molecular data to estimate the position of Gastrotricha on the 'animal tree'. The first multispecies molecular study (Wirz *et al.* 1999; but see also Littlewood *et al.* 1998) found the Gastrotricha to be a strictly monophyletic group along an evolutionary line quite distinct from that of Rotifera or Nematoda; the monophyly of the two orders was not supported and even more surprising a new view of the evolutionary history of the phylum was put forward in which Chaetonotida and not Macrotrichida appeared as the more plesiomorphic forms of the group. Zrzavý (2003), using the same sequences of Wirz and co-authors (plus *Turbanella*) and in conjunction with the available morphological datasets, found very different results when molecular and morphological data were analysed individually or in combination. In two out of three cases (molecular and combined data) Macrotrichida appeared as paraphyletic with *Turbanella* as the most basal taxon, with the other macrotrichids separated from the Chaetonotida (combined analysis) or in alliance with some of them (molecular analysis alone). Only when morphological data were analysed alone were Macrotrichida and Chaetonotida constituted as separate clades, as implied by current systematization (e.g. Ruppert 1991; Hochberg & Litvaitis 2000). Differences between studies employing short partial 18S rRNA gene sequences (Wirz *et al.* 1999) and almost complete sequences (Todaro *et al.* 2003), where congruence between morphology and molecules was greater, further suggests that additional complete gene sequences of a higher diversity of taxa may provide a more reliable estimate of relationships to test this further.

In order to estimate the position of Gastrotricha among the Metazoa, we utilized complete and partial small subunit (SSU) ribosomal DNA (rDNA) from 15 species of Chaetonotida (eight genera) and 28 species of Macrotrichida (26 genera), and incorporated these in an alignment of complete SSU rDNA from 53 additional metazoan taxa representing 26 phyla. Of the gastrotrich SSU rDNA sequences, eight are

new and, along with published sequences and those available from GenBank, include the five most speciose marine gastrotrich families. Eight families of gastrotrichs are represented in total; 13 species in six genera are included in the 'unnatural' family Lepidodasyidae, allowing an assessment of this clade to be undertaken (see also Guidi *et al.* 2004). Also included in this study are partial SSU rDNA fragments from chaetonotidans and macrodasyidans available from GenBank, which were analysed in combination with the complete sequences.

## Materials and methods

In order to best estimate the position of Gastrotricha among the Metazoa we utilized complete and partial SSU rRNA from all species of Chaetonotida and Macrodasyida available from GenBank (accessed 4 February 2006) with the eight new complete sequences presented here, and included these in an alignment of 53 metazoan taxa representing 26 phyla, taking into consideration secondary structure elements of SSU rRNA in the alignment. Table 1 indicates the full list of taxa used, with a listing of GenBank/EMBL accession numbers.

### DNA extraction and gene amplification

Total genomic DNA was extracted from individual ethanol-preserved specimens using a DNeasy™ Tissue kit (Qiagen) according to the manufacturer's protocol. Twenty-five-microlitre amplifications were performed with 3–5 µL of genomic extract (~10 ng) using Ready-To-Go PCR beads (Amersham Pharmacia Biotech) each containing 1.5 U *Taq* polymerase, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 200 µM each dNTP and stabilisers including BSA; and 0.4 µM of each PCR primer. Almost complete 18S-like SSU rRNA genes were PCR-amplified with general metazoan-specific primers as described in Littlewood *et al.* (1998). PCR products were purified with Qiagen Qiaquick columns, cycle-sequenced directly using ABI BigDye chemistry, ethanol-precipitated and run on an ABI PRISM 377 automated sequencer. Both strands were sequenced using terminal and internal primers as described in Littlewood *et al.* (1998). Sequences were assembled and edited using Sequencher version 3.1.1 (GeneCodes Corp.) and submitted to GenBank/EMBL (see Table 1 for accession numbers).

### Alignment of SSU rDNA with reference to secondary structure

Metazoan SSU sequences were downloaded already aligned from the European Ribosomal RNA database (<http://oberon.fvms.ugent.be:8080/rRNA/>) in the 'DCSE' parenthetical format which indicates the stem base pairings of rRNA genes. Ystem (Telford *et al.* 2005) was used to extract the aligned sequences from DCSE format into a nexus file. The newly sequenced gastrotrich sequences were aligned to these using Clustal\_X profile alignment and the alignment was

refined 'by eye' using MacClade. Portions of the alignment where positional homology was uncertain were identified for eventual exclusion prior to phylogenetic analyses. Ystem was used to reinsert the DCSE parenthetical notation indicating the stem pairs into our updated alignment. Xstem (Telford *et al.* 2005) was then employed to convert our updated DCSE file into a MrBayes file with all conserved stem pairs identified in the format 'pairs 1 : 5 2 : 4' where in this example position 1 pairs with position 5 and 2 with 4.

### Phylogenetic analysis

MrBayes 3.0 was used to estimate the best tree for each of the different datasets. The data were partitioned into stem nucleotides and loop nucleotides following the rationale and results in Telford *et al.* (2005) who worked on a similar dataset of metazoan SSU rDNA. For loop nucleotides a six-parameter general time reversible (GTR) model of nucleotide substitution was used (nst = 6). For stem nucleotide pairs the doublet model was used with two substitution types (nst = 2) as this had been shown not to be significantly worse than the more complex six-substitution type model by Telford *et al.* (2005). For both stem and loop partitions independently a gamma parameter was estimated with four rate categories. Four simultaneous chains were run for 2 000 000 generations. Trees were sampled after 1 500 000 generation (considerably after the distribution of likelihoods was seen to have plateaued) and every thousandth tree was stored. Branch lengths of trees were also stored and the MrBayes sumt command used to produce a consensus tree for each analysis with posterior probabilities showing clade support. For tree drawing, triploblastic Metazoa were rooted against the diploblasts. Three analyses were conducted: (i) all Metazoa and Gastrotricha where only complete SSU rDNA was available, (ii) all Metazoa and all Gastrotricha, including partial fragments of SSU rDNA and (iii) only Gastrotricha maintaining the Chaetonotida and Macrodasyida as monophyletic groups.

## Results

The final alignment included 1479 unambiguously alignable positions, of which 559 were constant and 674 were phylogenetically informative under the principles of parsimony. The full alignment with an indication of excluded positions, stems and loops is available in EBI/EMBL under the accession number ALIGN\_000980 and is available from [http://www3.ebi.ac.uk/Services/webin/help/webin-align/align\\_SRS\\_help.html](http://www3.ebi.ac.uk/Services/webin/help/webin-align/align_SRS_help.html)

In all analyses nodal support throughout the trees was not particularly high. Bayesian inference resolved the Gastrotricha as monophyletic with weak nodal support [posterior probability (pp) = 0.77] and the Chaetonotida (except *Neodasyis*) with stronger support (pp = 1.00). The Macrodasyida were resolved as paraphyletic with many basal nodes poorly supported. In analyses using partial SSU rDNA one sequence appeared to have come from a contamination; the partial

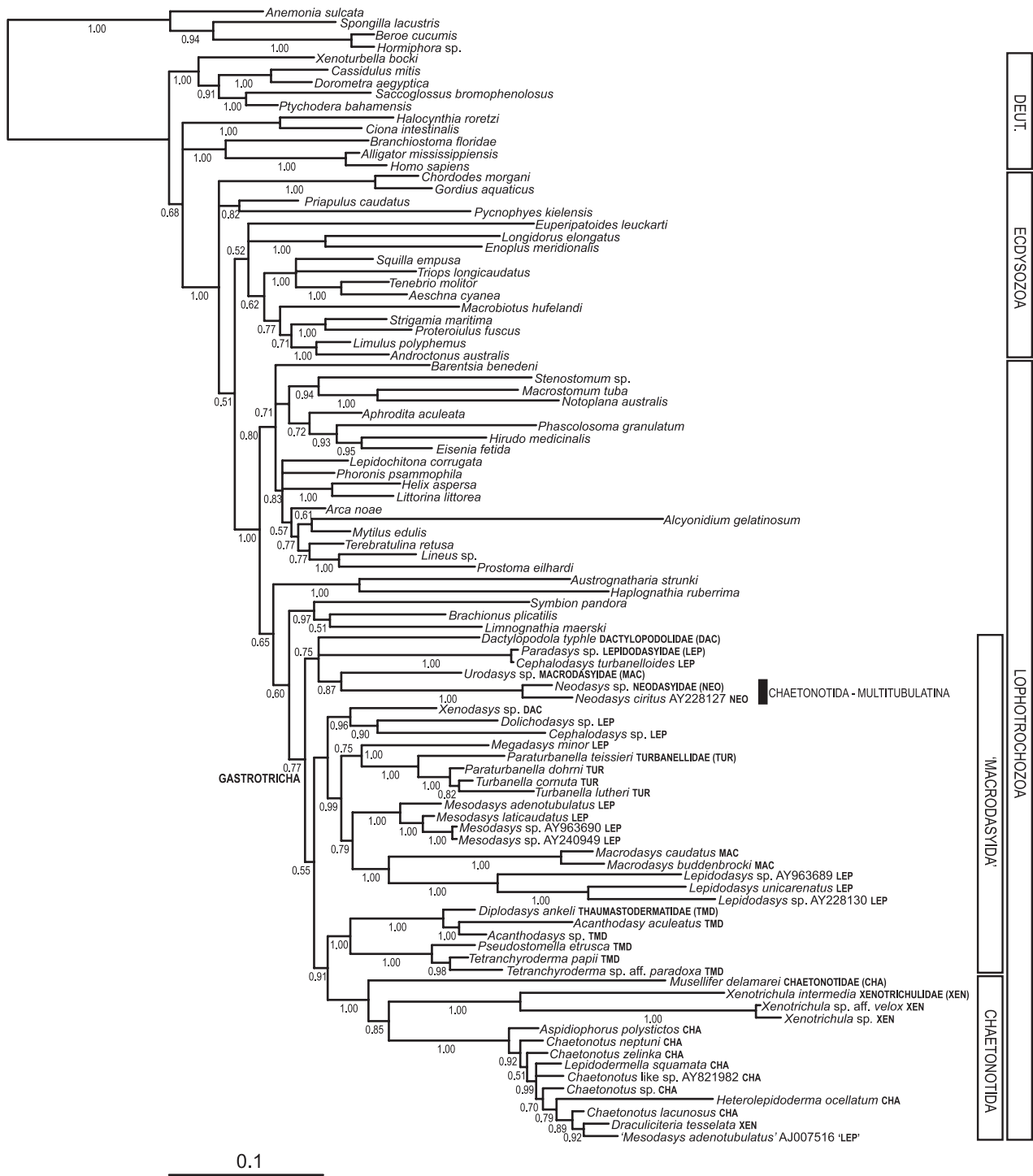
**Table 1** Taxa used in this study.

Classification	Species	Accession	Classification	Species	Accession
Cnidaria	<i>Anemonia sulcata</i>	X53498	Cycliophora	<i>Symbion pandora</i>	Y14811
Porifera	<i>Spongilla lacustris</i>	AF121112	Micrognathozoa	<i>Limnognathia maerski</i>	AJ487046
Ctenophora	<i>Beroe cucumis</i>	D15068	Rotifera	<i>Brachionus plicatilis</i>	U29235
	<i>Hormiphora</i> sp.	AF100944	Entoprocta	<i>Barentsia benedeni</i>	U36272
Chordata, Urochordata	<i>Halocynthia roretzi</i>	AB013016	Gastrotricha, Chaetonotida	<i>Aspidiophorus polystictos</i>	AY228126
	<i>Ciona intestinalis</i>	AB013017		<i>Chaetonotus lacunosus</i>	AJ007512*
Chordata, Cephalochordata	<i>Branchiostoma floridae</i>	M97571		<i>Chaetonotus neptuni</i>	AM231774†
Chordata, Craniata	<i>Alligator mississippiensis</i>	AF173605		<i>Chaetonotus</i> sp.	AJ001735
	<i>Homo sapiens</i>	U13369		<i>Chaetonotus zelinkai</i>	AJ007514*
Xenoturbellida	<i>Xenoturbella bocki</i>	AY291292		<i>Draculiciteria tessellata</i>	AJ007513*
Hemichordata	<i>Saccoglossus bromophenolosus</i>	AF236801		<i>Heterolepidoderma ocellatum</i>	AJ007517*
	<i>Ptychodera bahamensis</i>	AF236802		<i>Lepidodermella squamata</i>	U29198
Echinodermata	<i>Cassidulus mitis</i>	Z37148		<i>Musellifer delamarei</i>	AM231775†
	<i>Dorometra aegyptica</i>	AF088803		<i>Neodasys</i> sp.	AY963687
Priapulida	<i>Priapulius caudatus</i>	X87984	<i>Neodasys ciritus</i>	AY228127*	
Kinorhyncha	<i>Pycnophyes kielensis</i>	U67997	<i>Xenotrichula intermedia</i>	AY228128	
Onychophora	<i>Euperipatoides leuckarti</i>	U49910	<i>Xenotrichula</i> sp. aff. <i>velox</i>	AY963686/	
Nematomorpha	<i>Chordodes morgani</i>	AF036639	Gastrotricha, Macrodasysida	<i>Acanthodasys</i> sp.	AY239041*
	<i>Gordius aquaticus</i>	X80233		<i>Acanthodasys aculeatus</i>	AY228135
Nematoda	<i>Longidorus elongatus</i>	AF036594		<i>Cephalodasys</i> sp.	AY963691
	<i>Enoplus meridionalis</i>	Y16914		<i>Cephalodasys turbanelloides</i>	AM231776†
Arthropoda, Crustacea	<i>Triops longicaudatus</i>	AF144219		<i>Dactylopodola typhle</i>	AY228134*
	<i>Squilla empusa</i>	L81946		<i>Diplodasys ankei</i>	AM231777†
Arthropoda, Hexapoda	<i>Tenebrio molitor</i>	X07801		<i>Dolichodasys</i> sp.	AM231778†
	<i>Aeschna cyanea</i>	X89481		<i>Lepidodasys</i> sp.	AY963689
Arthropoda, Chelicerata	<i>Limulus polyphemus</i>	L81949		<i>Lepidodasys</i> sp.	AY228130*
	<i>Androctonus australis</i>	X77908		<i>Lepidodasys unicarenatus</i>	AY228129
Arthropoda, Myriapoda	<i>Strigamia maritima</i>	AF173265	<i>Macrodasys budenbrocki</i>	AY239040*/	
	<i>Proteroiulus fuscus</i>	AF173236	<i>Macrodasys caudatus</i>	AM231779†	
Tardigrada	<i>Macrobiotus hufelandi</i>	X81442	<i>Mesodasys</i> sp.	AY963689	
Nemertea	<i>Lineus</i> sp.	X79878	<i>Mesodasys minor</i>	AY228131	
	<i>Prostoma eilhardi</i>	U29494	<i>Mesodasys adenotubulatus</i>	AM231780†	
Mollusca, Polyplacophora	<i>Lepidochitona corrugata</i>	X91975	<i>Mesodasys adenotubulatus</i>	AJ007516*	
Mollusca, Bivalvia	<i>Arca noae</i>	X90960	<i>Mesodasys laticaudatus</i>	AY228132	
	<i>Mytilus edulis</i>	L33448	<i>Mesodasys</i> sp.	AY240949	
Mollusca, Gastropoda	<i>Helix aspersa</i>	X91976	<i>Paradasys</i> sp.	AM231781†	
	<i>Littorina littorea</i>	X91970	<i>Paraturbanella dohrmi</i>	AY228139	
Brachiopoda	<i>Terebratulina retusa</i>	U08324	<i>Paraturbanella teissieri</i>	AY228138	
	<i>Phoronis psammophila</i>	AF025946	<i>Pseudostomella etrusca</i>	AY228136	
Bryozoa	<i>Alcyonidium gelatinosum</i>	X91403	<i>Tetranchyroderma papii</i>	AY228137	
Annelida, Polychaeta	<i>Aphrodita aculeata</i>	Z83749	<i>Tetranchyroderma</i> sp. aff. <i>paradoxa</i>	AY240950*/	
Annelida, Clitellata	<i>Hirudo medicinalis</i>	Z83752	<i>Phascolosoma granulatum</i>	AY963688	
	<i>Eisenia fetida</i>	X79872	<i>Stenostomum</i> sp.	AF157007	
Sipuncula	<i>Phascolosoma granulatum</i>	X79874	<i>Macrostomum tuba</i>	AY963693	
Platyhelminthes	<i>Stenostomum</i> sp.	U95947	<i>Notoplana australis</i>	AY218102	
	<i>Macrostomum tuba</i>	D85091	<i>Austrognatharia strunki</i>	AY218110	
Gnathostomulida	<i>Notoplana australis</i>	AJ228786	<i>Haplognathia ruberrima</i>	AY218109	
	<i>Austrognatharia strunki</i>	AY218110			
	<i>Haplognathia ruberrima</i>	AY218109			

\* indicates partial sequence, † indicates new sequences.

fragment of *Mesodasys adenotubulatus* (AJ007516) was resolved as a member of the Chaetonotida whereas the complete new sequence of *M. adenotubulatus* was resolved within the Macro-dasyida along with other members of the same genus. In all analyses the interrelationships of the gastrotrichs with other

Metazoa and the interrelationships of the Gastrotricha themselves were consistently resolved with the same topologies whether or not partial sequences were included or excluded. As such, we present only the tree of the full analysis of all sequences analysed in a wider metazoan context (Fig. 1).



**Fig. 1** Phylogenetic relationships of the Gastrotricha inferred from Bayesian analysis of small subunit ribosomal RNA genes partitioned into stems and loops (50% majority-rule consensus tree) showing branch lengths. Membership of the Deuterostomia (Deut.), Ecdysozoa and Lophotrochozoa are indicated; for gastrotrichs membership of family and order are also indicated. GenBank numbers are shown for certain taxa to aid in interpretation. *Neodasys* is the only member of the Chaetonotida Multitubulitina and appears among the Macrodasysida. Numbers at nodes indicate posterior probability values > 50%.

Gastrotricha were resolved within a monophyletic Lophotrochozoa as part of a clade including Micrognathozoa, Rotifera and Cycliophora. The Gnathostomulida were sister to this clade. Nodal support was low ( $pp < 0.8$ ) for all of these relationships except the grouping of the Micrognathozoa, Rotifera and Cycliophora ( $pp = 0.97$ ). Relationships between lophotrochozoan phyla were not well supported although, where multiple exemplars were used, all phyla remained monophyletic with the exception of the Mollusca. The Gnathostomulida were resolved as the most basal members of the Lophotrochozoa. The Lophotrochozoa were supported as the sister group to a paraphyletic Ecdysozoa ( $pp = 1.00$ ).

Within the Chaetonotida, both suborders the Multitubulata and the Paucitubulata were sampled. Members of the monotypic Multitubulata family Neodasyidae were found in alliance with the macrodasyidan *Urodasyus* ( $pp = 0.87$ ) in an unresolved, unsupported clade ( $pp < 0.8$ ) including three additional Macrodasysida (i.e. *Dactylopodola*, *Cephalodasyus* and *Paradasys*). Gastrotricha Paucitubulata were found as forming a single, well-supported clade (see also below).

Within the Paucitubulata, only the members of the Chaetonotidae and Xenotrichulidae were sampled. Neither family was monophyletic due to the derived position of *Draculiciteria*, a xenotrichulid, within the Chaetonotidae. *Musellifer* was the most basal genus within the suborder in the full analyses, and the genus *Chaetonotus* was resolved as polyphyletic. Among the more densely sampled Macrodasysida the Lepidodasyidae and Macrodasysidae were each polyphyletic. Nodal support within the Gastrotricha was high ( $pp > 0.95$ ) for only a limited number of relationships, most notably the following: monophyly of the Turbanellidae and Thaumastodermatidae and interrelationships within these two families; monophyly of the genera *Xenotrichula*, *Lepidodasyus*, *Macrodasys* and *Mesodasyus*. The Chaetonotidae (except *Musellifer*) were also strongly supported but included the xenotrichulid *Draculiciteria* and the partial sequence AJ007516, suggesting the latter was a chaetonotid contamination or a misidentified *M. adenotubulatus*.

## Discussion

In general, the tree accords well with recent analyses of metazoan phylogeny, albeit with paraphyletic deuterostomes and ecdysozoans. The lophotrochozoans were well supported as a monophyletic clade and the Gastrotricha appeared as a relatively derived clade with Micrognathozoa, Rotifera and Cycliophora. However, even this clade is very poorly supported and a conservative assessment of relationships within the Lophotrochozoa suggests only that the gastrotrichs remain allied with these other phyla. There is a lack of resolution in some areas of the tree and this likely derives from the use of the more sophisticated separate stem and loop models for the analyses as these tend towards a conservative estimate of

phylogeny (Telford *et al.* 2005). This means that those aspects of our tree that are well supported are especially credible.

The evolutionary scenario in which Gastrotricha appear as lophotrochozoans, supported by most molecular studies (e.g. Giribet *et al.* 2000; Zrzavý 2003; Manylov *et al.* 2004) including this one, is in sharp contrast with the alternative hypothesis calling for a sister group relationship between Gastrotricha and Edysozoa that has received strong support from both morphology and combined morphological and molecular data (e.g. Schmidt-Rhaesa *et al.* 1998; Peterson & Eernisse 2001; Zrzavý 2003). Our analysis supports the current view (Hochberg & Litvaitis 2000) that sees the Gastrotricha as a phylum, although not with very strong support in terms of Bayesian posterior probabilities. This is in agreement with previous analysis based on partial SSU rRNA gene sequences (Wirz *et al.* 1999; Todaro *et al.* 2003; Zrzavý 2003); however, it is in contrast with studies from Manylov *et al.* (2004) and from Giribet *et al.* (2004) whose results found Gastrotricha as a polyphyletic taxon.

For morphologists, the uniqueness of the Gastrotricha as a monophylum rests on few, shared evolutionary novelties (found in all taxa so far examined) namely: (i) multilayered epicuticle, (ii) locomotory (and sensory) cilia enveloped by the (epi)cuticle, (iii) outlet of the duo-gland adhesive system covered by the cuticle which, in the absence of an anchor cell, provides tension transmitting forces between the animal and the environment during adhesion (Tyler & Rieger 1980; Ruppert 1991). An additional putative synapomorphy includes (iv) the recently discovered 'muscular double helix' (Hochberg & Litvaitis 2001a): a peculiar muscle arrangement (i.e. helicoidal muscles in right- and left-hand spirals) that was first described in nine species, and has subsequently been found in all the species investigated (Hochberg & Litvaitis 2001b, 2003; Leasi *et al.* 2005; F. Leasi & M. A. Todaro, unpublished data). These characters appear to be genuine synapomorphies by virtue of which the Gastrotricha can be readily recognized as a clade. Molecular results based on SSU rDNA support the monophyly of the Gastrotricha.

Within Gastrotricha, we did not find strong support for the monophyly of either Macrodasysida or of Chaetonotida. However, evidence of monophyly of the Chaetonotida Paucitubulata was very robust in terms of Bayesian support. The monophyly of the two orders implied by the current systematization is backed by results of the inclusive cladistic analysis based on 81 morphological characters by Hochberg & Litvaitis (2000). We would like to emphasize that weak nodal support among basal macrodasyidans in the present study does not preclude monophyly of the Macrodasysida, but strong nodal support of the Paucitubulata Chaetonotida might confirm the monophyly of the Chaetonotida.

This contrasts significantly with previous molecular studies. Wirz *et al.* (1999) found two species of Macrodasysida

examined nested within the paraphyletic Chaetonotida Paucitubulatina (Chaetonotida Multitubulatina were not included in the analysis). Zrzavý (2003), using the same sequences of Wirz *et al.* (1999) plus *Turbanella*, found the traditional Macrodasysida as a paraphyletic stemline of monophyletic Chaetonotida. Manylov *et al.* (2004), using the same sequences as Zrzavý (2003) plus five new, found the new four Macrodasysida clustered on a clade distinct from that of Chaetonotida, whereas all the gastrotrich sequences obtained by Wirz *et al.* (1999) still nested within the clade of Chaetonotida. Concerning these results, Manylov and coworkers concluded that 'the observed discrepancies suggests that the previous published sequences assigned by Wirz *et al.* (1999) as belonging to *Mesodasys adenotubulatus* and *Cephalodasys turbanelloides* might actually belong to some chaetonotid species'. Todaro *et al.* (2003) warned about possible artefacts burdening the molecular results of Wirz *et al.* (1999) and it appears now that the study by Zrzavý (2003) may have suffered from these artefacts to some degree.

In the present study we were able to demonstrate that the partial sequence of *Mesodasys adenotubulatus* (AJ007516) was indeed a likely contaminant, as was (not shown) the partial fragment of *Cephalodasys turbanelloides* (AJ007515) resolved now in its accepted position (i.e. among Macrodasysida). However, the unknown species of *Cephalodasys* from GenBank (AY963691) appears in a clade with *Dolichodasys* and *Xenodasys* suggesting another possible misidentification. Based on a few well-supported nonmonophyletic groupings of genera and families, other contaminations and misidentifications appear likely. Although some gastrotrich families are likely 'catch-all' taxa for difficult species to classify, and their nonmonophyly is not unexpected (e.g. Lepidodasyidae) new specimens of *Draculiciteria* need to be sequenced to verify its inclusion within the Chaetonotidae. The study highlights the problems in working with small species, the need for voucher specimens and the confused taxonomic status and membership of various gastrotrich families.

Within Chaetonotida we found *Neodasys* allied with the macrodasysidan *Urodasys*, well separated from the clade that includes all of the other chaetonotidans. This has some merit in terms of morphology. For instance, when first discovered, *Neodasys* was considered a macrodasysidan based on the worm-shaped appearance and possession of accessory reproductive organs (Remane 1927), while its current systematization relies on two characteristics shared with other Chaetonotida, i.e. orientation of the pharynx lumen and the absence of pharyngeal pores (Remane 1961). As *Neodasys* also bears several adhesive tubes along the body sides, in contrast with the remainder of Chaetonotida, it is included in the suborder Multitubulatina vs. Paucitubulatina (d'Hondt 1971). Recent ultrastructural investigations found the spermatozoa organization of *Neodasys* different from all the other models known

in gastrotrichs (Guidi *et al.* 2003), while the topography of its muscular system was found to be similar to basal species of Macrodasysida (Hochberg 2005a). Thus, additional studies are needed to support or disprove the position of *Neodasys* in the molecular phylogeny.

In the Chaetonotida Paucitubulatina branch (Fig. 1) we found *Musellifer* to be basal to the remainder of the examined chaetonotidans in good agreement with taxonomists and the results of the cladistic analysis based on morphological traits (see Hochberg & Litvaitis 2000; Guidi *et al.* 2004). Current systematization includes *Musellifer* among Chaetonotidae, although Hochberg & Litvaitis (2000) found the Chaetonotidae to be paraphyletic with, among others, *Musellifer* as sister taxon to the remainder Chaetonotida Paucitubulatina (i.e. all Chaetonotida except *Neodasys*). It is possible that the long branch with which *Musellifer* appears on our tree reflects a genuine deeper divergence of this taxon from other chaetonotids (i.e. Chaetonotidae *sensu stricto*) and not simply the fast evolving nature of its 18S rRNA gene. This hypothesis is born on a growing body of morphological evidence (e.g. shape and ultrastructure of the spermatozoa, Guidi *et al.* 2003; muscles organization, F. Leasi & M. A. Todaro, unpublished data) suggesting that, notwithstanding the general appearance, *Musellifer* is indeed very different from other chaetonotids and allies it more to the Xenotrichulidae; as suggested by the present analysis. However, as the likely sister taxon of *Musellifer* has recently been described (i.e. the new genus named *Diuronotus*, Todaro *et al.* 2005), further studies to investigate specifically the phylogenetic relationships of these taxa with the Xenotrichulidae and the Chaetonotidae *s.s.* are needed.

We are unaware of any morphological traits possessed by *Aspidiophorus* that might suggest this taxon to be perceived as 'primitive' among chaetonotids (all species examined belong to genera that have marine and freshwater representatives, all reproduce by parthenogenesis, etc.), with the possible exception of the stalked scales that make up the cuticular armature of this animals; a trait shared with many members of the more basal Xenotrichulidae (but also with *Polymerurus rhomboides*). So far, however, there is little evidence to confirm the homology of these two cuticular structures (see Hochberg 2005b) but the molecular data suggest a closer examination is required.

Additional issues concerning possible misidentifications and contaminations amongst the taxa included in the present analyses: Kisielowski (1991) gave convincing arguments for the transferal of *Chaetonotus zelinka* to a newly established genus named *Lepidochaetus*, the unorthodox topology set by the terminals in which *Chaetonotus* appears as paraphyletic in the present study (i.e. *C. neptuni*, *Lepidodermella squamata* and *Chaetonotus* sp.) is puzzling and may deserve to be investigated further as relationships within the extremely speciose genus *Chaetonotus* (> 180 spp.) are far from known. Furthermore, *C. neptuni* is a marine species whereas the other two are

freshwater. Among Macrodasysida, the family Lepidodasyidae was resolved as polyphyletic as expected. Two lepidodasyid taxa, *Cephalodasys* and *Paradasys* formed a well-supported clade; morphological similarity between these genera was highlighted by Remane (1934) and their close phylogenetic relationship has been inferred in a cladistic analysis based on 33 morphological characters (Hochberg & Litvaitis 2001c). The much disputed *Lepidodasys* (see Todaro *et al.* 2003) appears in a sister taxon relationship with *Macrodasys*, in contrast with current systematization and with the cladistic analysis based on morphology, which suggests a close alliance of *Lepidodasys* with the Thaumastodermatidae (Hochberg & Litvaitis 2000, 2001c). As species of both *Lepidodasys* and *Macrodasys* are readily identifiable, we tend to exclude that misidentification may burden the sequences analysed. On the other hand, similarity in several traits, e.g. general appearance, muscular organization and function of the accessory sexual reproductive organs, makes *Urodasy* the most likely candidate for the sister taxon of *Macrodasys*. This view is implied by the current systematization (both belong to the Macrodasysidae) and were resolved as sister taxa by the comprehensive cladistic analysis of the Gastrotricha based on morphology (Hochberg & Litvaitis 2000).

Phylogenetic novelties also include the close alliance of the lepidodasyid *Megadasys* and *Dolichodasy*, respectively, with Turbanellidae and *Xenodasy* (Dactylopodolidae) indicated, with strong Bayesian support, by our analysis; an evolutionary scenario for which, however, there are no morphological clues, so far.

The congruence of our results with those of the cladistic analysis based on morphological traits provides confidence about the value of each dataset, and calls for widening the research to include additional taxa. Of particular interest would be the inclusion of other taxa allegedly considered to be basal among macrodasysidans such as the Dactylopodolidae and chaetonotidans such as *Diuronotus*, *Heteroxenotrichula* and *Draculiciteria*. Incomplete and partial sequences of SSU rDNA were useful and usable, although in order to maximize the phylogenetic resolution available from this gene, whether or not stem and loop information is taken into consideration, full gene sequences from additional taxa should be determined. Finally, additional molecular markers and the broad sampling of accurately identified gastrotrichs, with the provision of voucher specimens for morphological reference will help to resolve the deep interrelationships of this monophyletic lophotrochozoan phylum.

#### Note on authorship

M.A.T. initiated the project, provided all samples and interpreted the results in the light of morphology. M.J.T. aligned the data and managed the phylogenetic analyses. A.E.L. provided the new sequences. D.T.J.L. designed and managed the

molecular component of the project and assisted with analyses and interpretation. M.A.T. and D.T.J.L. led manuscript completion.

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#### References

- Balsamo, M. & Todaro, M. A. (2002). Gastrotricha. In S. D. Rundle, A. L. Robertson & J. M. Schmid-Araya (Eds) *Freshwater Meio-fauna: Biology and Ecology* (pp. 45–61). Leiden: Backhuys Publishers.
- Coull, B. (1985). Long-term variability of estuarine meiobenthos: an 11 year study. *Marine Ecology Progress Series*, 24, 205–218.
- Garey, J. R. (2001). Ecdysozoa: the relationship between Cycloneuralia and Panarthropoda. *Zoologischer Anzeiger*, 240, 321–330.
- Giribet, G., Distel, D. L., Polz, M., Sterrer, W. & Wheeler, W. C. (2000). Triploblastic relationships with emphasis on the positions of Gnathostomulida, Cycliophora, Plathelminthes and Chaetognatha: a combined approach of 18S rRNA sequences and morphology. *Systematic Biology*, 49, 539–562.
- Giribet, G., Sorensen, M. V., Funch, P., Kristensen, R. M. & Sterrer, W. (2004). Investigations into the phylogenetic position of Micrognathozoa using four molecular loci. *Cladistics*, 20, 1–13.
- Guidi, L., Marotta, R., Pierboni, L., Ferraguti, M., Todaro, M. A. & Balsamo, M. (2003). Comparative sperm ultrastructure of *Neodasys cirritus* and *Musellifer delamarei*, two species considered to be basal among Chaetonotida (Gastrotricha). *Zoomorphology*, 122, 135–143.
- Guidi, L., Pierboni, L., Ferraguti, M., Todaro, M. A. & Balsamo, M. (2004). Spermatology of the genus *Lepidodasys* Remane, 1926 (Gastrotricha, Macrodasysida): toward a revision of the family Lepidodasyidae Remane, 1927. *Acta Zoologica*, 85, 211–221.
- Hochberg, R. (1999). Spatiotemporal size-class distribution of *Turbanella mustela* (Gastrotricha: Macrodasysida) on a northern California beach and its effect on tidal suspension. *Pacific Science*, 53, 50–60.
- Hochberg, R. (2005a). Musculature of the primitive gastrotrich *Neodasys* (Chaetonotida): functional adaptations to the interstitial environment and phylogenetic significance. *Marine Biology*, 146, 315–323.
- Hochberg, R. (2005b). First record of *Polymerurus* (Gastrotricha, Chaetonotida) from Australia with the description of a new species from Queensland and of cuticular ultrastructure in *P. nodicaudus*. *Invertebrate Biology*, 124, 119–130.
- Hochberg, R. & Litvaitis, M. K. (2000). Phylogeny of Gastrotricha: a morphology-based framework of gastrotrich relationships. *Biological Bulletin*, 198, 299–305.
- Hochberg, R. & Litvaitis, M. K. (2001a). A muscular double helix in Gastrotricha. *Zoologischer Anzeiger*, 240, 61–68.
- Hochberg, R. & Litvaitis, M. K. (2001b). The muscular system of *Dactylopodola baltica* and other macrodasysid gastrotrichs in a functional and phylogenetic perspective. *Zoologica Scripta*, 30, 325–336.
- Hochberg, R. & Litvaitis, M. K. (2001c). Macrodasysida (Gastro-



- tricha): a cladistic analysis of morphology. *Invertebrate Biology*, 120, 124–135.
- Hochberg, R. & Litvaitis, M. K. (2003). Organization of muscles in Chaetonotida paucitubulatina (Gastrotricha). *Meiofauna Marina*, 12, 47–58.
- d'Hondt, J. L. (1971). Gastrotricha. *Oceanography and Marine Biology Annual Review*, 9, 141–192.
- Hyman, L. H. (1951). *The Invertebrates*, Vol. I. New York: McGraw-Hill.
- Kisielewski, J. (1991). Inland-water Gastrotricha from Brazil. *Annales Zoologici (Warsaw)*, 43 (Suppl. 2), 1–168.
- Leasi, F., Rothe, B. H., Schmidt-Rhaesa, A. & Todaro, M. A. (2005). La muscolatura dei Gastrotrichi osservata al microscopio confocale. *Atti 66° Congresso UZI. 19-22 Settembre, Roma* (Abstract).
- Littlewood, D. T. J., Telford, M. J., Clough, K. A. & Rohde, K. (1998). Gnathostomulida: an enigmatic metazoan phylum from both morphological and molecular perspectives. *Molecular Phylogenetics and Evolution*, 9, 72–79.
- Manylov, O. G., Vladychenskaya, N. S., Milyutina, I. A., Kedrova, O. S., Korokhov, N. P., Dvoryanchikov, G. A., Aleshin, V. V. & Petrov, N. B. (2004). Analysis of 18S rRNA gene sequences suggests significant molecular differences between Macrodasyida and Chaetonotida (Gastrotricha). *Molecular Biology and Evolution*, 30, 850–854.
- Nielsen, C. (2001). *Animal Evolution of the Living Phyla*. Oxford: Oxford University Press.
- Peterson, K. J. & Eernisse, D. J. (2001). Animal phylogeny and the ancestry of bilaterians: inferences from morphology and 18S rDNA gene sequences. *Evolution and Development*, 3, 170–205.
- Remane, A. (1927). Neue Gastrotricha Macrodasyoidea. *Zoologische Jahrbucher Abteilung für Systematik, Ökologie und Geographographie der Tiere (Jena)*, 54, 203–242.
- Remane, A. (1934). Die Brackwasserfauna. 5. *Die Lebensgemeinschaften des Brackwassers. Verhandlungen der Deutschen Zoologischen Gesellschaft*, 36, 34–79.
- Remane, A. (1961). *Neodasyus uhdai* nov. spec., eine zweite *Neodasyus*-Art (Gastrotrich Chaetoida). *Kieler Meeresforschungen*, 17, 85–88.
- Rieger, G. E. & Rieger, R. M. (1977). Comparative fine structure study of the gastrotrich cuticle and aspects of cuticle evolution within the Aschelminthes. *Zeitschrift für Zoologische Systematik und Evolutionsforschung*, 15, 81–124.
- Rieger, R. M. (1976). Monociliated epidermal cells in Gastrotricha: significance for concepts of early metazoan evolution. *Zeitschrift für Zoologische Systematik und Evolutionsforschung*, 14, 198–226.
- Ruppert, E. E. (1982). Comparative ultrastructure of the gastrotrich pharynx and the evolution of myoepithelial foreguts in Aschelminthes. *Zoomorphologie*, 99, 181–220.
- Ruppert, E. E. (1991). Gastrotricha. In F. W. Harrison & E. E. Ruppert (Eds) *Microscopic Anatomy of Invertebrates, Vol. 4: Aschelminthes* (pp. 41–109). New York: Wiley-Liss.
- Schmidt-Rhaesa, A., Bartolomaeus, T., Lemburg, C., Ehlers, U. & Garey, J. R. (1998). The position of the Arthropoda in the phylogenetic system. *Journal of Morphology*, 238, 263–285.
- Sørensen, M. V. P., Funch, E., Willersley, A. J. & Hansen & Olsen, J. (2000). On the phylogeny of the Metazoa in the light of Cyclophora and Micrognathozoa. *Zoologischer Anzeiger*, 239, 297–318.
- Sterrer, W., Mainitz, M. & Rieger, R. M. (1985). Gnathostomulida: enigmatic as ever. In S. Conway Morris, J. D. George, R. H. Gibson & M. Platt (Eds) *The Origin and Relationships of Lower Invertebrates* (pp. 181–191). Oxford: Clarendon Press.
- Telford, M. J., Wise, M. J. & Gowri-Shankar, V. (2005). Consideration of RNA secondary structure significantly improves likelihood-based estimates of phylogeny: examples from the Bilateria. *Molecular Biology and Evolution*, 22, 1129–1136.
- Todaro, M. A., Littlewood, D. T. J., Balsamo, M., Herniou, E. A., Cassanelli, S., Manicardi, G., Wirz, A. & Tongiorgi, P. (2003). The interrelationships of the Gastrotricha using nuclear small rRNA subunit sequence data, with an interpretation based on morphology. *Zoologischer Anzeiger*, 242, 145–156.
- Todaro, A. M., Balsamo, M. & Kristensen, M. R. (2005). A new genus of marine chaetonotids (Gastrotricha), with a description of two new species from Greenland and Denmark. *Journal of the Marine Biological Association of the UK*, 85, 1391–1400.
- Tyler, S. & Rieger, G. E. (1980). Adhesive organs of the Gastrotricha. I. Duo-gland organs. *Zoomorphologie*, 95, 1–15.
- Winnepenninckx, B., Backeljau, T., Mackey, L. Y., Brooks, J. M., De Wachter, R., Kumar, S. & Garey, J. R. (1995). 18S rRNA data indicate that Aschelminthes are polyphyletic in origin and consist of at least three distinct clades. *Molecular Biology and Evolution*, 12, 1132–1137.
- Wirz, A., Pucciarelli, S., Miceli, C., Tongiorgi, P. & Balsamo, M. (1999). Novelty in phylogeny of Gastrotricha: evidence from 18S rRNA gene. *Molecular Phylogenetics and Evolution*, 13, 314–318.
- Zrzavý, J. (2003). Gastrotricha and metazoan phylogeny. *Zoologica Scripta*, 32, 61–81.
- Zrzavý, J., Hypsa, V. & Tietz, D. F. (2001). Myzostomida are not annelids: molecular and morphological support for a clade of animals with anterior sperm flagella. *Cladistics*, 17, 170–198.
- Zrzavý, J., Milulka, S., Kepka, P., Bezdek, A. & Tietz, D. F. (1998). Phylogeny of the Metazoa based on morphological and 18S ribosomal DNA evidence. *Cladistics*, 14, 249–285.