Phylogeny of Thaumastodermatidae (Gastrotricha: Macrodasyida) Inferred from Nuclear and Mitochondrial Sequence Data

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Abstract

Background: Phylogenetic relationships within Gastrotricha are poorly known. Attempts to shed light on this subject using morphological traits have led to hypotheses lacking satisfactory statistical support; it seemed therefore that a different approach was needed.

Methodology/Principal Findings: In this paper we attempt to elucidate the relationships within the taxonomically vast family Thaumastodermatidae (Macrodasyida) using molecular sequence data. The study includes representatives of all the extant genera of the family and for the first time uses a multi-gene approach to infer evolutionary liaisons within Gastrotricha. The final data set comprises sequences of three genes (18S, 28S rDNA and COI mtDNA) from 41 species, including 29 thaumastodermatids, 11 non-thaumastodermatid macrodasyidans and a single chaetonotidan. Molecular data was analyzed as a combined set of 3 genes and as individual genes, using Bayesian and maximum likelihood approaches. Two different outgroups were used: Xenotrichula intermedia (Chaetonotida) and members of the putative basal Dactylopodola (Macrodasyida). Thaumastodermatidae and all other sampled macrodasyidan families were found monophyletic except for Cephalodasyidae. Within Thaumastodermatidae Diplodasyinae and Thaumastodermatinae are monophyletic and so are most genera. Oregodasy reveals to be the most basal group within Thaumastodermatinae in analyses of the concatenated data set as well as in analyses of the nuclear genes. Thaumastodermatina appears as the sister taxon to the remaining species. Surprisingly, Tetranychyderma is non-monophyletic in our analyses as one group of species clusters with Ptychostomella while another appears as the sister group of Pseudostomella.

Conclusions/Significance: Results in general agree with the current classification; however, a revision of the more derived thaumastodermatid taxa seems necessary. We also found that the ostensible COI sequences from several species do not conform to the general invertebrate or any other published mitochondrial genetic code; they may be mitochondrially derived nuclear genes (numts), or one or more modifications of the mitochondrial genetic code within Gastrotricha.

Introduction

The approximately 760 described species of Gastrotricha are small aquatic metazoans. The group is cosmopolitan and a common component of the meiofauna. Gastrotrichs have been classified as Rotifera [1], Ciliata [2], Platyhelminthes [3] or closely related to Nematomorpha [4]. Hyman [5] and Ruppert [6] regarded the group as a class within Aschelminthes. Todaro et al. [7] considered a phylum [7], which based on molecular data, most likely has a basal position in the Platyzoa within Lophotrochozoa [e.g. 8–11]. Recent phylogenomic studies by Dunn et al. [12] and Hejnol et al. [13] also place Gastrotricha within Platyzoa. A competing hypothesis regards the group as affiliated to Ecdysozoa based on morphology [e.g. 14,15] and morphology together with molecular data [16].

Gastrotricha currently contains two orders, Chaetonotida and Macrodasyida, based on morphological data [17]. Members of Chaetonotida are tent-rim-shaped and contain both marine and freshwater species. Macrodasyidans are worm-shaped and almost exclusively marine. The two orders were both considered monophyletic on morphological grounds by Hochberg & Litvaitis [18]. The recent comprehensive morphological study by Kiencke et al. [19] hypothesizes Macrodasyida, with the exclusion of the enigmatic freshwater Redudasy and Marinellina, to be monophyletic. However, molecular and morphological analyses are not fully congruent since Macrodasyida and Chaetonotida are often resolved as non-monophyletic groups [e.g. 11,16,20,21]. Manylov et al. [22] investigated the differences between the two gastrotrich orders based on 18S rDNA and suggested a monophyletic Macrodasyida and a monophyletic Paucitubulatina (a suborder...
together with Multitubulatina within Chaetonotida). However these groups clustered with different bilaterian taxa and Gastrotrich was considered a polyphyletic taxon.

The evolutionary relationships among and within the lower ranking taxa (e.g. families and genera) of Gastrotrichia, including the ones that from a morphological point of view are quite well investigated, remain virtually unknown. A good example in this regard is the marine Thaumastodermatidae, the most speciose family within Macrodasysida, with more than 130 species [7]. Thaumastodermatid gastrotrichs are geographically widespread and live interstitially in coarse shelly and medium to fine grained subtidal or intertidal sand. These marine gastrotrichs can easily be identified because of their relatively large mouth, two posterior adhesive pedicles and especially their extraordinary cuticle, which forms spines, sculpted plates, bowl-shaped scales or multi-spined scales. Other, less-immediate, characteristics include the pharynx with reduced radial musculature and small pharyngeal pores, the lack of somatic circular muscles posterior to the head region, an internal connection of the vasa deferentia or vas deferens to the caudal organ and multiciliated epidermal cells [23]. Within the family eight genera are currently recognized and distributed into two subfamilies: Acanthodasys and Diplodasys (Diplodasyinae) vs Hemidasys, Oregodasys ( = Platydasys), Pseudostomella, Ptychostomella, Tetranychyderma and Thaumastodera (Thaumastodermatinae). Hemidasys was described by Claparède [24] and is now considered extinct [7].

Members of Diplodasyinae may be distinguished because they have, among other characters, paired testes and frontal and caudal organ that are anatomically and functionally disjunct i.e. the frontal organ (= frontal sac) is located at mid-body, in front of the largest egg, whereas the caudal organ is located in the posterior trunk region. In contrast, species of Thaumastodermatinae possess a single testis on the right side of the body and the frontal- and caudal organs are anatomically (and functionally?) close to (attached) each other, in the posterior trunk region [23].

Since gastrotrichs rather recently gained attention in molecular studies, most phylogenetic hypotheses dealing with the group are based on morphology only. For instance, Hochberg & Litvaitis [18] using a parsimony analysis of 81 morphological characters found Thaumastodermatidae to be monophyletic, suggesting two autapomorphies for the family: (i) sperm ducts that internally connect to the caudal organ, (ii) a wide bulging buccal cavity. Moreover, the two subfamilies introduced by Ruppert [23] were found monophyletic. Also Kienke et al. [19] using a parsimony analysis of 135 morphological characters found a monophyletic Thaumastodermatidae, but the monophyly of the two subfamilies was not recovered in their phylogenetic tree. It should be highlighted however that the topology obtained by Kienke et al. [19] was plagued by low bootstrap support at most nodes.

The monophyly of Thaumastodermatidae and the family’s internal relationships have never been purposely tested with a molecular approach, and in general, taxon sampling with regard to this family has been very poor in the previous gastrotrich molecular studies i.e. 1–2 species involved. One possible exception is the study by Todaro et al. [11], where six of the 43 gastrotrich taxa examined were thauramastodermatid species. In that study, the analysis of near complete and partial 18S rDNA yielded Thaumastodermatidae and the two subfamilies as monophyletic [11]. These preliminary encouraging results seemed to call for a widening of the molecular study in order to get a complete picture about relationships within the family. Consequently, we arranged to obtain specimens belonging to species of all the extant thauramastodermatid genera, and in an attempt to provide robustness to the outcomes, we planned the phylogenetic analyses to be based on comparison of multi-gene sequences.

**Materials and Methods**

**Selection of taxa**

To estimate the interrelationships within the family Thaumastodermatidae, complete 18S rDNA, partial 28S rDNA and COI mtDNA genes were sequenced from 29 single specimens, representative of all eight extant genera and including at least two species per genus (24 spp in total), with the exception of Pseudostomella for which only sequences of a single species were obtained (Figs. 1–3). In an attempt to determine possible intrageneric relationships, we decided to include several species of Tetranychyderma (the far most speciose genus in the family), which may form 2–3 groups based on morphological traits such as type of cuticular covering, the presence and/or shape of cephalic sensorial organs etc., all characteristics that are widely used in dichotomous keys for species identification [e.g. 25].

The identity of the sister group of Thaumastodermatidae is not known. Based on similarity of some morphological traits, Ruppert [23] indicated Lepidodasys as the potential sister taxon of Thaumastodermatidae; this suggestion found some support by the cladistic analysis of Hochberg & Litvaitis [18]. However, Ruppert’s hypothesis has not been substantiated by past molecular analyses, including that of Todaro et al. [11], which found the family in a sister group relationship with a cluster of chaetonotids, although with low statistical support. In order to get some insights in this regard, we sampled representatives of five additional macrodasidan families for a total of 19 specimens belonging to 16 species in seven genera.

Finally, a representative of the order Chaetonotida, Xenotrichula intermedia (Xenotrichulidae), was chosen as the out-group in the analyses. On morphological grounds, xenotrichulid gastrotrichs possess characteristics that are perceived to be plesiomorphic (e.g. solely marine, hermaphroditic sexual apparatus, functional spermatozoa, etc.) hence they are empirically considered among the basal taxa within Chaetonotida. Moreover, they are readily available in contrast with members of two other possible basal chaetoniotidan families i.e., Neodasyidae and Muselliferidae, which are infrequent or rare [e.g. 26,27]. To look for congruence, in some analyses members of the genus Dactylopoda (Dactylopodidae) were used as outgroup since these gastrotrichs are thought to possess the ancestral macrodasidan features and the family has always resulted to be a putative primitive lineage within the Macrodasida in analyses based on morphology [e.g. 18,28–31].

All of the specimens used in this study were found during a number of faunistic surveys headed by the senior author; no special permission/permits were needed to collect the animals under study. Soon after sampling, gastrotrichs were extracted from the sandy substrata using a 7% MgCl2 solution [17], fixed in 95% Ethanol and stored at −20°C until further treatment. Full list of specimens, together with sampling location as well as geographic coordinates and GenBank accession numbers are presented in Tables 1,2.

**DNA extraction and amplification**

DNA was extracted from single whole specimens using the QIAamp DNA mini kit (Qiagen) with columns from the QIAamp DNA micro kit (Qiagen) according to the manufacturer’s instructions. The extraction yielded extracts of 20 and 40 μl respectively for each specimen. DNA was amplified using the 0.2 ml PuReTaq Ready-To-Go PCR beads (GE Healthcare). For ribosomal 18S and 28S rDNA ~1700 bp and ~2500 bp were
amplified respectively and for mitochondrial COI ~660 bp. For amplification 0.5 μl of each primer, 2 μl of DNA and 22 μl of purified water were assembled in the RTG-PCR tubes yielding a final volume of 25 μl. Primer sequences and PCR-programs are presented in Table 3. Polymerase chain reactions were made in a Gene Amp PCR System 9700 (Applied Biosystems). For some COI sequences a reamplification was necessary to get a sufficient amount of DNA. PCR products were checked on a 0.8% ethidium-bromide gel. In some cases the PCR-product had to be purified with the QIAquick PCR Purification Kit (QIAGEN) according to the manufacturer's instructions.

To remove excess nucleotide fragments EXO and SAP (Fermentas) were mixed in proportions 1:4 and subsequently 5.5 μl EXOSAP added to all PCR-products. EXOSAP-reactions were run at 37°C for 30 min and 80°C for 15 min. Sequence reactions were made according to the BigDye® Terminator v3.1 Sequencing Standard Kit (Applied Biosystems) following the manufacturer’s instructions. An ABI3130XL Automated DNA sequencer (Applied Biosystems, Hitachi) was used to produce chromatograms. Samples which yielded unreadable sequences were cloned using the TOPO TA for Sequencing Cloning Kit (Invitrogen) according to the manufacturer’s instructions.

Alignment and dataset
Contigs were assembled using Staden v 1.6.0 [32]. The consensus sequences were blasted so that contaminations could be discovered. 18S and 28S rDNA sequences were aligned using Muscle [33] with maximers set to 9999 and maxtrees set to 9999. We used the invertebrate mitochondrial code as a guide in order to only infer gaps between codons. Nucleotide sequences were used in all phylogenetic analyses. However it should be pointed out that when searching for stop codons in our COI sequences using the recent software Translator X [34] the ostensible COI sequences from the two Oregodosys species, the two specimens of Tetranchyroderma thysanophorum, Tetranchyroderma esarabophorum, Ptychostomella sp.1, and P. tyrrenica do not conform to the general invertebrate or any other published mitochondrial genetic code.

Aligned 18S and 28S rDNA were processed so that positions that contained more than 10% gaps were removed using the software Filter (Wallberg unpublished). The combined dataset consisted of 4366 nucleotide characters (1664, 1993 and 654 nucleotide characters for 18S, 28S and COI respectively). For 18S rDNA gene all of the 49 taxa were represented, for 28S 42 taxa were represented and for COI 31 taxa were represented. The dataset was subsequently converted into an interleaved nexus formatted file.

Phylogenetic analyses and statistics
The combined dataset was analyzed with MrBayes 3.1.2 [35,36]. Evolutionary models were tested for each of the sequenced genes with FindModel available from the HIV sequence database [37]. The best evolutionary model for each of the sequenced genes were the six parameter general time reversible (GTR) model of nucleotide substitution (nst = 6, rates = invgamma). Two runs with four simultaneous chains were run for 40,000,000 generations. Trees were processed every 100th generation after a burn in of 10,000,000 generations. The MrBayes analyses were carried out on the Bioportal at Oslo University [38]. For tree drawing Figtree v1.1.2 [39] was used. Consensus trees, as well as filtered groups of trees, were produced with Mesquite [40]. Xenotrichula intermedia (Chaetonotida) or species of Dactylopoda were used as outgroups.
Moreover a combined analysis of 18S and 28S rDNA was run and compared with the gene tree of COI according to the settings presented above. Gene trees for 18S and 28S rDNA were also obtained by running each individual data set. All analyses were run according to the above settings but for 10,000,000 generations with a burn-in of 2,500,000 generations. Dactylopodola was used as outgroup for these analyses.

A maximum likelihood analysis using RaxML v7.0.3 [41,42] with 1000 bootstrap replicates under the GTRCAT approximation was run on the combined dataset to check for congruence with the Bayesian analysis. The optimal tree topology from the ML analysis was subsequently tested against an alternative hypothesis (monophyly of Tetranchyroderma) using the approximately unbiased (AU) test [43] using Tree-Puzzle v5.2 [44] and Consel v0.1i [45].

**Results**

The final alignment of the combined data set consisted of 4366 characters (positions). The phylogenetic analysis with Xenotrichula intermedia as outgroup yielded Thaumastodermatidae as strictly monophyletic and both the subfamilies, Thaumastodermatinae and Diplodasyinae, were well supported (Figure 4). Within Diplodasyinae, Diplodasys appear non-monophyletic, due to the uncertain position of a single species of Diplodasys out of five terminals. Within Thaumastodermatinae, the genus Oregodasys, here represented by the three species, O. ocellatus, O. ruber and O. tentaculatus, was monophyletic and turned out as basal to all other taxa within the group. The monophyly of Thaumastoderma represented by three specimens from two species, was highly supported as well. The genus appears here as the sister taxon of the remaining taxa. The more densely sampled Tetranchyroderma, was non-monophyletic and formed two well supported clades where one assemblage of species appeared as the sister-group to Pseudostomella and the other clustered with Ptychostomella. The first clade contains Tetranchyroderma cirrophorum, T. hirtum, T. pachysomum, T. thysoanophorum and Tetranchyroderma sp. 1 as a sister group to Pseudostomella eurasica. The second clade has Ptychostomella sp. 1 and P. tyrhenica nested within a group containing T. cf. antennatum, T. esaralophorum, T. papii, T. quadritentaculatum, Tetranchyroderma sp. 3 and Tetranchyroderma sp. 4.

The phylogenetic analysis of the combined data set with Dactylopodola species as outgroup was in general accordance with the analyses where Xenotrichula intermedia served as outgroup. The non-monophyly of Tetranchyroderma is retained in this analysis as well and the within-family groupings are the same as in the Xenotrichula intermedia outgroup-bearing phylogeny (Figure 5). In general, statistical support at nodes are higher in this analysis, whereas slight differences regard alliances among non-Thaumastodermatidae taxa.

The combined data set of the nuclear genes contained 3663 positions; the results were in general accordance with the species tree based on the combined nuclear and mitochondrial data set. The only notable exception was that the two specimens of T. quadritentaculatum were in a sister group relation to Thaumastoderma (Figure S1).

![Figure 2. Gastrotircha, Thaumastodermatidae, Thaumastodermatinae. SEM photomicrographs showing the general body shape and aspects of the cuticular covering of representatives of the genera Oregodasys, Tetranchyroderma and Thaumastoderma. A, B, Oregodasys ocellatus dorsal and ventral view respectively; C, E Tetranchyroderma cf. antennatum, C, habitus dorsal view, E, close-up of the anterior end in a ventral view showing the ample mouth, adhesive tubes of the anterior series and cephalic sensory organ; D, F Thaumastoderma ramuliferum, D, habitus in ventral view; F, close-up of the anterior end in a dorsal view showing the cuticular armature made up of tetrancres, cephalic sensory organs and the cirrata tubes. Scale bars A–C = 50 μm, D–F = 20 μm. doi:10.1371/journal.pone.0017892.g002](https://www.plosone.org/figure/2-gastrotircha-thaumastodermatidae-thaumastodermatinae-sem-photomicrographs-showing-the-general-body-shape-and-aspects-of-the-cuticular-covering-of-representatives-of-the-genera-oregodasys-tetranchyroderma-and-thaumastoderma-a-b-oregodasys-ocellatus-dorsal-and-ventral-view-respectively-c-e-tetranchyroderma-cf-antennatum-c-habitus-dorsal-view-e-close-up-of-the-anterior-end-in-a-ventral-view-showing-the-ample-mouth-adhesive-tubes-of-the-anterior-series-and-cephalic-sensory-organ-d-f-thaumastoderma-ramuliferum-d-habitus-in-ventral-view-f-close-up-of-the-anterior-end-in-a-dorsal-view-showing-the-cuticular-armature-made-up-of-tetrancres-cephalic-sensory-organs-and-the-cirrata-tubes-scale-bars-a-c-50-μm-d-f-20-μm-doi101371journalpone0017892g002)
Analyses of individual gene trees indicated that the nuclear genes (18S rDNA and 28S rDNA) have little, if any, conflicts. The 18S gene tree is better resolved than the 28S gene tree. In both trees Thaumastodermatidae emerge as a well supported group and within this clade the subfamilies Diplodasyinae and Thaumastodermatinae were also well supported. Within Thaumastodermatinae, Oregodasys has a basal position in both gene trees. The large genus Tetranchyroderma is non-monophyletic in both trees (Figures S2, S3).

In the 18S gene tree, P. etrusca is a sister group to all other Tetranchyroderma except T. quadriventriculatum. Ptychostomella is nested within Tetranchyroderma in a clade together with T. cf. antennatum, T. esarabdophorum, T. papii, T. sp. 3 and T. sp. 4.

In the 28S gene tree, P. etrusca is nested within a poorly resolved clade together with Tetranchyroderma and Thaumastoderma. Ptychostomella is nested in a poorly resolved clade with the same species as in the 18S gene tree (Figures S2, S3).

The basal parts of the COI gene tree are very poorly resolved. Tetranchyroderma is non-monophyletic in a clade together with Oregodasys, Ptychostomella and Pseudostomella. Moreover, the COI phylogeny is in conflict with the nuclear gene trees for example regarding the position of Oregodasys, which is not basal in Thaumastodermatidae but nested within Tetranchyroderma (Figure S4). Nevertheless, including COI in the concatenated phylogenetic analysis increased support values for several clades compared to the 18S and 28S gene trees.

Figure 3. Gastrotricha, Thaumastodermatidae, Thaumastodermatinae. DIC (A, C) and SEM (B,D,E) photomicrographs showing the general body shape and aspects of the cuticular covering of representatives of the genera Pseudostomella and Ptychostomella. A, B, Pseudostomella etrusca, A, habitus, B, close-up of the anterior end showing the impressive oral palps; C, Ptychostomella sp 1, habitus; D, Ptychostomella mediterranea, habitus, dorsal view; E, Ptychostomella sp., habitus ventral view. The latter two species were not involved in the molecular study. Scale bars A, C = 50 μm, B, D, E = 20 μm.

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With regard to the other taxa, two families are here represented by more than one genus: Turbanellidae, which appeared to be monophyletic, and Cephalodasyclidae, which was resolved as polyphyletic, with Megadasyx shown in alliance with Turbanellidae, and Mesodasyx standing alone. All the other single genera formed a polytomy (Figure 5). Our analyses failed to find the sister group of Thaumastodermatidae among the species/taxa examined here.

The ML phylogeny was congruent with the Bayesian phylogeny. Bootstrap support values were high for Thaumastoderma and Diplodasyx. The basal position of Oregadosys within Thaumastodermatinae was strongly supported and so was the sister group relationship of Thaumastoderma with the remaining taxa. Likewise Tetranchydroderma was non-monophyletic with the same groupings as in the Bayesian analysis. The approximately unbiased test based on the ML analysis rejected a constraint tree where Tetranchydroderma was kept monophyletic (p<0.01) compared to the best scoring ML-tree (Table 4).

### Discussion

In analysis of the concatenated dataset two different outgroups were used: (i) Xenotrichula intermedia (Chaetonotida, Xenotrichulidae) and (ii) Dactylopodola cf. baltica, D. mesophyle and D. typhe (Macrodasyida, Dactylopodolidae). Use of these different outgroups did not alter the general topology of the tree, although for the concatenated tree resolution was better and support values were a little higher with Dactylopodola as outgroup. Because of this, Dactylopodola was also used as outgroup for the individual gene trees.

The combined phylogeny of 18S, 28S and COI gives the same general results as the combined phylogeny of the two nuclear genes as well as the gene trees of 18S rDNA and 28S rDNA. Also, the maximum likelihood analysis is congruent with these results, so
was the result of a parallel analysis conducted using a Maximum Parsimony method (Figure S5).

All of this suggest that the phylogenetic scenario recovered by the topology in the concatenated analysis (Figure 4) is extremely robust (i.e. very likely).

The hypothesis that Thaumastodermatidae - the largest and one of the most morphologically diverse macrodasyidan families - is monophyletic gains strong support in our study. Rieger & Rieger [46] also confirm monophyly of the family based on cuticular ultrastructure, as does Ruppert [23] based on the structure/function of the reproductive organs.

In this framework, it seems interesting to discuss the in-group evolutionary hypotheses put forward by previous authors in the light of our results. For instance, considering the features of the cuticular covering together with the traits of the reproductive apparatus, the two subfamilies Diplodasynae and Thaumastodermatinae gain high support as monophyletic groups, having a sister group relationship [e.g. 18,30]. Moreover, morphology seems not to leave doubts about the monophyly of the currently recognized genera and so far taxonomists have not raised concerns in this regard.

In our analyses, the two subfamilies are resolved as monophyletic with high statistical support and their sister group relationship is corroborated.

Regarding the monophyly of the genera, within Diplodasynae both genera in the group were represented in our analysis containing seven terminals in total: two putative species for Acanthodasys and three species and 5 specimens for Diplodasys. In our study Acanthodasys appears monophyletic while Diplodasys is unresolved due to the uncertain position of Diplodasys sp., a new species collected in Kuwait (Figs. 1, 2). Considering that the other four Diplodasys terminals cluster together with a high statistical support and knowing that only minor taxonomic characters (e.g. number of adhesive tubes, presence of cephalic sensorial organs etc) distinguishes the Kuwaiti specimens from other known Diplodasys species, there seem to be realistic reasons to consider Diplodasys as a monophyletic taxon.

According to Hochberg & Litvaitis [18,30], the subfamily Thaumastodermatinae, which includes five extant genera, may be defined by a single morphological autapomorphy: the loss of the left testis. However, other characteristics may distinguish Thaumastodermatinae from Diplodasynae e.g., by the presence of caudal and frontal organs in posterior trunk region and adjacent to each other, and an extensively modified cuticular covering. Differences in these traits have been used to infer in-group phylogeny; results of these studies however have not always been congruent with each other [e.g. 18 vs 19]. One exception is the

### Table 2. Non-Thaumastodermatidae taxa used in this study.

| Taxon                  | Sampling location   | Coordinates                  | GenBank accession number |
|------------------------|---------------------|------------------------------|--------------------------|
|                        |                     |                              | 18S | 28S | COI |
| Cephalodasyidae        |                     |                              |                |       |     |
| Megadasya sp.          | Grotta del Cioio, Italy | 39°50'38"N; 18°23'09"E      | JF357655 | JF357703 | JF432040 |
| Megadasya sp. 1        | Porto Cesarea, Italy | 40°15'33"N; 17°53'53"E      | JF357656 | JF357704 | JF432041 |
| Mesodasya latcaudatus  | Albini, Italy        | 42°29'29"N; 11°11'28"E      | JF357657 | JF357705 | JF432042 |
| Mesodasya latcaudatus  | Bohuslan, Sweden     | See [59]                     | JF357668 | NA   | JF432050 |
| Mesodasya littoralis   |                     |                              | JF357658 | JF357706 | JF432043 |
| Dactylopodolidae       |                     |                              |                |       |     |
| Dactylopodola cf. baRica | Kuwait              | 29°20'53"N; 48°06'02"E      | JF357650 | JF357698 | NA   |
| Dactylopodola mesotyphile | Punta Ala, Italy    | 42°48'42"N; 10°44'46"E      | JF357651 | JF357699 | JF432036 |
| Dactylopodola typhile  | Bou Ficha, Tunisia   | 36°16'50"N; 10°29'41"E      | JF357652 | JF357700 | JF432037 |
| Dactylopodola typhile  | Torre Civette, Italy | 42°50'42"N; 10°46'31"E      | JF357653 | JF357701 | JF432038 |
| Lepidodasyidae         |                     |                              |                |       |     |
| Lepidodasya unicarenatus | Pianosa, Italy      | 42°37'04"N; 10°05'21"E      | JF357665 | NA   | JF432048 |
| Macrodyidae            |                     |                              |                |       |     |
| Macrodyas sp. 1        | Torre Civette, Italy | 42°50'42"N; 10°46'31"E      | JF357654 | JF357702 | JF432039 |
| Macrodyas sp. 2        | Bohuslan, Sweden    | See [59]                     | JF357670 | JF357714 | JF432052 |
| Turbanellidae          |                     |                              |                |       |     |
| Paraturbanella dohrni  | Punta Ala, Italy    | 42°48'42"N; 10°44'46"E      | JF357659 | JF357707 | NA   |
| Paraturbanella pallida | Capri, Italy        | 43°00'53"N; 09°49'24"E      | JF357660 | JF357708 | JF432044 |
| Paraturbanella tessieri | Punta Ala, Italy    | 42°48'42"N; 10°44'46"E      | JF357661 | JF357709 | NA   |
| Turbanella bocqueti    | Tramore, Ire land   | 52°09'24"N; 07°08'12"W      | JF357662 | JF357710 | JF432045 |
| Turbanella cornuta     | Chioggia, Italy     | 45°12'57"N; 12°17'57"E      | JF357663 | JF357711 | JF432046 |
| Turbanella cornuta     | Åhus, Sweden        | 55°54'22"N; 14°17'41"E      | JF357666 | NA   | JF432051 |
| Turbanella katheri     | Torö, Sweden        | 58°48'30"N; 17°48'20"E      | JF357669 | NA   | JF432051 |
| Xenotrichulidae*       |                     |                              |                |       |     |
| Xenotrichula intermedia | Mahdia, Tunisia     | 35°30'57"N; 11°03'00"E      | JF357664 | JF357712 | JF432047 |

*Order Chaetonotida.

Sampling locations together with their respective coordinates are given as well as GenBank accession number. NA, Not available.

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sub-clade that includes *Pseudostomella* as the sister group of *Tetranchyroderma* plus *Thaumastodera* that has consistently been recovered in the cladistic analyses, and at least twice with a reasonably high statistical support [e.g. 18,30]. Species of this recovered in the cladistic analyses, and at least twice with a reasonable high statistical support [e.g. 18,30]. Species of this sub-clade that includes *Pseudostomella* as the sister group of *Tetranchyroderma* plus *Thaumastodera* that has consistently been recovered in the cladistic analyses, and at least twice with a reasonably high statistical support [e.g. 18,30]. Species of this recovered in the cladistic analyses, and at least twice with a reasonable high statistical support [e.g. 18,30]. Species of this

### Primers & Regime

| Primers & Regime | Direction | Primer sequence (5’-3’ ) | Usage | Reference |
|------------------|-----------|--------------------------|-------|-----------|
| **18S/SSU Primers** | | | | |
| S30 | Forward | GCTTGTCTCAAGATAAGGCC | PCR/Sequencing | [60] |
| SFK | Reverse | TTCTGGCAAGGCTTCGCC | PCR/Sequencing | [60] |
| PCR regime S30/SFK | 95°C at 2 min, 40×(94°C at 30 s, 52°C at 30 s, 72°C at 30 s), 75°C at 10 min | | | |
| 4FB | Forward | CAGTACGGCGGATAATTCAG | PCR/Sequencing | [60] |
| 1806R | Reverse | CTCGTAGCCTTTTCCTTCCT | PCR/Sequencing | [60] |
| PCR regime 4FB/1806R | 95°C at 2 min, 40×(94°C at 30 s, 52°C at 30 s, 72°C at 30 s), 75°C at 10 min | | | |
| S7 | Forward | GCCAAAGGATTTGCAAGGA | Sequencing | [60] |
| 7F | Forward | GCAATACAGGCTGTGATGC | Sequencing | [60] |
| 7FK | Reverse | GCATCAGAAGCTTATTG | Sequencing | [60] |
| **28S/LSU Primers** | | | | |
| U178 | Forward | GACCCCGCTGAAYTAAG | PCR/Sequencing | [61] |
| 1634L | Reverse | ATTCGGCAGGTGATGTTACA | PCR/Sequencing | This study |
| PCR regime U178/1634L | 95°C at 2 min, 40×(94°C at 30 s, 56-54°C at 30 s, 72°C at 1 min), 36×(94°C at 30 s, 52°C at 30 s, 72°C at 1 min), 72°C at 10 min | | | |
| 1200F | Forward | CCCGGAAAGATGTGCAACTG | PCR/Sequencing | [61] |
| 2450R | Reverse | GCTTGTITTTAATTTAGCAGCTCGGA | PCR/Sequencing | [61] |
| PCR regime 1200F/2450R | 95°C at 2 min, 40×(94°C at 30 s, 52°C at 30 s, 72°C at 1 min), 72°C at 10 min | | | |
| 300F | Forward | CAAGTACCAGTGAGGAAGTTG | Sequencing | [61] |
| 300R | Reverse | CAACTTTGCCCACGACTTG | Sequencing | [61] |
| 1200R | Reverse | GCATAGTTCACCATCTTGG | Sequencing | [61] |
| UJR2176 | Reverse | GGATACCAATTTGCAGCCTCCTTA | Sequencing | [62] |
| 1600F | Forward | AGCAGAGGGTGGCCATGGAAG | Sequencing | [61] |
| **COI Primers** | | | | |
| LCO1490 | Forward | GGTCAACAAATCTAAAGATATTG | PCR/Sequencing | [63] |
| HCO2198 | Reverse | TAAACCTCAGGGTGACAAAAATCA | PCR/Sequencing | [63] |
| PCR regime LCO1490/HCO2198 | 95°C at 2 min, 40–45×(94°C at 30 s, 46°C at 30 s, 72°C at 30 s), 72°C at 10 min | | | |

PCR regimes for primer pairs are also given. DOI:10.1371/journal.pone.0017892.t003
also future morphology-based phylogenetic analyses, the result of which, due to different characters and character state scoring, have so far yielded contrasting results with regard to the position of Oregodasys: e.g., a basal position in Hochberg and Litvaitis [18], more derived line in Hochberg & Litvaitis [30] and in Kieneke et al [19].

Our study also suggests that future phylogenetic studies based on morphology should consider the absence of scales/spines in Oregodasys and Ptychostomella not to be a homologous trait and consequently score this characteristic accordantly; this hypothesis is further corroborated by the differences that exist between the
The basal position of *Oregodasys* along the Thaumastoderma-tinae branch may raise a question about the ancestral state of the cuticle in Thaumastodermatidae: armoured or smooth? Several evidences point to the first as the ancestral state, among others: (i) scales and spines are present in members of most genera of the family, including *Acanthodasys* and *Diplodasys* that possess other ancestral characteristics e.g., paired testes; (ii) abundance of epidermal glandulocytes in *Oregodasys* [50]. Ruppert [6] hypothesized a repugnatorial function for the glandulocytes, so vicarising the protective function of scales and spines by producing toxic and/or repellent material as defence against predation. This hypothesis is supported by the subsequent discovery of *Tetranchyrodema* species that are characterized by a reduction of the cuticular armature (bikini-trix, see below) and an abnormal high number epidermal glands.

Our study found a well-supported monophyletic *Thaumastoderma* to be the second-most basal taxon along the Thaumastoderma-tinae phylogenetic branch, an evolutionary hypothesis never formulated before. Our results shed light on the origin and evolution of the one of the most striking morphological features of these gastrotrichs, i.e., the ancestral shape of the anchored spines, whose appearance is responsible for the taxon name, *Thaumastoderma* (Latin: = miraculous skin).

The position of *Thaumastoderma* as basal to the reminder of the ancre-bearing taxa implies that their ancestor probably had a...
tetrancreous covering (i.e., tetrancre as primary states) with tentancrees evolving secondarily in the common ancestor of Tetranchyroderma and Pseudostomella. In this scenario the central prong that characterizes the tentancrees is a secondary acquisition and therefore not homologous with the central spine of species of Diplodasyinae as previously hypothesized (see above). Furthermore, while the central spine, and hence tentancre, may be a synapomorphy of Tetranchyroderma and Pseudostomella, its seemingly distribution throughout both genera may also imply independent evolution in both taxa.

Our study also indicates that the speciosus genus Tetranchyroderma is non-monophyletic, a result strongly supported in both Bayesian and ML analysis; of the 600000 trees that were sampled during the analyses none contained a monophyletic Tetranchyroderma. The alternative hypothesis (monophly of Tetranchyroderma) was rejected by the approximately unbiased test (p<0.01). It appears from our results that the genus should be split into at least two groups, of which one should be affiliated to Pseudostomella and the other should encompass also Ptychostomella (Figure 1).

A sister group relationship between Pseudostomella and Tetranchyroderma has been shown in several studies [e.g., 11,18,30]. However, it is difficult to compare our findings with the results from previous phylogenetic analyses due to differences in methodologies and taxonomic sampling. For instance the studies based on morphology [18,30] have considered the two taxa as single terminals while only two Tetranchyroderma species were involved in the molecular study of Todaro et al. [11]. All considered, it is realistic to believe that the past results may have been biased by the poor taxonomic sampling; the poorness regards both the number but also the type of the species involved in the past studies. This statement finds support considering that there is a conflict between our study and Todaro et al. [11] regarding the relative position of Pseudostomella and T. popii, which are mutually exclusive in the two studies. It should be highlighted that the position of Pseudostomella was unstable in additional analyses performed with a subset of the taxa in our dataset (not shown).

In the species-based cladistic analysis of Gastrotricha by Kienke et al. [19] that included two Pseudostomella and three Tetranchyroderma species, a monophyletic Pseudostomella was found basal to most thaumastodermatids, except Acanthodasys and Thaumastodermatidae while Tetranchyroderma appeared paraphyletic; again bootstrap values at nodes was in all cases very low, leaving little confidence on these results.

In our opinion, a sister-group relationship between Pseudostomella and at least some Tetranchyroderma species is most likely, based for example on the potential to evolve five- and three-pronged scales, starting from the ancestral tetrancretes. A monophyletic Pseudostomella also is credible, based on the impressive autoapomorphy constituted by the oral palps, a characteristic without equivalent in any other gastrotrich taxa (Figure 3).

Our most surprising result is the splitting of Tetranchyroderma into two clades. This is because we cannot think of any morphological synapomorphies of the two groups that can be used as diagnostic features, except perhaps the fact that species allied with Pseudostomella lack cephalic tentacles, whereas cephalic tentacles, rod- and/or knob-like are present in the species that cluster with Ptychostomella. It is also possible that the importance of the phylogenetic signal of other characters (e.g. arrangement of the anterior adhesive tubes, structure of the caudal pedicles, shape and structure of the fronto-caudal organ complex etc.) may have thus far been overlooked.

In comparison, the relationship of certain Tetranchyroderma species with Ptychostomella may be simpler to explain. The monophly of Ptychostomella is supported by our analysis; however, morphologically the group is distinguished on a negative character i.e., the absence of scales and/or spines (Figure 3). A possible explanation for the rise of the Ptychostomella lineage would be that the group shares a common ancestor with a subset of the Tetranchyroderma species characterized by a tendency toward reduction and loss of the cuticular hooks. This hypothesis receives support based on the overall resemblance of their external and internal ananomies to small species of Tetranchyroderma than to any other thaumastodermatids. Moreover, the reduction of the cuticular covering is a phenomenon all but infrequent in Tetranchyroderma as testified by the presence of the so-called “bikini-trix” [41,52] a complex of 5-6 species characterized by incomplete cuticular covering which in T. hypopsilacrum, for instance, may be restricted to some epauletts in the pharyngeal region [cf 25]. If corroborated by further studies this hypothesis would make Tetranchyroderma paraphyletic, creating even more conflicts with the current classification.

To summarize, the concatenated phylogeny is congruent with other studies dealing with in-group relationships in Macrodasyida. Thaumastodermatidae has been found to be monophyletic based on morphology by Hochberg & Litvaitis [18,30] and Kienke et al. [19], Ruppert [23] states that the possibility of polyphyly is remote and gives the following apomorphies for a monophyletic Thaumastodermatidae: (i) complex cuticle, (ii) structure of the pharynx, (iii) lack of circular muscles in the lateral body regions, (iv) internal connection of vas deferens or vas deferentia to the caudal organ, and (v) multiliculated epidermal cells. Molecular studies are concordant with morphology and the group has been found monophyletic based on 18S rDNA by Todaro et al. [11] and Petrov et al. [21]. The only other investigation where some of the internal relationships within the family were studied is Todaro et al. [11], which found high support for the monophyly of Diplodasyinae and Thaumastodermatinae. Moreover the same study also found a sister group relationship between Pseudostomella etrusca and two species of Tetranchyroderma. A similar sister group relation is also presented in this study where approximately half of the sampled Tetranchyroderma form a sister group relation to P. etrusca.

Traditionally the number of prongs on scales of Tetranchyroderma has been used to discriminate between species and subgroups within the genus. In our analysis taxa with different number of prongs clusters together. T. antenatum, T. eiaedsdophorum, T. hirtum, T. popii, T. quadrientalaculatum, T. sp. 1, T. sp. 4 and T. thysonophorum all have five pronged scales [51,52–56, M.A. Todaro unpublished] while T. cirrophorum, T. pacchysorum, T. sp. 3 have four pronged scales [51,57, M. A. Todaro unpublished]. Hence, the number of prongs on scales is not a good morphological character to use for determining relationships within Tetranchyroderma, although it is extremely useful in dichotomous keys [cf 25].

Regarding the relationships of other families exclusive of Thaumastodermatidae, a few comments can be made. Turbanellidae are monophyletic and congruent with traditional classification within Macrodasyida. The non-monophyly of the Lepidodasyidae sensu Remane [58] has been known for some time [23]; our results support the recent separation of Lepidodasyidae from other genera previously affiliated with the family [7]; however, it seems that the revisional work is not finished as the position of Megadasys and Mesodays in our tree suggests that also the new erected family (i.e. Cephalodasyidae) may be non-monophyletic.

Finally, the ostensible COI sequences from the two Oregadosys species, the two specimens of Tetranchyroderma thysonophorum, Ptychostomella spl and P. tyrhenica do not conform to the general invertebrate or any other published mitochondrial genetic code. We re-examined all COI chromatograms and re-amplified and sequenced the six specimens with identical results. The sequences
we have obtained (Accession numbers: JF432020, JF432021, JF432022, JF432027, JF432025, JF432033, JF432034) may be mitochondrially derived nuclear genes (nnumts), or there may have been one or more modifications of the mitochondrial genetic code within Gastrotricha.

Supporting Information

Figure S1  Phylogenetic relationships of Thaumastodermae inferred from Bayesian analysis of 18S rDNA and 28S rDNA (95% consensus tree). The outgroup is represented by members of Dactylopoda. Number at nodes represent posterior probabilities. (TIF)

Figure S2  Phylogenetic relationships of Thaumastodermae inferred from Bayesian analysis of 18S rDNA (95% consensus tree). The outgroup is represented by members of Dactylopoda. Number at nodes represent posterior probabilities. (TIF)

Figure S3  Phylogenetic relationships of Thaumastodermae inferred from Bayesian analysis of 28S rDNA (95% consensus tree). The outgroup is represented by members of Dactylopoda. Number at nodes represent posterior probabilities. (TIF)

References

1. Ehrenberg CG (1838) Die Infusionsthierchen als Vollkommene Organismen. In: Voss L, ed. Ein Blick in des tiefere organische Leben der Natur. Leipzig: Nebst Atlas. pp 386–390.
2. Dujardin F (1841) Histoire Naturelle des Zoophytes, Infusoriae. Paris: Libraire Encyclopédique de Roret, pp 694.
3. Schultz M (1853) Über Chaetonotus und Ichthydium (Ehrl.) und eine neue verwandte Gattung Turbaulites. Müller’s Archiv für Anatomie und Physiologie 6: 241–254.
4. Ludwig H (1875) Über die Ordnung Gastrotricha. Mutschirn für Wissenschaftliche Zoologie 26: 193–226.
5. Hyman LH (1951) Gastrotricha. In: The Invertebrates, Vol. III, Acanthocephala, Aschelminthes and Ectoprocta. New York: McGraw-Hill. pp 53–59, 131–170.
6. Ruppert EE (1991) Gastrotricha. In: Harrison FW, Ruppert EE, eds. Microscopic Anatomy of Invertebrates, Vol. 4, Aschelminthes. New York: Wiley-Liss. pp 41–109.
7. Hummon WD, Todaro MA (2010) Analytic taxonomy and notes on marine, brackish-water and estuarine Gastrotricha. Zootaxa 2392: 1–32.
8. Winnepermincks B, Backeljau T, Mackey LY, Brooks JM, De Wachter R, et al. (2009) Molecular phylogeny of Gastrotricha on the basis of a comparison of the 18S rRNA genes: Rejection of the hypothesis of a relationship between Gastrotricha and Nematoda. Mol Biol Evol 41: 445–452.
9. Manylov OG, Vladychenskaya NS, Milyutina IA, Korokhov NP, et al. (2004) Analysis of 18S rRNA gene sequences suggests significant molecular differences between Macrodasysida and Chaetonotida (Gastrotricha). Mol Phylog Ecol 30: 850–854.
10. Ruppert EE (1978) The reproductive system of gastrotricha. 3. Genital organs of Macrodasyidae subfam. n. and Diplodasyinae subfam. n. with discussion of reproduction in Macrodasysidae. Zool Ser 7: 93–114.
11. Claparède E (1867) Monographies Zoologiques. III. Type d’un nouveau genre de gastrotriches. Ann Soc Nat Zool 8: 16–23.
12. Todaro MA, Hummon WD (2008) An overview and a dichotomous key to genera of the phylum Gastrotricha. Meiofauna Mar 16: 3–20.
13. Hochberg R, Litvak MK (2000) Phylogeny of Gastrotricha: A morphology-based framework of gastrotrich relationships. Biol Bull 198: 299–305.
14. Kiencke A, Riemann O, Ahrlich WH (2000) Novel implications for the basal internal relationships of Gastrotricha revealed by an analysis of morphological characters. Zool Ser 37: 429–460.
15. Wis A, Pucciarelli S, Miceli C, Tomigori P, Balsamo M (1999) Novelty in phylogeny of Gastrotricha: evidence from 18S rRNA gene. Mol Phylog Evol 13: 314–316.
16. Petrov NB, Pegova AN, Manylov OG, Vladychenskaya NS, Mague NS, et al. (2007) Molecular phylogeny of Gastrotricha on the basis of a comparison of the 18S rRNA genes: Rejection of the hypothesis of a relationship between Gastrotricha and Nematoda. Mol Biol Evol 12: 1132–1137.
17. Todaro MA, Hummon WD (2008) An overview and a dichotomous key to genera of the phylum Gastrotricha. Meiofauna Mar 16: 3–20.
18. Hochberg R, Litvak MK (2000) Phylogeny of Gastrotricha: A morphology-based framework of gastrotrich relationships. Biol Bull 198: 299–305.
19. Kiencke A, Riemann O, Ahrlich WH (2000) Novel implications for the basal internal relationships of Gastrotricha revealed by an analysis of morphological characters. Zool Ser 37: 429–460.
20. Wis A, Pucciarelli S, Miceli C, Tomigori P, Balsamo M (1999) Novelty in phylogeny of Gastrotricha: evidence from 18S rRNA gene. Mol Phylog Evol 13: 314–316.
21. Petrov NB, Pegova AN, Manylov OG, Vladychenskaya NS, Mague NS, et al. (2007) Molecular phylogeny of Gastrotricha on the basis of a comparison of the 18S rRNA genes: Rejection of the hypothesis of a relationship between Gastrotricha and Nematoda. Mol Biol Evol 12: 1132–1137.
22. Manylov OG, Vladychenskaya NS, Milyutina IA, Korokhov NP, et al. (2004) Analysis of 18S rRNA gene sequences suggests significant molecular differences between Macrodasysida and Chaetonotida (Gastrotricha). Mol Phylog Ecol 30: 850–854.
23. Ruppert EE (1978) The reproductive system of gastrotricha. 3. Genital organs of Macrodasyidae subfam. n. and Diplodasyinae subfam. n. with discussion of reproduction in Macrodasysidae. Zool Ser 7: 93–114.
24. Claparède E (1867) Monographies Zoologiques. III. Type d’un nouveau genre de gastrotriches. Ann Soc Nat Zool 8: 16–23.
25. Todaro MA (2002) An interesting new gastrotrich from littoral meiohobenthos (Long Beach Island, USA), with a key to species of Tetanychyonema (Gastrotricha: Macrodasyida). J Mar Biol Assoc UK 82: 555–563.
26. Guidi L, Marcotta R, Pierboni L, Ferraguti M, Todaro MA, et al. (2003) Comparative sperm ultrastructure of Neodasys ciritus and Musellifis delamarei, two species considered to be basal among Chaetonotida (Gastrotricha). Zoomorphology 122: 135–143.
27. Leasi F, Todaro MA (2010) The gastrotrich community of a north Adriatic Sea site, with a redescription of Musellifis profundus (Chaetonotida: Muselliferidae). J Mar Biol Assoc UK 90: 645–654.
28. Ruppert EE (1982) Comparative ultrastructure of the gastrotrich pharynx and the evolution of myoepithelial foreguts in Aschelminthes. Zoomorphology 99: 181–220.
29. Travis PB (1983) Ultrastructural study of body wall organization and Y-cell composition in the Gastrotricha. Zool Syst Evolutionsforsch 21: 52–68.
30. Hochberg R, Litvak MK (2001) Macrodasysida (Gastrotricha): A cladistic analysis of morphology. Invertebr Biol 120: 124–133.
31. Marcotta R, Guidi L, Pierboni L, Ferraguti M, Todaro MA, et al. (2005) Sperm ultrastructure of Macrodasyidae (Gastrotricha: Macrodasysida) and a sperm-based phylogenetic analysis of Gastrotricha. Meiofauna Mar 14: 9–21.
32. Staden R (1996) The Staden sequence analysis package. Mol Biotechnol 5: 233–241.
33. Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 32: 1792–1797.
34. Abascal F, Zardoya R, Telford MJ (2010) TranslatorX: multiple alignment of nucleotide sequences guided by aminoacid translation. Nucleic Acids Res; doi: 10.1093/nar/gkq291.
35. Ronquist F, Huelsenbeck JP (2003) MRBAYES 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19: 1572–1574.
36. Huelsenbeck JP, Ronquist F (2005) Bayesian analysis of molecular evolution using MrBayes. In: Nielsen R, ed. Statistical Methods in Molecular Evolution. New York: Springer. pp 183–232.
37. HIV sequence database, accessed September, 15, 2009. (http://www.hiv.lanl.gov/content/findmodel/findmodel.html).
38. University of Oslo Bioportal accessed during September to December 2009. (http://www.bioportal.uio.no).
39. Rambaut A (2006–2008) Figtree v1.1.2. Available from http://tree.bio.ed.ac.uk. Accessed March, 2, 2008.
40. Maddison WP, Maddison DR (2010) Mesquite: a modular system for evolutionary analysis. Version 2.73 http://mesquiteproject.org.
41. Stamatakis A (2006a) Phylogenetic models of rate heterogeneity: A high performance computing perspective. Proceedings of International parallel and distributed processing symposium, HICOMB Workshop, Proceedings on CD, Rhodos, Greece (2006).
42. Stamatakis A (2008b) RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics 22: 2688–2690.
43. Shimodaira H (2002) An approximately unbiased test of phylogenetic tree selection. Syst Biol 51: 492–508.
44. Schmidt HA, Strimmer K, Vingron M, von Haeseler A (2002) TREE-PUZ:le: maximum likelihood phylogenetic analysis using quartets and parallel computing. Bioinformatics 18: 502–504.
45. Shimodaira H, Hasegawa M (2001) CONSEL: for assessing the confidence of phylogenetic tree selection. Bioinformatics 17: 1246–1247.
46. Rieger GE, Rieger RM (1977) Comparative fine structure study of the gastrotrich cuticle and aspects of cuticle evolution within Aschelminthes. Z Zool Syst Evolutionsforsch 15: 81–124.
47. Dal Zotto M, Ghiviriga S, Todaro MA (2010) A new Tetranchyroderma (Gastrotricha, Thaumastodermatidae) with triancres from the Mediterranean Sea. Meiofauna Mar 18: 41–48.
48. Lee JM, Chang CY (2003) Two new marine gastrotrichs of the genus Psychostomella (Macrotrichida, Thaumastodermatidae) from South Korea. Zool Sci 20: 481–489.
49. Hochberg R (2010) Two new species of Oregodasys (Gastrotricha: Macrotrichida, Thaumastodermatidae) from Carrie Bow Cay, Belize with ultrastructural observations of the epidermal glandular system. Zootaxa. pp 1–17.
50. Rothe BH, Schmidt-Rhaesa A (2010) Oregodasys curatus, a new species of Gastrotricha (Macrotrichida) from Terrefie (Canary Islands), with a description of the muscular and nervous system. Meiofauna Mar 16: 49–66.
51. Hummon WD, Todaro MA, Tongiorgi P (1993) Italian marine Gastrotricha: II. One new genus and ten new species of Macrotrichida. B Zool 69: 109–127.
52. Hummon WD, Todaro MA (2009) Italian marine Gastrotricha: VI. Seven new species of Macrotrichida. Zootaxa 2278: 47–60.
53. Gerlach SA (1953) Gastrotrichien aus dem Kuestengrundwasser des Mittelmeeres. Zool Anz 150: 203–211.
54. Luporini P, Magagnini G, Tongiorgi P (1970) Gastrotrichi macrodasioidei delle costa della Toscana. Pubb Staz Zool Napoli 38: 267–288.
55. Tongiorgi P, Balsamo M (1984) A new Tetranichyndrena species (Gastrotricha, Macrotrichidae) from the Adriatic coast. Boll Zool 51: 335–338.
56. Todaro MA, Balsamo M, Tongiorgi P (1992) Marine gastrotrichs from the Tuscan archipelago (Tyrrhenian Sea). I. Macrotrichida, with description of three new species. Boll Zool 59: 471–485.
57. Levi C (1950) Contribution a l’etude des gastrotriches de la region de Roscoff. Arch Zool Exp Gen 87: 31–42.
58. Remane A (1927) Gastrotrichia. In: Grimpe G, ed. Die Tierwelt der Nord- und Ostsee. Leipzig: Akademische Verlagsgesellschaft. pp 1–56.
59. Willemis WR, Currin-Galletti M, Ferreiro TJ, Fontaneto D, Heiner I, et al. (2009) Meiofauna of the Koster-area, results from a workshop at the Sven Lovén Centre for Marine Sciences (Tjärnö, Sweden), Meiofauna Mar 17: 1–34.
60. Norén M, Jondelius U (1999) Phylogeny of the Prolecithophora (Platyhelminthes) Inferrred from 18S rDNA Sequences. Cladistics 15: 103–112.
61. Telford MJ, Lockyer AE, Cartwright-Finch C, Littledow DTJ (2003) Combined large and small subunit ribosomal RNA phylogenies support a basal position of the acelomorph flatworms. P Roy Soc Lond B 270: 1077–1083.
62. Wallberg A, Currin-Galletti M, Ahmdadzadeh A, Jondelius U (2007) Dismissal of Acelomorpha: Acoela and Nemertodermatida are separate early bilaterian clades. Zool Scr 36: 509–523.
63. Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotech 3: 294–299.