Zool. Anz. **242** (2003): 145–156

http://www.urbanfischer.de/journals/zoolanz

ZOOLOGISCHER ANZEIGER

The Interrelationships of the Gastrotricha Using Nuclear Small rRNA Subunit Sequence Data, with an Interpretation Based on Morphology

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Abstract. Gastrotrichs are meiobenthic invertebrates of obscure origin and unclear phylogenetic alliances. Uncertainties also plague the intra-group relationship with major contrasts between the evolutionary scenarios inferred from morphology or molecules. In this study we analysed partial sequences of the 18S rDNA gene of 18 taxa (14 new and 4 published) to test morphological estimates of gastrotrich phylogeny and to verify whether controversial interrelationships from previous molecular data are due to poor sampling. Data were analysed using both maximum parsimony and maximum likelihood. MP topology was then forced to reflect published morphological estimates and the most parsimonious solutions from each constraint analysis was statistically compared against the unconstrained solution. MP analysis yielded a single tree with few nodes well supported by bootstrap resampling. These included the monophyly of the Chaetonotidae and the internal relationships of the members of this family, with *Aspidiophorus* appearing as the most basal member. The monophyly of the Turbanellidae was also well supported with some suggestion that its sister group might be *Mesodasys*. Lepidodasyidae was found to be an unnatural taxon with *Lepidodasys* forming a separated clade but unrelated also to the Thaumastodermatidae. With the exception of genera *Lepidodasys* and *Neodasys*, the Macrodasyida appeared to be resolved separately from the Chaetonotida, and *Dactylopodola* was resolved as the most basal macrodasyid. ML analysis yielded a tree not too dissimilar from MP, although *Dactylopodola* and *Xenodasys* were resolved as a clade. Statistics indicate that the output from our MP analysis is compatible with the classical view placing representatives of the two orders within two distinct evolutionary lines. Most of the constrained solutions, except the shortest, corroborate the monophyly of the two orders, whereas all five constrained solutions support also the notion that sees *Neodasys* as an early divergent clade along the Chaetonotida branch. Thus, results are generally compatible with the hypothesised evolutionary scenario based on morphological data, but are in contrast with previous findings from molecules. Future research should consider using the complete SSU rDNA gene sequence in their analysis and additional genes for deeper resolution.

Key words. Lower Metazoa, meiofauna, molecular phylogeny, 18S rDNA gene.

1. INTRODUCTION

Gastrotricha are microscopic (0.06–3.0 mm in body length) free-living aquatic worms, characterised generally by a meiobenthic life style. In marine habitats they are mainly interstitial, whereas in fresh waters they are a ubiquitous component of periphyton and benthos and to a more limited extend also of the plankton. In marine sediments, gastrotrich density may reach 364 individuals/10 cm^2 (TODARO 1998); typically they rank third in abundance following the Nematoda and the harpacticoid Copepoda, although in several instances they have been found to be first or the second most abundant meiofaunal taxon (COULL 1985; TODARO et al. 1995; TODARO 1998; HOCHBERG 1999). In freshwater ecosystems population density may reach 158 ind/10 cm^2 making the taxon rank among the top 5 most abundant groups (STRAYER & HUMMON 1991). In

0044-5231/03/242/02-145 $ 15.00/0

aquatic environments the ecological role of the gastrotrichs is realised within the microphagous, detritivorous, benthic community. Like free-living nematodes, gastrotrichs swallow their food, which is made up of microalgae, bacteria and small protozoans, by means of the powerful sucking action of the muscular pharynx (RUPPERT 1991), and in turn they are preyed upon by turbellarians and small macrofauna (BALSAMO & TODARO 2002).

The phylum is cosmopolitan with about 690 species grouped into two orders: Macrodasyida, with 240 strap-shaped species, all but two of which are marine or estuarine, and Chaetonotida with 450 tenpin shaped species, two thirds of which are freshwater (BALSAMO & TODARO 2002). RUPPERT (1988) listed six families and 27 genera in the Macrodasyida and seven families and 18 genera in the Chaetonotida. However due to the non-inclusion of some genera, the subsequent description of seven new genera (KISIELEWSKI 1987a, 1991; EVANS & HUMMON 1991; HUMMON et al. 1993) and the major systematic revisions of freshwater taxa carried out later on (SCHWANK 1990; KISIELEWSKI 1991), these statistics should be considered as very conservative, particularly for the Chaetonotida (see also HOCHBERG & LITVAITIS 2000; BALSAMO & TODARO 2002).

Despite their diversity and abundance, the phylogenetic relationships of the Gastrotricha are still unclear. Based on morphology, most researchers, though considering the evolutionary connections of these worms to be quite obscure, regard them as close allies of the Rotifera (WALLACE et al. 1996), or Nematoda (SCHMIDT-RHAESA et al. 1998). On the other hand, a re-examination of the "Aschelminthes" phylogeny based on the SSU rRNA gene sequence analysis showed the Gastrotricha as the sister taxon of the Platyhelminthes (WINNEPENNINCKX et al. 1995), while later studies placed them close to the Ecdysozoa, the Lophotrochozoa, or neither one (e.g., PETERSON & EERNISSE 2001). Such discrepancies between the traditional and the modern views on the gastrotrich phylogeny suggest that further research in this direction is necessary.

Uncertainty also troubles the assessment of the intragroup relationships of the Gastrotricha. To mention a few problems, briefly: representatives of the two orders Macrodasyida and Chaetonotida are so different in their morphology and ultrastructure that they could be considered paraphyletic relative to the Nematoda or even as two different phyla (RIEGER & RIEGER 1977; RUPPERT 1982); the monophyly of some families of Macrodasyida (e.g., Lepidodasyidae and Planodasyidae) is all but certain (TRAVIS 1983; RUPPERT 1991), the affiliation with the Chaetonotida of the unusual marine genus *Neodasys* Remane, 1927, may be doubtful (RUPPERT 1991); and finally, the evolutionary relationships within and among most families are poorly known because of the incomplete descriptions and a lack of knowledge about the microscopic anatomy of taxa perceived to be 'primitive', or at least possessing features perceived to be plesiomorphic.

These problems are highlighted also by two recent phylogenetic reconstructions of Gastrotricha based on morphological traits (HOCHBERG & LITVAITIS 2000, 2001a). These studies, in confirming the monophyly of phylum, orders and most families, acknowledge that the current systematization displays good phylogenetic congruence, except among the Lepidodasyidae and Planodasyidae and perhaps also the Dactylopodolidae and Chaetonotidae on which future research should be focused (HOCHBERG & LITVAITIS 2000, 2001a). Although phylogenetic inference on morphology-based frameworks will certainly benefit from additional surveys of insufficiently known taxa, (e.g., FERRAGUTI & BALSAMO 1995; BALSAMO et al. 1999; HOCHBERG & LITVAITIS 2001b, c, d), different approaches to evolutionary reconstruction are needed to test independently the current morphological evidence and to help recognize plesiomorphy in these morphologically diverse animals. Sequences of SSU rRNA gene have been recognised as an important source of information for inferring phylogenetic relationships of many taxa, providing at the same time an independent tool to evaluate hypotheses based on morphological characters (e.g., PETERSON & EERNISSE 2001). Consequently, WIRZ et al. (1999) used partial sequences of the 18S rRNA gene from seven species, five Chaetonotida and two Macrodasyida, in an attempt to shed light on some controversial points. The study found the Gastrotricha to be a 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 appear as the most primitive forms of the group. In this study we widen the SSU rDNA gene analysis to include a greater number of taxa and a longer sequence region to test morphological estimates of phylogeny and to verify whether controversial interrelationships from previous molecular data are better resolved by additional taxa and informative sites.

2. MATERIAL AND METHODS

2.1. Sampling of taxa

A reconstruction of gastrotrich evolution based on molecular data has been attempted only once (WIRZ et al. 1999). Whilst shedding light on the monophyly of the phylum, this study left puzzling results and several questions unanswered, perhaps due to the low number of species involved (seven) and the

short region available for analysis (~600 bp of the 18S rRNA gene). Therefore, we decided to increase the number of species and, where possible, to at least double the length of the 18S rRNA gene region used. To complement four published sequences, we sequenced approximately ~1200bp of 14 additional taxa. At the outset, based on strong nodal support in the trees of WIRZ et al. (1999), we considered this region to be of sufficient length and cost effective for the goal we were pursuing. As most of the intriguing phylogenetic questions concern the marine representative of both orders (see RUPPERT 1991; HOCHBERG & LITVAITIS 2000, 2001a) we concentrated our effort on these. The full species list with region of collection, and EMBL accession numbers is reported in Tab. 1.

2.2. DNA extraction, gene amplification, cloning and sequencing

DNA extraction, amplification and gene sequencing was conducted in two different laboratories (Italy and UK). For the majority of taxa, the extraction of genomic DNA was performed by using a chelating resin (Chelex, Biorad, CA) starting from frozen samples (except *Neodasys* preserved in 95% ethanol), containing from two to five specimens each. Briefly, after thawing, each sample was centrifuged at 12,000 g, for 5 min, at 4 °C. The pellet was rinsed once in 300 μl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8) and then resuspended by vortexing in 100 μl of Chelex (20% w/v in 10 mM Tris buffer pH 7.5). The suspension was incubated at 55 °C for 1 hour, flicking occasionally, vortexed for 30 sec and boiled for 8 min. The resulting homegenate, containing the genomic DNA, was collected by spinning the sample at 12,000 g for 1 min at RT, and stored at – 20 °C for future DNA amplifications. To amplify a 18S rDNA fragment two primers (5′ GGCTCATTAAATCAGTTATGG 3′ as forward primer and 5′ ACCACCACCCACCGAATCA 3′ as reverse primer) were designed, relying on a region of this gene conserved in a number of species including *Turbanella cornuta* Remane, 1925, *Chaetonotus* sp., and *Lepidodermella squamata* Dujardin, 1841 available in GenBank/EMBL (Tab. 1). For each sample, 5 μl of genomic DNA was amplified in a total reaction volume of 50 μl by using Taq DNA polymerase (Promega, Madison, WI, USA), according to the manufacturer's recommendation, through the temperature profile consisting in 35 cycles of 94 °C 45 sec, 53 °C 45 sec and 72 °C 1.5 min. PCR products were loaded on an agarose gel, purified by using a gel extraction kit (Agarose Gel DNA Extraction kit, Roche, Indianapolis, IN, USA) and cloned in pGem-T Easy vector (Promega) as described by standard protocols. Cloning was necessary because of the small amount of the PCR product. Sequencing reactions were performed with purified plasmid (High Pure Plasmid Isolation Kit, Roche), employing vector specific primers, flanking the polylinker region (T7, SP6), by means of BigDye Terminator mix (PE Applied Biosystem, Foster, CA, USA) and analysed on a Genetic Analyzer 310 system (PE Applied Biosystem). At least two clones per species were sequenced and analysed for congruence.

Genomic DNA of *Paraturbanella dohrni* Remane, 1927, *Tetranchyroderma papii* Gerlach, 1953 and *Pseudostomella etrusca* Hummon, Todaro & Tongiorgi, 1993 was extracted, and the complete 18S rRNA locus amplified and sequenced as described in LITTLEWOOD et al. (1998).

2.3. Choice of outgroups

The identity of the sister group to the Gastrotricha is not known for certain. Both molecular and morphological data suggest widely different possibilities. Indeed, although clearly protostomes, it is not known for certain as to whether they are close to the Ecdysozoa, Lophotrochozoa, or neither (for reviews and recent estimates see PETERSON et al. 2000; PETERSON & EERNISSE 2001 and papers therein). Here we have selected a number of taxa that appear clustered with the Gastrotricha among the so-called Platyzoa, from a combined morphological and molecular analysis (GIRIBET et al. 2000). The Platyzoa includes Gnathostomulida, Cycliophora, Syndermata (Rotifera+Acanthocephala), Platyhelminthes and Gastrotricha and it is felt that this most recent and inclusive assessment at least offers the best estimate of likely sister group candidates. Considering the potential problems with acoelomorph Platyhelminthes (GIRIBET et al. 2000; PETERSON & EERNISSE 2001; see also RUIZ-TRILLO et al. 1999; TELFORD et al. 2000), and Gnathostomulida sequences (LITTLEWOOD et al. 1998), we restricted our sampling of plathelminthomorphs to Catenulida and basal Rhabditophora. A full listing of outgroup taxa used, and their accession numbers, is given in Tab. 1.

2.4. Sequence alignment and phylogenetic analysis

Partial and complete sequences were initially aligned using ClustalX, vers 1.8 with default multiple alignment parameters (THOMPSON et al. 1997); gap opening [10.00], gap extension [0.20], delay divergent sequences [30%], DNA transition weight [0.50]. The alignment was further adjusted by eye using MacClade (MADDISON & MADDISON 2000, ver. 4) and saved as NEXUS-formatted files so that all ambiguously alignable regions were excluded. The full alignment, including exclusion sets and which may be adapted as a NEXUS file, is available by anonymous FTP from FTP.EBI.AC.UK in directory/pub/databases/embl/align or via the EMBLALIGN database via SRS at http://srs.ebi.ac.uk, under the accession: ALIGN_000 509; the exclusion set is indicated as a note. We concentrated only on including positions where there was sequence data for each taxon.

All phylogenetic analyses were conducted using PAUP* (SWOFFORD 2001, ver. 4.0b8). We analysed the data using both maximum parsimony (MP) and maximum likelihood (ML). MP analyses were run using a heuristic search strategy with tree bisection-reconnection branch swapping, equally weighted characters and gaps treated as missing data. Bootstrap analysis was performed using a full heuristic search strategy and 1,000 replicates. We employed Modeltest vers 3.04 (POSADA & CRANDALL 1998) to estimate the best evolutionary model, based on likelihood scores, for the ML estimate. Under the likelihood ratio test implemented in Modeltest, the data were best described with the TrN+I+G model;

Tab. 1. List of taxa, their classification, collection details and accession numbers used in this study.

Taxon	Habitat and region of collection	EMBL No
PHYLUM GASTROTRICHA		
Order CHAETONOTIDA		
Suborder MULTITUBULATINA		
Family Neodasyidae		
Neodasys ciritus Evans, 1992	Marine, Western Atlantic, NJ, USA	AY228127§
Suborder PAUCITUBULATINA		
Family Chaetonotidae		
Aspidiophorus polystictos Balsamo and Todaro, 1987	Marine, Tyrrhenian Sea, Italy	AY228126§
Chaetonotus sp.	Freshwater, North England, UK	AJ001735
Heterolepidoderma ocellatum (Metschnikoff, 1865)	Freshwater, Tuscany, Italy	AJ007517
Lepidodermella squamata (Dujardin, 1841)	Freshwater, Carolina Biological supply, USA	U29198
Family Xenotrichulidae		
Xenotrichula intermedia Remane, 1934	Marine, Tyrrhenian Sea, Italy	AY228128§
ORDER MACRODASYIDA		
Family Dactylopodolidae		
Dactylopodola typhle Remane, 1927	Marine, Tyrrhenian Sea, Italy	AY228134§
Xenodasys sp.	Marine, Ionian Sea, Italy	AY228133§
Family Turbanellidae		
Turbanella cornuta Remane, 1924	Marine, White Sea, Russia	AF157007
Paraturbanella teissieri Swedmark, 1954	Marine, Tyrrhenian Sea, Italy	AY228138§
Paraturbanella dohrni Remane, 1927	Marine, Tyrrhenian Sea, Italy	AY228139§
Family Thaumastodermatidae		
Subfamily Thaumastodermatinae		
Tetranchyroderma papii Gerlach, 1953	Marine, Tyrrhenian Sea, Italy	AY228137§
Pseudostomella etrusca Hummon et al., 1993	Marine, Tyrrhenian Sea, Italy	AY228136§
Subfamily Diplodasyinae		
Acanthodasys aculeatus Remane, 1927	Marine, Eastern Mediterranean, Crete	AY228135§
Family Lepidodasyidae		
Lepidodasys unicarenatus Balsamo et al., 1994	Marine, Sardinia, Italy	AY228129§
Lepidodasys sp.	Marine, Elba Island, Italy	AY228130§
Megadasys minor Kisielewski, 1987	Marine, Ionian Sea, Italy	AY228131§
Mesodasys laticaudatus Remane, 1951	Marine, Tyrrhenian Sea, Italy	AY228132§
OUTGROUPS (Platyzoa)		
Phylum CYCLIOPHORA		
Symbion pandora Funch and Kristensen, 1995		Y14811
Phylum SYNDERMATA		
*Brachionus platus**		AF154568
Philodina roseola Ehrenberg, 1832		AF154567
Phylum PLATHYELMINTHES		
Stenostomum leucops aquariorum Luther, 1960		AJ012519
Suomina sp.		AJ012532
Stylochus zebra (Verrill, 1882)		AF342801
Pseudoceros tristriatus Hyman, 1959		AJ228794
Paromalostomum fusculum Ax, 1952		AJ012531
Macrostomum hystricinum Beklemischev, 1951		AF051329
Macrostomum tuba Graff, 1882		U70080

Classification of Gastrotricha according to RUPPERT (1988); § represents new sequence generated for the present study; * after an intensive search we could not find the authority for this species, it is therefore possible that the specific epithet reported in EMBL/GeneBank is misspelled.

i.e. a time-reversible model of nucleotide evolution incorporating estimates of invariant sites and among-site rate variation. ML analysis was performed using 10 replicate heuristic searches under the model. Bootstrap resampling was not performed for the ML analysis as each replicate would take over 24 h.

Constraint analyses were performed using MP, beginning with a constraint that forced the topology to reflect a previously published morphological estimate (HOCHBERG & LITVAITIS 2000), and then others with more relaxed constraints. The lengths, and character changes, of the most parsimonious solutions from each constraint analysis were then compared against the unconstrained MP solution and both Kishino-Hasegawa and Templeton's tests were run as implemented in PAUP*.

3. RESULTS

3.1. General

The DNA extraction-to-cloning process carried out on 18 different species yielded 14 reliable sequences of the pursued length; these represented 11 genera and eight families including among others the much debated genera *Neodasys* (Neodasyidae), *Lepidodasys* Remane, 1926 (Lepidodasyidae) and *Xenodasys* Swedmark, 1967 (Dactylopodolidae); when the four published sequences were added, the number of genera involved increased to 14 while the number of families remained unchanged (Tab. 1). We were unable to obtain reliable sequences from the following species: *Crasiella* sp. (Planodasyidae), *Macrodasys caudatus* Remane, 1927 (Macrodasyidae), *Cephalodasys turbanelloides* (Boaden, 1960) (Lepidodasyidae), *Heteroxenotrichula squamosa* Wilke, 1954 (Xenotrichulidae); some were clearly contaminant (fungal) sequences, based on BLAST searches, and others were simply impossible to amplify from fixed specimens. Sequences of the first two species would have allowed the analysis of the entire set of families in which the order Macrodasyida is currently divided (RUPPERT 1988).

Of the ~1200 bp long gene fragment obtained from each species, 959 sites were alignable, across the 18 ingroup and the 10 outgroup taxa. Of the unambiguously alignable positions, 516 were constant and 254 parsimony-informative. In spite of the potentially large evolutionary distance between ingroup and outgroup we found that alignable sites among the gastrotrichs were also alignable across outgroup phyla. In other words, the few regions of high sequence conservation among the gastrotrichs were also conserved across phyla, and overall diversity among gastrotrich SSU rDNA appeared to be relatively high.

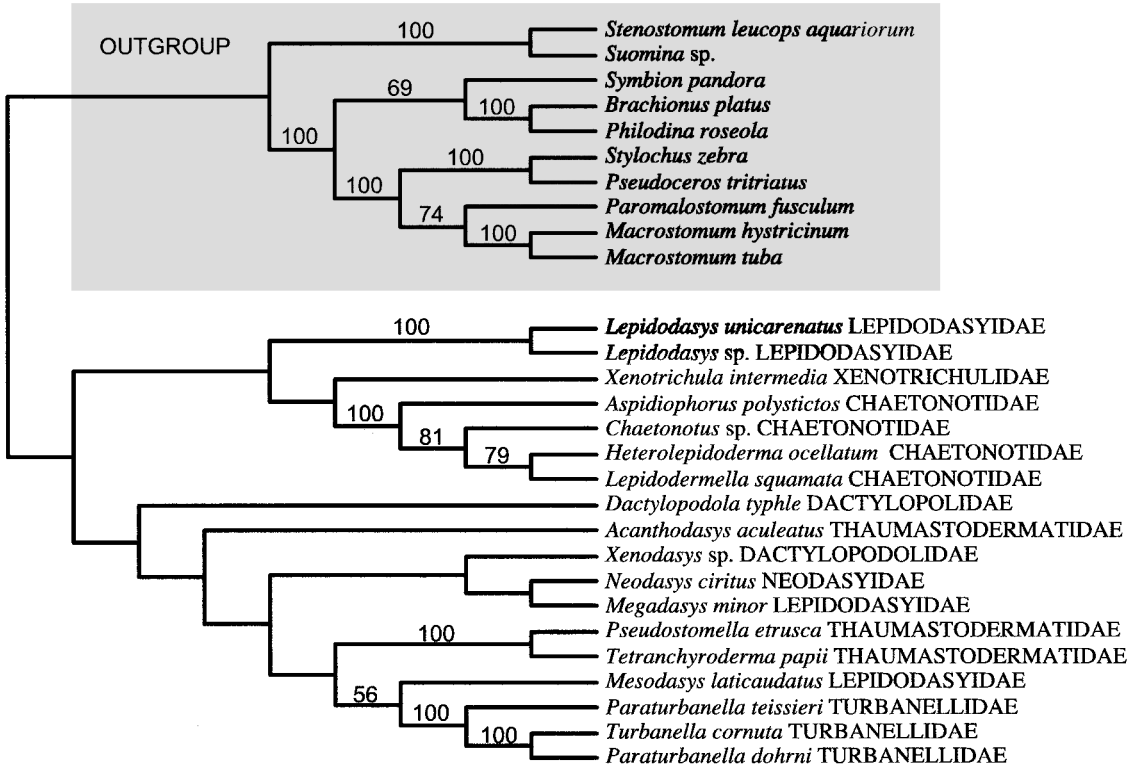
MP analysis yielded a single tree (Fig. 1a), found in each of the 10 replicate searches, of length 1034 steps, with few nodes well supported by bootstrap resampling (n = 1000). Well-supported nodes included the monophyly of the Chaetonotidae and the internal relationships of the members of this family, with *Aspidiophorus* Voigt, 1904 appearing as the most basal member. The monophyly of the Turbanellidae was also well supported with some suggestion that its sister group might be *Mesodasys* Remane, 1951. Few other taxa, where multiple exemplars were available (Thaumastodermatinae and *Lepidodasys*) were resolved as monophyletic. With the exception of genera *Lepidodasys* and *Neodasys*, the Macrodasyida appeared to be resolved separately from the Chaetonotida but low bootstrap values left little confidence in the resolving power of this fragment of SSU rDNA at basal nodes. *Dactylopodola* (Remane, 1927) was resolved as the most basal macrodasyid. ML analysis yielded a tree largely congruent with the MP solution, although Dactylopodolidae were resolved as monophyletic. Short internal branches at the base of the Gastrotricha reflect the inability to resolve deeper relationships, and some longer branches, notably those leading to *Lepidodasys* species and *Xenotrichula* Remane, 1926, indicate the need to sample these taxa more densely in order to break up the branches. Internal relationships among the outgroup taxa appear more likely in the ML analysis, based on previously published topologies, with Catenulida+Rhabditophora being resolved as a clade.

3.2. Constraints

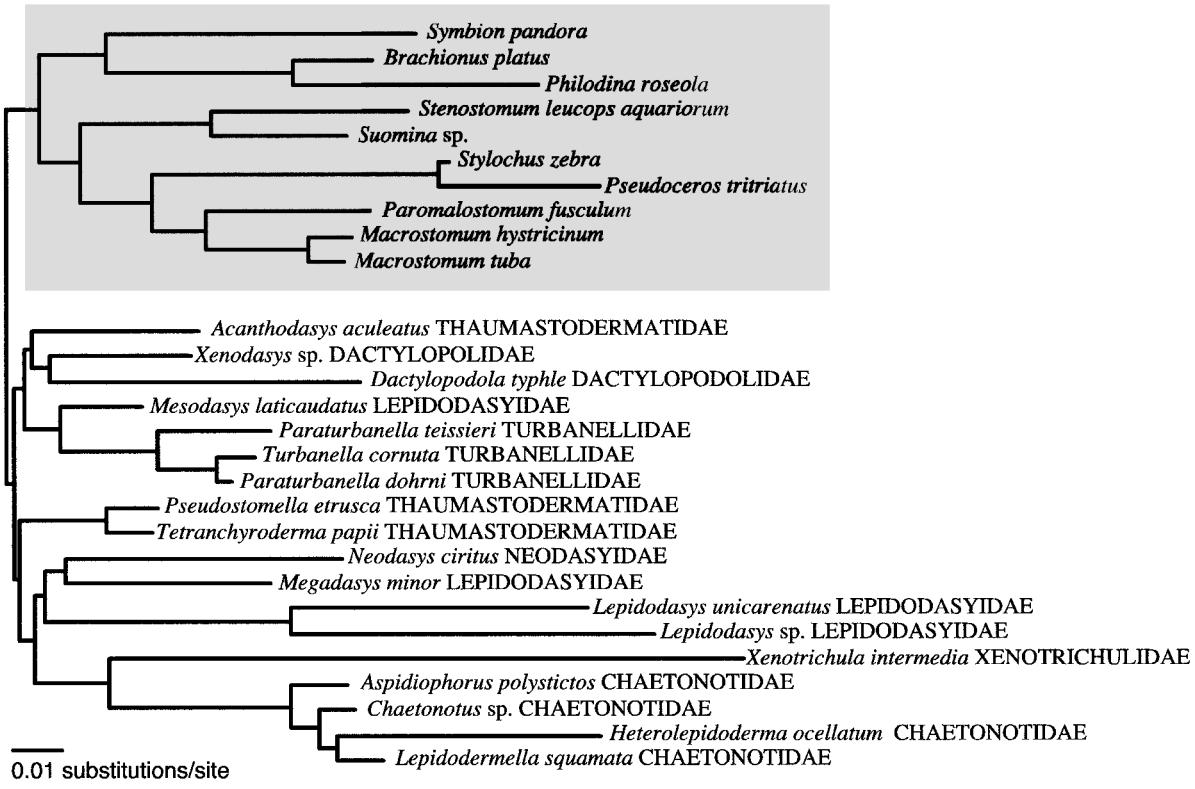
The topology of the trees resulting from the MP and ML analysis may appear odd to gastrotrich taxonomists, as in some instances it does not reflect the relationships implied by the current systematic position of taxa within the phylum (e.g., compare position of *Neodasys*, *Acanthodasys* and *Xenodasys* in Fig. 1 vs Tab. 1). It should be stressed however that the low bootstrap value of "critical" nodes suggests that they should be considered with caution. In order to test our data in the light of morphological analyses, using MP alone we constrained the heuristic searches to find trees compatible with five different topologies, Con1–Con5, which reflect hypothesised phylogenetic trends based on biologically meaningful hypotheses, at least in terms of a morpho-functional point of view. MP trees under each search constraint were saved and then compared against the unconstrained solution to evaluate statistically the possible differences. Fig. 2 shows the different constraints proposed while details are given in Tab. 2, and below.

Con1. This constrained the topology (Fig. 2a) to reflect the morphology-based estimate provided by HOCHBERG & LITVAITIS (2000), which in general terms

a. Maximum parsimony



b. Maximum likelihood



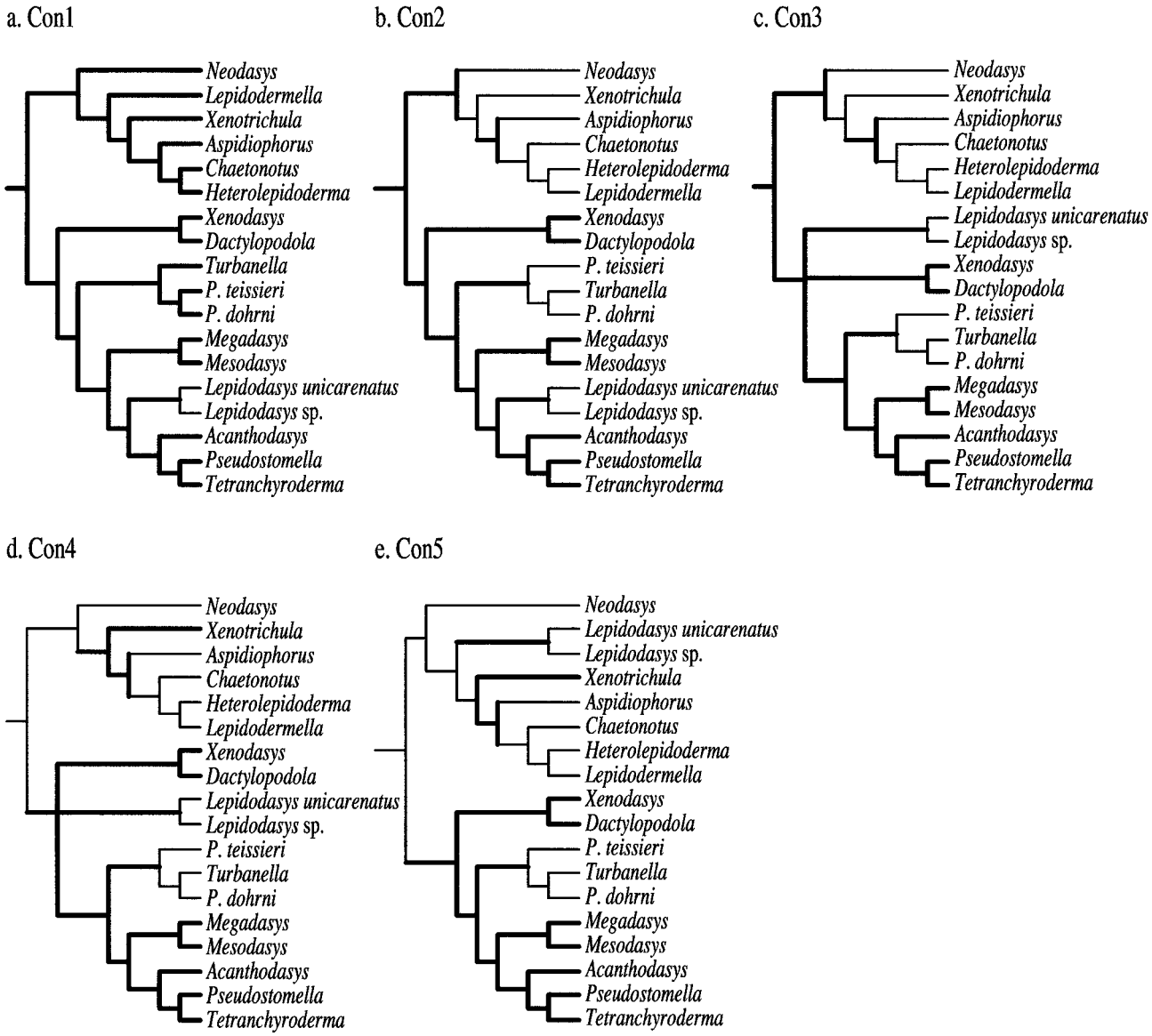


Fig. 2. MP tree topologies employing various constraints. **a.** Con1 represents the fully constrained tree and represents the topology estimated from morphology by HOCHBERG & LITVAITIS (2000). **b–d.** Con2–Con5 represents progressively more relaxed constraints, indicated in Tab. 2 and explained further in the text. Emboldened lines and nodes indicate the groups constrained for each analysis. In Con2–Con5 trees represent strict consensus solutions of two trees but only in Con3 and Con4 were the differences among the two trees appearing in the ingroup; polytomies among the outgroup taxa appeared in Con2 and Con5 and outgroup relationships are not shown here.

Fig. 1. Phylogenetic analyses of the Gastrotricha based on partial 18S rDNA sequences with trees rooted against various 'platyzoan' taxa (shaded boxes). **a.** Single most parsimonious solution showing bootstrap support where ≥ 50%; length = 1034, CI = 0.601, RC = 0.338, **b.** Phylogram based on maximum likelihood analysis using the TrN+I+G model of substitution; base frequencies A = 0.290 C = 0.191 G = 0.264 T = 0.256, rate matrix [A–C] = 1.0 [A–G] = 2.2 [A–T] = 1.0 [C–G] = 1.0 [C–T] = 4.3 [G–T] = 1.0, proportion of invariant sites = 0.224, gamma shape parameter = 0.752.

Tab. 2. Results of constraint analyses. The unconstrained solutions for MP and ML are shown in Fig. 1. The MP solutions under Con1–Con5 inclusive are shown in Fig. 2. See text for further details. $P < 0.05$ indicates that the MP solutions are significantly different from the unconstrained solution.

Constraint	Length	P (K–H)	P (Templetons)
Unconstrained (MP; Fig. 1a)	1034	–	–
Con1 – after HOCHBERG and LITVAITIS (2000); Fig. 2a	1082	<0.001	<0.001
Con2 – as Con1 but with *Neodasys* and *Xenotrichula* unconstrained in a clade with Chaetonotidae; Fig. 2b	1047	0.042	0.136
Con3 – as Con2 but with *Lepidodasys* unconstrained in the Macrodasyida; Fig. 2c	1047	0.048	0.151
Con4 – as Con3 but with *Neodasys* unconstrained; Fig. 2d	1047	0.053	0.164
Con5 – as Con4 but with *Lepidodasys* unconstrained; Fig. 2e	1045	0.078	0.248

also represents the ingroup evolutionary scenario hypothesised by early researchers (e.g., RIEGER 1976; RUPPERT 1978, 1979, 1982, 1991;TYLER & RIEGER 1980; TYLER et al. 1980). One exception in the HOCHBERG & LITVAITIS's tree is perhaps the position of *Xenotrichula* that makes the Chaetonotidae paraphyletic.

Con2. Under this condition (as Con 1 but with *Neodasys* and *Xenotrichula* unconstrained with the Chaetonotidae) the resulting tree (Fig. 2b) reflects the current systematization and a more likely topology within the Chaetonotida branch, with the Chaetonotidae monophyletic, the Xenotrichulidae as their sister taxon and the Neodasyidae as the most basal taxon.
In fact such a scenario corroborates several hypothesised trends in morpho-functional traits, like the changes in reproductive biology (from hermaphroditism to a substantial parthenogenesis) that seem to have take place during the shift of Chaetonotida from marine to freshwater environments (BALSAMO 1992).

Con3. This condition (*Lepidodasys* unconstrained within the Macrodasyida) was set up to see if molecular data could provide a solution for the unsolved debate regarding the phylogenetic position of *Lepidodasys*. The inclusion of the latter genus within the Lepidodasyidae (i.e., *Mesodasys* and *Megadasys*) has been questioned mainly on the basis of different direction of oocyte growth (caudo-cephalic in *Lepidodasys* and opposite in other Lepidodasyidae), the presence of miofilaments in the Y-cell system and the absence of circular muscles from the lateral region of the body, which makes *Lepidodasys* closer to the Thaumastodermatidae (RUPPERT 1978). A *Lepidodasys*-Thaumastodermatidae relationship is highlighted also by the consensus trees found from the morphological matrices set up by HOCHBERG & Litvaitis (2000, 2001a). The output of our analysis (Fig. 2c) shows in all cases *Lepidodasys unicarenatus* Balsamo, Fregni & Tongiorgi, 1994 and *Lepidodasys* sp. join together in a separate clade, and in a more basal position with respect to the other two families.

Con4. *Neodasys* possesses intermediate characters between the two orders (e.g., like the Macrodasyida, it exhibits an elongate strip-shaped body, lateral adhesive elements, epidermal epithelium of cellular type, and epidermal cell monociliated, whereas like the Chaetonotida it has a pharynx with a Y-shaped lumen and no pores. Thus this relaxed constraint solution (*Neodasys* unconstrained) was chosen to test whether molecular data could support the current systematization and the phylogenetic estimate based on morphology which recognizes the monogeneric family *Neodasyidae* in alliance with the Chaetonotida but in the separate suborder Multitubulatina. The topology of the new tree (Fig. 2d) resolves *Neodasys* along the Chaetonotida branch as the sister taxon of the Paucitubulatina clade.

Con5. according to RUPPERT (1978), *Lepidodasys* has at least two characteristics that are unique in Macrodasyida: the absence of pharyngeal pores and the presence of a non-striated pharynx myoepithelium. Both traits are unknown elsewhere in Macrodasyida but are similar to the pharynx construction of Chaetonotida. Furthermore *Lepidodasys* shares additional characters with selected taxa of Chaetonotida and Macrodasyida e.g., absence of circular muscles from the lateral region of the body (also true for *Neodasys*, most Chaetonotida and the Thaumastodermatidae); complex protective cuticular ornamentations (also true for the Chaetonotida except *Neodasys* and the Thaumastodermatidae). This constraint condition (as Con4 but with *Lepidodasys* unconstrained) was set up to validate the current systematization that sees *Lepidodasys* as a macrodasyidan gastrotrich. The resulting tree (Fig. 2e)

shows *Lepidodasys* allied with Chaetonotida in a more derived position with respect to *Neodasys* and as sister taxon of all remainder Chaetonotida (Xenotrichulidae and Chaetonotidae).
Both Kishino-Hasegawa and Templeton's tests indicated that the fully constrained solution Con1, which was 48 steps longer, was significantly different from the unconstrained MP solution. However, Con2 and Con3 were 13 steps longer and only significantly different ($P < 0.05$) for the Kishino-Hasegawa test and not for Templeton's. Con4, which was also 13 steps longer, and Con5 which was only 11 steps longer, were not significantly different topologies from the unconstrained tree regardless of test.

4. DISCUSSION

Most authors consider the Gastrotricha as a monophyletic taxon. This well established notion was challenged by RUPPERT (1982) who, considering the ultrastructural organization of the pharynx, concluded that Chaetonotida Paucitubulatina should be considered as the sister taxon of Nematoda, thus making the phylum paraphyletic. Several recent studies have ruled out this possibility, grouping the Nematoda with other moulting animals within the Ecdysozoa (e.g., AGUINALDO et al. 1997; VALENTINE & COLLINS 2000; ZRZAVÝ 2001). These data corroborate the findings of the first multispecies molecular study of Gastrotricha (WIRZ et al. 1999), which showed the group as monophyletic along an evolutionary line distinct from other phyla. In turn the Gastrotricha has been considered to be related variously with the Nematoda, Rotifera and Platyelminthes. The monophyly of the Gastrotricha is apparently retained in our more extensive study in which among others, basal taxa, such us *Neodasys*, *Dactylopodola* and *Lepidodasys*, are analysed together with a number of the their potentially most close allied, including the short branched plathelminthomorphs, Catenulida and basal Rhabditophora. We acknowledge however, that our result are partially due to the selected choice of the outgroups used in the analysis and the outcome could be different if other (additional) taxa are included.
The monophyly of the two orders Macrodasyida and Chaetonotida is another well-recognised notion among researchers, although the problematic inclusion among the Chaetonotida of the macrodasyidan-looking *Neodasys* needs confirmation (HOCHBERG & LITVAITIS 2000). Along with the general notion of the phylum split in the two orders, the commonly held view of the Chaetonotida being comprised of more derived forms (except perhaps *Neodasys*) endures. In a cladistic framework the latter notion is consistent with an evolutionary scenario that sees Chaetonotida as a derived clade within a paraphyletic Macrodasyida. Against these frameworks were the results of the molecular study by WIRZ et al. (1999), which showed the two Macrodasyida studied (i.e., *Mesodasys* and *Cephalodasys* Remane, 1926) as a sub-set within the Chaetonotida and the genus *Chaetonotus* Ehrenberg, 1830 as the most basal of all. The output from our MP analysis is in part compatible with the classical view placing representatives of the two orders within two distinct evolutionary lines. Yet, four out of five constrained solutions corroborate the monophyly of the two orders whereas five out of five constrained solutions support also the notion that sees *Neodasys* as an early divergent clade along the Chaetonotida branch (Fig. 2). The puzzling position of *Lepidodasys* among Chaetonotida in alliance with *Xenotrichula* (Figs. 1a, 2e) may be considered either as a potential evolutionary scenario in which current Macrodasyida should be considered polyphyletic, having *Lepidodasys* and not *Neodasys* as the sister taxon of the Chaetonotida Paucitubulatina, or, more likely as an artifact perhaps due to the fast evolving nature of the gene in these taxa, that in the ML is seen as a possible effect of long-branch attraction (FELSENSTEIN 1978). MP analysis also suggests a basal position for *Dactylopodola* among Macrodasyida. However the low bootstrap values left little confidence in the resolving power of 18S rDNA at basal nodes.
Results from ML analysis are more contrasting. Taxa of Macrodasyida are shown to occupy a more basal position while the monophyly of the two orders cannot be authenticated. It is noteworthy however that, as in the MP analysis, the ML account shows *Mesodasys* allied with other Macrodasyida (i.e., Turbanellidae) not with Chaetonotida as reported in WIRZ et al. (1999), suggesting that previous molecular results against the monophyly of the orders may have been burdened by artifacts; e.g. too few sites analysed or the use of erroneous sequences. Support for the latter hypothesis is low sequence similarity (data not shown) between the two different *Mesodasys* species analysed by WIRZ et al. (1999) and us. Once again the inability to resolve deeper relationships in our ML analysis, as testified by short internal branches at basal nodes, may be attributed to the low number of informative sites in the sequence region we investigated, or more likely, a deep divergence between the orders and an ancient radiation within each.
Morphological data support the monophyly of most families of Gastrotricha. Notable exceptions are found among Macrodasyida, namely Lepidodasyidae and perhaps the Planodasyidae and the Dactylopodolidae. It should be stressed however that the widespread monophyly within Chaetonotida may, in fact, be an artifact due to the inadequate data available on these

animals, for which information are often dated and/or derived from light microscopy only. Therefore, it should not come as a surprise if new studies (based either on morphology or molecules) may change the actual outline, even that regarding Chaetonotidae and Xenotrichulidae for which data are more inclusive and robust. Recent discoveries of sperm in a plethora of freshwater species (WEISS 2001), previously believed to reproduce by obligate parthenogenesis, stands as testimony to this (i.e., indicating that features bearing phylogenetic significance have been overlooked). In this framework, restricting comments to some debated points and to the taxa (families and genera) under study, our analysis (MP) confirms for the most part the scenario highlighted by HOCHBERG & LITVAITIS (2000, 2001a) on morphological data. For instance, the SSU gene found the Lepidodasyidae to be a polyphyletic taxon with *Lepidodasys* forming a separated clade but unrelated also to the Thaumastodermatidae as suggested by some authors (e.g., RUPPERT 1978). Moreover *Lepidodasys* occupies a more basal position, unveiling a possible different evolutionary scenario which future workers should investigate. The clustering of *Xenodasys* with *Dactylopodola* (Fig. 1b) sheds light on phylogenetic relationships of these bizarre macrodasyids that have long been associated with both Dactylopodolidae or Turbanellidae (e.g., HUMMON 1974; RIEGER et al. 1974; KISIELEWSKI 1987b; HUMMON 1982; RUPPERT 1988).

In accordance with the current opinion and the current systematization (e.g., TRAVIS 1983; RUPPERT 1982, 1988) we found the Chaetonotidae to be monophyletic, with *Aspidiophorus* as the most basal taxon, and Xenotrichulidae as their sister group. These findings are in disagreement with the findings of WIRZ et al. (1999) and HOCHBERG & LITVAITIS (2000), in which the family was resolved variously as paraphyletic. The consensus between MP and ML results of our study, together with the suspicious positioning of Macrodasyida among Chaetonotida found by WIRZ et al. (1999), and the low bootstrap value reported in HOCHBERG & LITVAITIS (2000) should probably be taken into account when assessing the most likely evolutionary scenario between families. While supporting other undisputed clades (i.e., Turbanellidae and Thaumastodermatinae; Fig. 1) our study failed to show a sister-taxon relationships between *Acanthodasys* Remane, 1927 and other Thaumastodermatidae (*Tetranchyroderma* Remane, 1927 and *Pseudostomella* Swedmark, 1956) as the current systematization should imply (Tab. 1).

To summarise, our results are generally compatible with the envisioned evolutionary scenario based on morphological data, unveiling perhaps unsuspected avenues for future investigations (e.g., paraphyly of Macrodasyida, *Neodasys*-Chaetonotida relationships etc). On the other hand the present study does not corroborate previous findings based on molecular data, indicating that inferences of Gastrotricha phylogeny based on SSU rDNA may be reliable only if the analysis involves many species and an ample region of this gene: even with 1200 bp we were unable to solve most basal relationships. In light of this future workers should consider using the complete SSU rDNA gene sequence in their analysis, and possibly the need of additional genes for deeper resolution. A denser sampling also will help to break up longer branches of selected clades. Additionally, our study highlights the need for morphological character coding at the species, or at least genus, level to perform either 'total evidence' analysis or to be able to properly map morphological characters onto the molecular tree, since so many families appear paraphyletic.

Acknowledgments. This research was supported by grants from MIUR (Rome) to MB (Cofin Project 2000/2001 'Evoluzione molecolare e marcatori di processi di filogenesi e di adattamento') and to PT (Cofin Project 1999/2000 'Aspetti descrittivi e metodologici della biodiversità animale in Italia'). DTJL and EAH were funded by a Wellcome Senior Research Fellowship to DTJL (Wellcome 043965). The study benefited also from a grant to MAT (MIUR, Scientific Research Fund 2000–2001). We wish to thank Dr. G. Giribet and an anonymous reviewer for their helpful comments on an early draft of the manuscript.

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Received: 19. 07. 2002
Reviewed: 17. 01. 2003
Accepted: 05. 02. 2003
Corresponding Editor: R. M. KRISTENSEN