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Short Communication

Analysis of 18S rRNA gene sequences suggests significant molecular differences between Macrodasyida and Chaetonotida (Gastrotricha)

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Abstract

Partial 18S rRNA gene sequences of four macrodasyid and one chaetonotid gastrotrichs were obtained and compared with the available sequences of other gastrotrich species and representatives of various metazoan phyla. Contrary to the earlier molecular data, the gastrotrich sequences did not comprise a monophyletic group but formed two distinct clades, corresponding to the Macrodasyida and Chaetonotida, with the basal position occupied by the sequences of *Tetranchyroderma* sp. and *Xenotrichula* sp., respectively. Depending on the taxon sampling and methods of analysis, the two clades were separated by various combinations of clades Rotifera, Gnathostomulida, and Platyhelminthes, and never formed a clade with Nematoda. Thus, monophyly of the Gastrotricha is not confirmed by analysis of the presently available molecular data.

Keywords: 18S rRNA; Molecular phylogeny; Gastrotricha; Macrodasyida; Chaetonotida; Nematoda; Bilateria

Gastrotrichs are small, mostly meiobenthic acoelomate animals, traditionally considered within the aschelminth assemblage (Hyman, 1951; Ruppert, 1991a). The relationships of the phylum Gastrotricha to other metazoan taxa remain obscure, despite the significant role assigned to this group in some large-scale reconstructions of the phylogeny of protostomes (Garey and Schmidt-Rhaesa, 1998; Schmidt-Rhaesa, 2002). Historically, the Gastrotricha has been either placed at the base of aschelminths close to Rotifera (Hyman, 1951) or regarded as the sister group of Nematoda (Malakhov, 1994; Remane, 1936; Ruppert, 1982) or, together with the latter, as the sister group of Gnathostomulida (Boaden, 1985).

The systematics of Gastrotricha is based on the structure of the pharynx and genital organs, the number and shape of cuticular spines and scales, and the number and position of adhesive tubes (Ruppert, 1991b). The phylum includes two orders Macrodasyida and Chaetonotida, distinguished by a number of morphological and developmental features, and the monophyly of both orders is supported by the cladistic analysis of morphological characters (Hochberg and Litvaitis, 2000, 2001). Despite the significant morphological differences between the two orders, the first cladistic analysis involving 71 morphological characters from nearly all known genera showed the phylum as a whole to be monophyletic (Hochberg and Litvaitis, 2000).

At present, molecular data on the 18S rRNA gene sequences of the Gastrotricha are fairly scarce. Until recently, complete 18S rRNA sequences of only two chaetonotid species, *Lepidodermella squammata* and

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Chaetonotus sp. (Winnepenninckx et al., 1995; Littlewood et al., 1998), as well as partial sequences of two macrodasyid and four chaetonotid species (Wirz et al., 1999) have been published and used in molecular phylogenetic analyses. The 18S rRNA and combined (18S rRNA + morphology) analyses either place the gastrotrichs (represented by the chaetonotid *L. squammata*) at the base of the bilaterian tree after acoels and gnathostomulids (Peterson and Eernisse, 2001), or show their platyzoan affinity (Giribet et al., 2000; Giribet, 2002). Treating molecular data as testifying the basal position of Gastrotricha among Protostomia and considering the presence of pharyngeal pores in macrodasyids, Dewell (2000) attributes the presence of gill slits to a bilaterian stem group. In short, the Gastrotricha appears to be a group of utmost phylogenetic importance.

The first analysis of partial 18S rRNA gene sequences showed the Gastrotricha as a whole to be a monophyletic and highly homogenous group, equally separated from Rotifera and Nematoda (Wirz et al., 1999), but their relationships with Gnathostomulida were not assessed due to the absence of molecular data on the latter taxon at that time. At the same time, this phylogenetic analysis led to a surprising conclusion about the nested position of Macrodasyida within Chaetonotida (Wirz et al., 1999). This result contradicted both the traditional morphology-based view concerning the primitiveness of Macrodasyida (summarized in Ruppert, 1991b), and the results of cladistic analysis of morphological characters (Hochberg and Litvaitis, 2000). Recently, Zrzavý (2003) reanalyzed the phylogeny of Gastrotricha, using the previously published morphological dataset of Hochberg and Litvaitis (2001) and the same 18S rRNA gene sequences which were used by Wirz et al. (Wirz et al., 1999), with addition of the complete sequence of *Turbanella cornuta*. The combined analysis resulted in the monophyletic Chaetonotida and paraphyletic Macrodasyida, showing again the discrepancy between the morphological and molecular data. It is evident, therefore, that additional molecular data are required in order to reassess the monophyly of Gastrotricha and to analyze the relationships within this group. To address this problem, we obtained partial 18S rRNA gene sequences of three macrodasyid and one chaetonotid species and analyzed them together with other gastrotrich sequences presently known.

Specimens of gastrotrichs were collected near the Biological Station of St. Petersburg State University (Kandalaksha Bay, the White Sea) and fixed in 95% ethanol (more than 15 animals per sample for each species). DNA was extracted from ethanol-fixed tissues as described by Arrighi et al. (1968) and purified by phenol/chloroform/isoamyl alcohol and chloroform/isoamyl alcohol extractions followed by ethanol precipitation (Sambrook et al., 1989). 18S ribosomal RNA coding regions were amplified in polymerase chain reactions using two primers (5′ GGCTCATTAAAT CAGTTATGG 3′ and 5′ CACCTCTAACGGCGCAA TAC 3′) designed by Wirz et al. (1999). PCR products were purified by agarose gel electrophoresis, cloned in pBluescript KS+ plasmid, and sequenced on both strands using Promega fmol cycle sequencing kit, a set of specific 18S rRNA internal primers, and a universal M13 sequencing primer.

Partial 18S rRNA gene sequences obtained were submitted to GenBank under the following Accession Nos.: *Macrodasys buddenbrocki* Remane, 1927 (AY239040); *Mesodasys* sp. (probably a new species, distinct from *M. adenotubulatus* Hummon, Todaro and Tongiorgi, 1993) (AY240949); *Tetranchyroderma* sp. aff. *T. paradoxa* Tongiorgi, 1974 (AY240950); and *Xenotrichula* sp. aff. *X. velox* Remane, 1927 (AY239041). These sequences as well as the previously published partial sequences of gastrotrichs (Wirz et al., 1999) and the corresponding part of the complete 18S rRNA sequence of *T. cornuta* Remane, 1925 (AF157007) were manually fitted into an original alignment of small subunit rRNA sequences. Since a sister group of the Gastrotricha remains unknown, representatives of various protostome and deuterostome phyla, including those of Diploblastica, were used as a multiple outgroup.

Maximum likelihood (ML) trees (Felsenstein, 1981) were inferred using the fastDNAml program (Olsen et al., 1994) with global branch exchange and randomization of input order as well as fastDNAml implementing a GTR model of the sequence evolution (Korber et al., 2000). Bayesian inferences were performed using MrBayes version 2.01 program (Huelsenbeck and Ronquist, 2001). Six simultaneous Markov chain Monte Carlo (MCMC) chains were run for 1,100,000 generations with sampling every 10 generations for the total of 110,000 samples per run. The states of the chain before it reached stationarity (10,000) were discarded as the burn-in and inferences from each run were based on a total of 100,000 sampled trees. The likelihood parameters for BI corresponded to the General Nonreversible Model (nst = 12, ncat = 8, rates = invgamma, shape = estimate, and basefreq = estimate). Confidence in the ML trees derived by fastDNAml was determined using a consensus tree inferred from 36,000 suboptimal trees, which were selected under the Kishino and Hasegawa (1989) test from 200,000 trees generated in 20 bootstrap replicates.

ML and bayesian inferences (BI) from the partial 18S rRNA gene sequences of 32 species produced phylogenetic trees of similar topology (Fig. 1A), demonstrating an early protostome/deuterostome split and a subsequent split into the Ecdysozoa clade (represented by Priapulida, Arthropoda, and Nematoda) and a clade consisting, in the order of branching off the main stem, of Lophotrochozoa (composed of Brachiopoda, Annelida, Nemertea, and Mollusca) + Macrodasyida

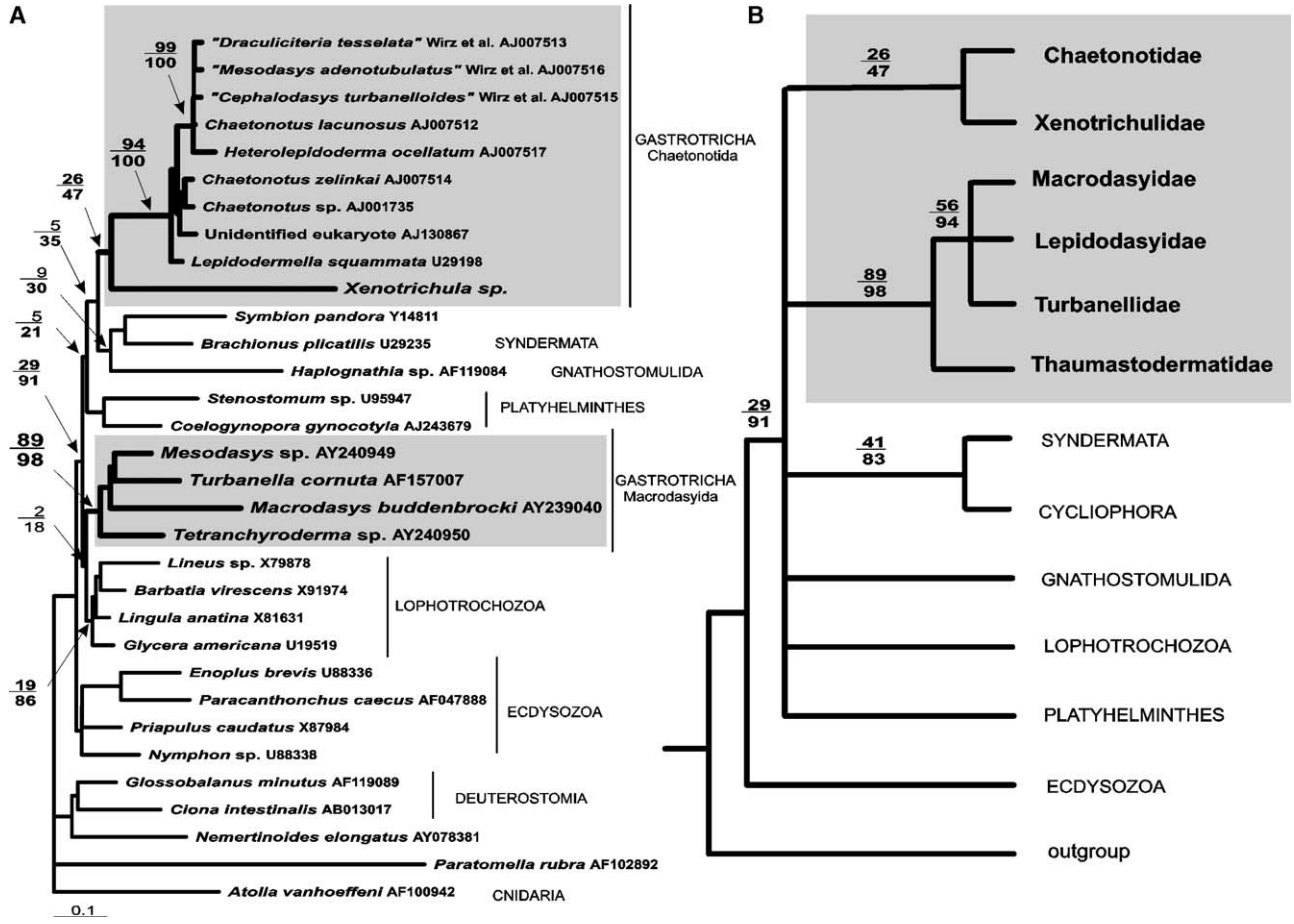

Fig. 1. Phylogenetic tree of partial 18S rRNA gene sequences of the Gastrotricha. Gastrotrich clusters are shaded, and branches leading to gastrotrich species are shown as bold lines. The scale bar indicates 0.1 changes per site. (A) This tree is a summary of 20 multiple bootstrap replicates with fastDNAml and Bayesian inference to infer the tree topology. (B) The strict consensus of the same tree. Numbers at nodes represent the percentage support of major clusters derived from ML suboptimal trees generated in 20 bootstrap replicates and selected by Kishino–Hasegawa test (above lines) and percentages of posterior probabilities from Bayesian analysis (below lines).

(Gastrotricha), Platyhelminthes, Gnathostomulida + Rotifera, and Chaetonotida (Gastrotricha). In the BI tree (not shown), Lophotrochozoa and Macrodasyida did not form a separate clade but branched consecutively off the main stem. In these trees, gastrotrichs did not constitute a monophyletic group: macrodasyid gastrotrichs formed a clade with *Tetranchyroderma* sp. at the base, strongly supported in bayesian analysis (98% posterior probability), whereas chaetonotid gastrotrichs comprised a much less compact clade with *Xenotrichula* sp. at the base, only weakly supported in bayesian analysis (47% PP).

None of the two Gastrotricha clades showed a close affinity to any invertebrate group included in our analyses. In particular, the clade (Platyhelminthes + (Gnathostomulida + Rotifera) + Chaetonotida) was supported only by 21% PP, and the clade uniting Chaetonotida with (Rotifera + Gnathostomulida) was supported by 35% PP. The alliance of Macrodasyida and Chaetonotida, with Platyhelminthes and Gnathostomulida + Rotifera nested between them, was supported by 34% PP. In its turn, this clade showed a relatively strong affinity (91% PP) to the clade Lophotrochozoa, represented by the phyla Brachiopoda, Annelida, Nemertea, and Mollusca. Thus, the phylogenetic relationships within the clade Lophotrochozoa + Macrodasyida + Platyhelminthes + Syndermata + Chaetonotida still appear to be poorly resolved (Fig. 1B). It is notable that neither of the gastrotrich clades is clustered with that of Nematoda, contrary to the traditional view of close relations between the two phyla.

In our analyses, none of the *Chaetonotus* spp. sequences showed an early and sharp divergence, as it was observed in the analysis of Wirz et al. (1999). *Chaetonotus* sp. (AJ001735) formed a clade with *Chaetonotus zelinkai* (AJ007514) and with an unidentified sequence (AJ130867), whereas *Chaetonotus lacunosus* (AJ007512) formed a clade with *Heterolepidoderma* (AJ007517), *Draculiciteria* (AJ007513), and with the sequences assigned by Wirz et al. (1999) to the macrodasyid genera

Cephalodasys and *Mesodasys*. These clades are nested within the clade which can be conditionally named Chaetonotida and is strongly supported in both bayesian and ML trees. None of our macrodasyid sequences, including that of *Mesodasys* sp., was nested within the Chaetonotida clade. Instead, they composed their own rather distinct clade, branching off the main stem of invertebrates at a more basal level.

Our results strongly disagree with the previously published molecular data (Wirz et al., 1999) in several points. First, 18S rRNA gene sequences of gastrotrichs proved to be much more heterogeneous than it followed from the earlier data. Second, the partial 18S rRNA gene sequences of macrodasyids (including *Mesodasys* sp.) obtained by us comprise a clade distinct from that of chaetonotids, whereas all gastrotrich sequences obtained by Wirz et al. (1999) are still nested within the clade Chaetonotida. As our results are in good agreement with the monophyly of Macrodasyida inferred from analysis of morphological characters (Hochberg and Litvaitis, 2001), the observed discrepancy suggests that the previously published sequences assigned by Wirz et al. (1999) as belonging to *Mesodasys adenotubulatus* and *Cephalodasys turbanelloides* might actually belong to some chaetonotid species. Third, sequences from the genus *Chaetonotus* do not display an early divergence from sequences of all other gastrotrich genera in our analyses. Instead, they are nested within the clade Chaetonotida. This agrees with the cladistic analysis of morphology (Hochberg and Litvaitis, 2000), in which both Macrodasyida and Chaetonotida were shown to be monophyletic. Fourth, in our analyses, none of the chaetonotid sequences (except that of *Xenotrichula* sp.) appears to be much more divergent than any other gastrotrich sequences, whereas in the previous analysis (Wirz et al., 1999), the internal branch leading to the clade *Chaetonotus zelinkai* + *Ch. lacunosus* was by far the longest, exceeding even the branches leading to rhabditian nematodes. This suggests that the sequences of *Chaetonotus* spp. might have been shifted in relation to other sequences in the alignment used by Wirz et al. (1999).

To summarize, our analysis demonstrates that gastrotrichs do not comprise a monophyletic group but form two distinct and well-supported clades, corresponding to the traditional orders Macrodasyida and Chaetonotida. Therefore, neither of these two clades can be considered ancestral for the phylum as a whole. It should be emphasized that the idea of Gastrotricha being non-monophyletic is really difficult to accept in view of the numerous morphological data. Equally, in the light of data presented above conclusions on the paraphyly of Macrodasyida (Zrzavý, 2003) and the ancestral state of Chaetonotida relative to Macrodasyida (Wirz et al., 1999) can hardly be accepted. More extensive molecular research, including a broader taxon sampling and analysis of complete rRNA gene sequences, appears to be necessary to resolve this controversy.

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