An Integrated Morphological and Molecular Approach to the Description and Systematisation of a Novel Genus and Species of Macrodasyida (Gastrotricha)

M. Antonio Todaro¹ *, Matteo Dal Zotto¹,², Francesca Leasi³

¹ Department of Life Sciences, University of Modena and Reggio Emilia, Modena, Italy, ² Consorzio Interuniversitario per il Centro di Biologia Marina ed Ecologia Applicata ‘G. Bacci’, Livorno, Italy, ³ National Museum of Natural History, Smithsonian Institution, Washington, D.C., United States of America

* antonio.todaro@unimore.it

Abstract

Background

Gastrotricha systematics is in a state of flux mainly due to the conflicts between cladistic studies base on molecular markers and the classical systematisation based on morphological traits. In sandy samples from Thailand, we found numerous macrodasyidan gastrotrichs belonging to an undescribed species of difficult taxonomic affiliation. The abundance and original nature of the specimens prompted us to undertake a deep survey of both morphological and molecular traits aiming at a reliable systematisation of the new taxon.

Methodology/Principal Findings

Using several microscopical techniques we investigated the external and internal anatomy, including the muscular and nervous systems of the new species. Additional specimens were used to obtain the 18S rRNA gene sequence; molecular data was analysed cladistically in conjunction with data from additional species belonging to the near complete Macrodasyida taxonomic spectrum. Specimens are vermiform, up to 806 μm in total length, and show a well-defined head equipped with peculiar leaf-like sensorial organs and a single-lobed posterior end. The adhesive apparatus includes anterior, ventrolateral, dorsal and posterior tubes. Pharynx is about 1/4 of the total length and shows pores at its posterior 3/4. Adult specimens exhibit maturing eggs and a bulky, muscular caudal organ, but do not show sperm nor the frontal organ. Musculature and nervous system organisation resemble the usual macrodasyidan plan; however, the somatic circular muscles of the intestinal region surround all other muscular components and a third FMRFamide-IR commissure ventral to the pharyngo-intestinal junction appear to be an autopomorphic traits of the new species.
Conclusions/Significance

While the anatomical characteristics of the Asian specimens appear so unique to grant the establishment of a new taxon, for which the name Thaidasys tongiorgii gen. et sp. nov. is proposed, the result of phylogenetic analyses based on the 18S rRNA gene unites the new genus with the family Macrodasyidae.

Introduction

Gastrotricha is a phylum of aquatic microinvertebrates, which contains about 820 accepted species (as of April 2015) divided into two orders: Chaetonotida that includes tenpin-shaped, hermaphroditic and/or parthenogenetic species found in marine, brackish or freshwater habitats, and Macrodasyida, a group of vermiform, hermaphroditic species that live interstitially, mostly in marine sand (e.g., [1, 2]). Knowledge on the alpha biodiversity of the entire phylum is growing at a fast pace due to the continual description of new species (e.g., freshwater: [3–7]; marine: [8–22]), whereas recent cladistics studies challenging the phylogenetic congruence of the classical systematisation have notably increased the number of recognised genera and families [21, 23–26]. Currently, the order Chaetonotida is subdivided into 8 families and 31 genera, while the order Macrodasyida includes 10 families and 34 genera (e.g., [27]). However, the effort to make systematisation more congruent with the results of phylogenetic studies is far from completed, as best testified by the recent work on the largest family of the phylum [28].

One of the problems that makes revision difficult is that the anatomical ground patterns of taxa putatively belonging to the different evolutionary lines are still not well-known (e.g., [1, 29–32]). Since the process of re-systematisation benefits from additional surveys of insufficiently known taxa [33], the discovery of new species with novel characteristics could help to identify plesiomorphy in these morphologically diverse animals, thus providing a more solid ground for their natural grouping [1, 21].

Herein, a new interesting macrodasyidan species sampled from a beach at Phuket Island, Thailand is described. Using several microscopical techniques, namely Differential Interference Contrast (DIC), Scanning Electron (SEM) and Confocal Laser Scanning Microscopy (CLSM), we investigated the external and internal anatomy including the muscular and nervous systems. While the external morphology and traits of the reproductive system of the new species appear so unique among Gastrotricha to grant the creation of a new genus, reliable clues about its phylogenetic alliances at a higher taxonomic (family) levels only came from cladistic analyses based on the 18S rRNA gene that included all of the relevant taxa of the order Macrodasyida.

Material and Methods

Sampling

Sampling took place in February 2010 at Kata beach (Phuket island, Thailand); during high tide about 500 ml of sand was collected by hand at a depth of 0.5 m using a plastic jar [34]; thereafter, sand was kept refrigerated in an insulated bag and brought to the laboratory in Modena, Italy within 48 hrs. No special permission/permits were needed to collect these animals as gastrotrichs are microscopic, non-pathogenic organisms; field studies did not involve endangered species and sampling was carried out on a public beach.
Morphological analysis

**Gastrotrich extraction and Differential Interference Contrast microscopy.** In the laboratory, the specimens were extracted daily with the narcotisation-decantation technique using a 7% magnesium chloride solution within one week of collection; the supernatant was poured into plastic Petri dishes (3 cm diameter) and scanned for gastrotrichs at a maximum magnification of 50 x under a Wild M8 stereomicroscope [35]. When located, each individual gastrotrich specimen was mounted on a glass slide and observed in vivo with Nomarski differential interference contrast optics using a Nikon Eclipse 90i microscope. During observation, eight specimens were photographed with a DS-5M Nikon digital camera and measured using the Nikon ACT-2U software v.1.4. Additional specimens were identified and prepared for scanning electron- or confocal microscopy while three more were fixed in 95% ethanol and stored for DNA analysis.

**Scanning Electron Microscopy.** Four formalin-fixed specimens were rinsed in in 0.1 M PBS buffer, dehydrated through a graded ethanol series, critical point-dried using CO2, mounted on aluminium stubs, sputter-coated with gold-palladium, and observed with a Philips XL 30 scanning electron microscope [36].

The description of the new species follows the scheme adopted by Hummon et al. [37], where the locations of some morphological characteristics along the body are given in percentage units (U) of total body length measured from the anterior to posterior.

**Confocal Laser Scanning Microscopy.** Up to fifteen mature, relaxed specimens were incubated at 4°C for 1 hour in 4% formaldehyde solution (freshly made from paraformaldehyde in 0.1M Phosphate Buffered Saline (PBS); pH 7.4) and subsequently prepared for survey of the muscle and nervous systems. For musculature observations, five fixed specimens were washed several times with 0.1M PBS, permeabilised for 1hr in a PBT preincubation solution (0.2% Triton X-100, 0.25% Bovine Serum Albumin (BSA), and 0.05% NaN₃ in PBS 0.1M), incubated in TRITC-phalloidin (Sigma) (8 μl 38 μM solution in 200 μl preincubation solution) for 1 hour, rinsed again in PBS and embedded in 3% DABCO (Sigma, Italy) on microscope slides [38]. For surveys of the nervous system, ten fixed specimens were blocked for 2–3 h with normal goat serum (50% in PBS) at room temperature. The serum was previously deactivated at 55°C for 30 min. The specimens were then transferred to a rabbit polyclonal serotonin (5-HT) antibody or a rabbit polyclonal FMRFamide antibody together with a goat polyclonal α-tubulin antibody (Sigma-Aldrich, 1:200 PBT), and kept on an orbital shaker overnight at 4°C. After being rinsed in 0.1 M PBS, the animals were transferred to BSA (1% in) PBS and then into the secondary antibody (anti-rabbit, Alexa Fluor 488 for serotonin and FMRFamide; anti-goat Alexa Fluor 633 for x-tubulin; 1:500 in PBT) overnight at 4°C. Samples were then rinsed in 0.1 M PBT. Some of the processed animals were stained with DAPI (4′,6-diamidino-2-phenylindole; Sigma, Italy). Two additional control groups of two specimens were each processed to assess the specificity of the immune-cytochemical response; one control group had the primary antibody omitted and another was incubated with preabsorbed antibody; finally, specimens were mounted in 3% DABCO (Sigma, Italy) [39]. Observation of both the muscular and nervous systems was performed using a Leica DM IRE 2 Confocal Laser Scanning Microscope. A series of optical sections were projected in one maximum-projection (MPJ) image, or visualized as a simulated fluorescence projection (SFPJ) for three-dimensional appearance.

**Granulometry and Abundance**

Granulometric analysis of the substrata was carried out according to Todaro et al. [40]. Mean grain size, sorting coefficient, kurtosis, and skewness were calculated by a computerised programme based on the equation of Seward-Thompson & Hails [41]. The rationale for
abundance of the species among other species of a sample is as follows: rare, less than 1% of a sample; scarce, 3–5% of a sample; numerous, 10–20% of a sample (often a sub-dominant); and prevalent, more than 30% of a sample (usually dominant or co-dominant) [42].

Molecular analysis

To estimate the phylogenetic relationships of the new taxon within the order Macrodasyida, the near complete 18S rRNA sequence of 44 species (45 specimens) belonging to 24 genera within the ten currently recognised families were used (Table 1). A representative of the order Chaetonotida, Xenotrichula intermedia (Xenotrichulidae), was chosen as the out-group in the analyses. The sequences used are the same as in Todaro et al. [21].

With regard to the new taxon, DNA was extracted from a single whole specimen using the QIAamp DNA mini kit (QIAGEN), with columns from the QIAamp DNA micro kit (QIAGEN), according to the manufacturer’s instructions. The extract was then used as a template for the subsequent amplifications. A 1712 bp fragment of DNA was amplified using the 0.2 ml PuReTaq Ready-To-Go PCR beads (GE Healthcare). For amplification, 0.5 ml of each primer, 2 ml of DNA and 22 ml of purified water were assembled in the RTG-PCR tubes, yielding a final volume of 25 ml. Primer sequences and PCR-programs are from Todaro et al. [45], with the polymerase chain reactions carried out in a Biometra personal thermocycler. The PCR-products were purified using the QIAquick PCR Purification Kit (QIAGEN) according to the manufacturer’s instructions and sent for sequencing to Macrogen, Korea (www.macrogen.co.kr). Contigs were assembled using Staden v1.6.0 [48]. The 45 sequences were aligned with MUSCLE (Multiple sequence comparison by Log-Expectation), as implemented in MEGA 6 [49], using the default parameters. The data set, which consisted of 1895 nucleotide characters, was subsequently converted into both interleaved Nexus and Fasta formatted files and analysed phylogenetically using three different approaches: 1) Maximum Parsimony (MP, MEGA 6), 2) Maximum Likelihood (ML, MEGA 6) and 3), Bayesian inference (BI, MrBayes 3.1.2) [50]. For the analyses carried out with ML and BI the evolutionary model of nucleotide substitution GTR+G+I was used, which is favoured by both the AICc and the lnL criteria in MrModeltest v2.3 [51] and Mega 6. For both the ML and MP analyses, the “use-all sites” data treatment option was selected, and node support was generated using 1000 bootstrap replicates. For the Bayesian analysis, two independent runs, each with four simultaneous chains were run for 6,000,000 generations; trees were sampled every 100th generation, and posterior probabilities were determined after a burn-in of 15000 generations. A 50% consensus tree was produced with TreeView [52].

Nomenclatural acts

The electronic edition of this article conforms to the requirements of the amended International Code of Zoological Nomenclature, and hence the new names contained herein are available under that Code from the electronic edition of this article. This published work and the nomenclatural acts it contains have been registered in ZooBank, the online registration system for the ICZN. The ZooBank LSIDs (Life Science Identifiers) can be resolved and the associated information viewed through any standard Web browser by appending the LSID to the prefix “http://zoobank.org/”. The LSID for this publication is: urn:lsid:zoobank.org:pub:D2086FAC-1B6F-4DA4-B080-702A05BE9E9B. The electronic edition of this work was published in a journal with an ISSN, and has been archived and is available from the following digital repositories: PubMed Central, LOCKSS.
Table 1. Gastrotrich taxa involved in the molecular analyses. Origin, reference and GenBank accession number are provided.

| Taxon                        | Origin          | Reference | Accession |
|------------------------------|-----------------|-----------|-----------|
| Cephalodasyidae              |                 |           |           |
| Cephalodasys sp              | White Sea, Russia | [43]     | AY963691 |
| Dolichodasys sp              | San Isidoro, Italy | [44]    | AM231778 |
| Mesodasys laticaudatus       | Albinia, Italy  | [45]     | JF357657 |
| Mesodasys littoralis         | Bou Ficha, Tunisia | [45]   | JF357658 |
| Paradasys sp.                | Ionian sea, Italy | [44]  | AM231781 |
| Pleurodasys helgolandicus    | Ibiza, Spain    | [24]     | JN203486 |
| Dactylopodolidae            |                 |           |           |
| Dactylopodola cf. baltica    | Ras Alard, Kuwait | [45]    | JF357650 |
| Dactylopodola mesotyphle     | Punta Ala, Italy | [45]    | JF357651 |
| Dactylopodola typhle         | Bou Ficha, Tunisia | [45] | JF357652 |
| Dactylopodola typhle         | Torre Civette, Italy | [45] | JF357653 |
| Hummondasyidae              |                 |           |           |
| Hummondasys jamaicensis      | Negril, Jamaica | [21]     | KM083602 |
| Lepidodasyidae              |                 |           |           |
| Lepidodasys unicarenatus     | Pianosa, Italy  | [45]     | JF357655 |
| Macrodasyidae               |                 |           |           |
| Macrodasys sp. 1             | Torre Civette, Italy | [45] | JF357654 |
| Macrodasys sp. 2             | Bohuslän, Sweden | [45]    | JF357670 |
| Thaidasys tongjorgii         | Phuket island, Thailand | Present study | KR072683 |
| Urodasys sp.                 | NA              | [46]     | AY218102 |
| Urodasys sp.1                | Florida, USA    | [47]     | DQ079912 |
| Planodasyidae               |                 |           |           |
| Crasiella sp.                | Ilha Bela, Brazil | [24]    | JN203488 |
| Megadasys sp.                | Grotta del Ciolo, Italy | [45] | JF357655 |
| Megadasys sp. 1              | Porto Cesareo, Italy | [45] | JF357656 |
| Redudasysidae               |                 |           |           |
| Anandrodasys agadasys        | St. John Island, USA | [24] | JN203487 |
| Redudasys fornerise          | Represa do Broa, Brazil | [24] | JN203489 |
| Thaumastodermatidae         |                 |           |           |
| Acanthodasys sp. a           | Capraia, Italy  | [45]     | JF357638 |
| Acanthodasys aculeatus       | Capraia, Italy  | [45]     | JF357639 |
| Diplodasys ankei             | Meloria, Italy  | [45]     | JF357624 |
| Diplodasys meloriae          | Meloria, Italy  | [45]     | JF357640 |
| Oregodasys ocellatus         | Meloria, Italy  | [45]     | JF357642 |
| Oregodasys ruber             | Meloria, Italy  | [45]     | JF357625 |
| Oregodasys tentaculatus      | Meloria, Italy  | [45]     | JF357626 |
| Pseudostomella etrusca       | Albinia, Italy  | [45]     | JF357633 |
| Ptychostomella lameliphora (= sp1) | Ilha Bela, Brazil | [45] | JF357643 |
| Ptychostomella tyrhenica     | Albinia, Italy  | [45]     | JF357634 |
| Tetranchyroderma papii       | Sardegna, Italy | [45]    | JF357637 |
| Tetranchyroderma esarabdomophorum | Mahdia, Tunisia | [45] | JF357627 |
| Tetranchyroderma hirtum      | Capraia, Italy  | [45]     | JF357628 |
| Tetranchyroderma thyisanophorum | Albinia, Italy | [45] | JF357630 |
| Thaumastoderma moebjergi     | Bohuslän, Sweden | [45] | JF357671 |

(Continued)
**Table 1. (Continued)**

| Taxon                        | Origin          | Reference | Accession |
|------------------------------|-----------------|-----------|-----------|
| *Thaumastoderma ramuliferum* | Meloria, Italy  | [45]      | JF357631  |
| *Paraturbanella dohrni*      | Punta Ala, Italy| [45]      | JF357659  |
| *Paraturbanella pallida*     | Capraia, Italy  | [45]      | JF357660  |
| *Paraturbanella teissieri*   | Punta Ala, Italy| [45]      | JF357661  |
| *Turbanella bocqueti*        | Tramore, Ireland| [45]      | JF357662  |
| *Turbanella comuta*          | Chioggia, Italy | [45]      | JF357663  |
| *Turbanella lutheri*         | Torö, Sweden    | [45]      | JF357669  |
| *Xenodasyidae*               |                 |           |           |
| *Xenodasys riedli*           | St. John Island, USA | [24] | JN203490  |
| *Xenotrichulidae*            |                 |           |           |
| *Xenotrichula intermedia*    | Mahdia, Tunisia | [45]      | JF357664  |

* Order Chaetonotida; NA, Data not available.

doi:10.1371/journal.pone.0130278.t001

**Results**

**Taxonomic treatment**

Order *Macrodasyida* Remane, 1925 [Rao & Clausen, 1970]
  
  Family *Macrodasyidae* Remane, 1924
  
  *Genus Thaidasys* gen. nov.
  
  urn:lsid:zoobank.org:act:4920EC03-B601-4C6E-80B3-C90354097FCA
  
  **Diagnosis.** Body elongate, up to 806 μm in total length (LT), and rather narrow, up to 68 μm in width, flattened ventrally and vaulted dorsally, with numerous epidermal glands. Cuticular covering smooth, devoid of scales and/or spines. Head consisting of a well demarcated anterior region showing a peculiar leaf-like structure on each posterior side, and a posterior region, hosting the brain, comprised between two evident constrictions. Posterior body region unilobed, ovoidal in shape. Sensory hairs arranged singly in lateral and dorsolateral columns along the body, sparsely on the lateral sides of the head but forming a dense, semi-circular fringe on the head. Ventral locomotor ciliature in the form of two bands running separately from under the head to the posterior trunk region; cilia in the bands appear rather sparse especially in the rear end. Anterior adhesive tubes (TbA), 2–3 per side, forming diagonal columns, which insert directly on the body surface and project forward; ventral adhesive tubes (TbV), absent; ventrolateral adhesive tubes (TbVL), up to 24 per side; five-six along the pharyngeal region and the remaining along the intestinal region; dorsal adhesive tubes (TbD), up to 15 per side, three of which are present on the posterior half of the pharyngeal region; dorsolateral adhesive tubes (TbDL), absent; posterior adhesive tubes (TbP), up to 6, surrounding the caudum. Mouth terminal, of mid-size (up to 15 μm in diameter), leading to a short buccal cavity (8–10 mm in length), which opens into a 192–209 μm long and 15–22 μm wide pharynx; pharyngeal pores far-off from the base with ventrolateral openings. Pharyngo-intestinal junction (PhiJ) at about U26. Intestine increases in width from the PhiJ to mid-body and gradually narrows toward the posterior body end; anus ventral at U96. Testes and/or sperm absent/not seen. Female gonad unpaired, in the second third of the trunk, showing oocytes maturing in a caudo-cephalic direction with largest egg dorsal to the mid intestine. A noticeable muscular organ in the posterior trunk region (U83), following the ovary is present. The structure is
ventral to the intestine, tube-like, up to 76 μm in length and 24 μm in width, with a strongly muscularised wall and a throughout canal, 6–8 μm in diameter; it is interpreted as the caudal organ.

**Etymology.** The genus is named after the country where these animals were first found

*Thaidasys tongiorgii* sp. nov.
urn:lsid:zoobank.org:act:26CF6C3E-5975-450F-82A5-E7333E99732B (Figs 1–11)

**Diagnosis.** Same as the genus

**Etymology.** The species is named after Paolo Tongiorgi, master and friend, in recognition of his valuable contributions to the field and the endless support to the Italian Gastrotricha research group. The suffix “-dasys” is traditionally used in most genera of macrodasyidan gastrotrichs and alludes to their dense ciliation.

**Examined material.** The description of *Thaidasys tongiorgii* gen. et sp. nov. is mainly derived from eight specimens, seven adults and a single juvenile, observed under DIC optics. The holotype, LT = 782 μm, is illustrated in Fig 2A and 2B (International Code of Zoological Nomenclature, Articles 73.1.1, 73.1.4). After observation the physical specimen was fixed in 95% ethanol and later used for DNA analysis (GenBank accession Number KR072683).

The juvenile and the six additional studied adults are no longer extant. Some details about the external morphology are derived from two adults observed with a SEM. Data about musculature and the nervous system came from fifteen (5 + 10) adults observed with CLSM. Three further identified specimens were fixed in alcohol and are kept in the author’s collection together with the SEM prepared specimens. At least fifteen more specimens were examined, in vain, for sperm and/or the frontal organ, and subsequently discarded.

**Type locality.** The sediment sample was collected on 1st February 2010 from the south side of Kata beach, along the South-western coastline of Phuket Island, Thailand (Lat. 07° 48’ 12.35” N; Long. 98°17’55” E).

**Ecology.** Numerous in abundance (95% of a sample); intertidal at a water depth of 0.5 m in fine sand (2.34 phi), moderately well sorted (0.94 phi) carbonate sand (kurtosis = 3.43; skewness = -0.64). Values of salinity and temperature of the interstitial water at the time of sampling were 22‰ and 28°C respectively.

**Description.** Based mostly on the adult specimens with a total body length of 782 μm shown in Fig 2. Body elongate and rather narrow, flattened ventrally and vaulted dorsally, with gently undulating sides due to the presence of numerous epidermal glands; cuticular covering smooth, devoid of scales and/or spines (Figs 1A, 1B, 2A, 3D and 5A). Body attaining the maximum width in the mid- to hindgut, and then narrowing again to an ovoidal caudum (Figs 1A, 1B, 2A, 2B and 5A, 5E). Head consisting of two distinct portions. The anterior one, roughly trapezoidal, with rounded angles, appears clearly demarked by a posterior constriction and with a pair of peculiar leaf-like structures, interpreted as sensory organs at U03; it bears sparse sensory cilia on the lateral side, but neither piston pits nor eye spots are present (Figs 1, 2A, 2C, 3B and 4). The posterior portion of the head hosts the brain (see below), it appears slightly inflated and delimited to the rear by a second constriction at U10.

Widths of head,mid-pharyngeal region,mid-trunk\posterior-trunk are as follows: 41\48\64 \60 μm at U03\U13\U62\U94, respectively.

Epidermal glands: up to 30 pairs of noticeable epidermal glands irregularly spaced from the posterior end of the head and along the pharyngeal and intestinal regions (from U03 to U94; Figs 1B and 2A); glands are variable in shape, from round to elliptical, and range in size from 9 to14 μm in width and 10–22 μm in length (Fig 2A and 2B); external pores not discernible under DIC optics but visible on the dorsal side under SEM (Fig 4D).
Ciliation: Sensory hairs, up to 22 μm in length, arranged in lateral and dorsolateral columns that are regularly spaced along the body with others, 14–24 μm in length, loosely packed more along the lateral sides of the head. Additional sensory hairs, 15–24 μm in length, forming a dense, semi-circular fringe on the dorsal side of the head (Fig 1).

The ventral locomotory ciliation is in the form of two bands that run separately from under the head (U02) to the posterior end of the trunk (U94). Individual cilia are 12–16 μm in length and appear sparsely packed, especially in the posterior region (Fig 3A).

Adhesive tubes: TbA, 3 per side, 5–8 μm long, forming diagonal columns on each side, and inserting directly on the body surface, just posterior to the oral opening from U01 to U02 (Figs 1C and 3A); TbV, absent; TbVL, 20 per side, 8–10 μm long; six along the pharyngeal region and the remaining along the intestinal region (Fig 1A and 1B); TbD, up to 14 per side, 4–6 μm long, two of which along the posterior half of the pharyngeal region (Fig 1A and 1B); TbDL, absent; TbP, 5–6 in total, 6–10 μm long, surrounding the posterior edge of the caudum (Fig 1A, 1B and 2A).

Digestive tract: Mouth terminal, of medium size (15 μm in diameter, Fig 4A), leading to a short buccal cavity (8 μm in length, Fig 3B); pharynx is 192 μm long, measured from the frontal edge of the head and slightly increasing in width from anterior (15 μm) to posterior (22 μm); pharyngeal pores far from the base at U22, with ventrolateral openings (Figs 1B, 2C and 3C). Pharyngo-intestinal junction at about U26 (Fig 2C). Intestine increases in width from the PhIJ to mid-body and gradually narrows up to the posterior body end; anus ventral at U96 (Fig 1B).

Reproductive tract: Testes and/or sperm absent. Female gonad unpaired, in the second third of the trunk, showing oocytes maturing in a caudo-cephalic direction with largest egg, 128 μm long and 56 μm wide, dorsal to the mid intestine, centered at U62 (Figs 1B, 2A, 2B and 4A). A putative accessory sexual organ in posterior trunk region (U83) is present (Figs 1B, 2A, 2B and 4). The bulky structure is ventral to the intestine, tube-like, up to 76 μm in length and 24 μm in width, with a strongly muscular wall and a canal, 6–8 μm in diameter, running through its length. The canal contains homogeneous, finely-grained material packed in the form of a cigar-like structure whose posterior end appears connected to a sclerotized, hook-like process (Fig 4). In very compressed animals the tip of the sclerotized process protrudes externally from the ventral side. The whole organ is best interpreted as the caudal organ present in other Macrodasyida (see discussion below). Frontal organ absent.

Musculature: The muscular system of T. tongiorgii gen. et sp. nov. consists of muscles, likely obliquely striated, arranged in internal circular, longitudinal, and helicoidal fibres in the splanchnic compartment, and external somatic longitudinal muscles (Figs 6 and 7). The putative caudal organ (see below) comprises numerous thin fibres spirally arranged (Figs 6C, 6E and 2F). The myoepithelial sucking pharynx is surrounded by numerous, serially arranged visceral muscle rings (1 μm width); outside those are visceral ventral (1–2 μm width) and dorsal (1–2 μm width) longitudinal muscles that stretch along the whole gut tube from the mouth opening to the posterior end (Figs 6A, 6B, 6D, 6E and 7). Two helicoidally arranged muscles insert in the mouth rim (1 μm width; Figs 6B and 7A, 7B) and surround the longitudinal fibres along the pharynx, up to the pharyngo-intestinal junction crossing seven times; no helicoidal muscles are seen in the intestine region (Fig 6A and 6B). Each pharyngeal ventral pore is
surrounded by a thin sphincter muscle (1 μm width; Fig 6A). In the intestinal region, posterior to the PhIJ, the visceral longitudinal muscles are surrounded by regularly spaced visceral muscle rings (Fig 7C). Hence, the spatial arrangement of the splanchnic longitudinal and circular muscles is inverted from that of the pharyngeal region that is circular muscles innermost along
Fig 3. *Thaidasys tongiorgii* gen. et sp. nov. Differential interference contrast photomicrographs. A, close-up of the anterior region, ventral view, showing the locomotor ciliation and the anterior adhesive tubes (arrow); B, close-up of the internal view of the anterior region of a different specimen, showing the pharynx, pharyngeal pores (arrows) and pharyngo-intestinal junction (arrowhead). C, D. Posterior region of the trunk of two subadult specimens, showing the caudal organ (arrows) at different development stages. E, a juvenile. Scale bars A, B, E = 100 μm, C, D = 50 μm.

doi:10.1371/journal.pone.0130278.g003
the pharynx, outermost along the intestine (Fig 7). Thicker somatic ventrolateral paired muscles (2–3 μm width) insert in the mouth rim, extend along the entire body to the rear end, where they join the splanchnic longitudinal muscles (Figs 6A, 6B, 6D, 6E and 7).
Somatic circular muscle (1 μm width) were detected along the intestinal region, especially in the area of the caudal organ; these muscles appeared as serially arranged complete (closed) rings surrounding the somatic longitudinal muscles and all other muscular components (Figs 6E, 6F, and 7C). The caudal organ, ventral to the intestine, shows a series of numerous (at least 70) and thin bands (1 μm width) of spirally arranged muscles enwrapping its canal (Fig 6E and 6F).

Nervous system: Staining of the nervous system of *T. tongiorgii* gen. et sp. nov. with DAPI and antibodies against tubulin, serotonin, and FMRFamide revealed a general pattern
consisting of 1) a cerebral ganglion involving at least 80 cells, 2) peripheral ventrolateral nerve cords, and 3) anterior sensory structures (Figs 8–11). The cerebral ganglion is centered at about U07; it occupies the posterior region of the head located between the two anterior body constrictions (Figs 8A, 9A, 10B and 11A).

Tubulin immunoreactivity shows the cerebral ganglion as a pair of cluster of dorsal somata connected by a thick commissure. Neurites extend from the commissure in both anterior and posterior directions (Figs 8A, 10B and 11). The posterior neurites extend as the ventrolateral nerve cords that fuse at the posterior end (Figs 8A, 9, 10B and 11). Several perikarya are present along the length of the cords (Figs 8A and 9C).

A strong tubulin-IR signal is also visible at the anterior end in the sensory cilia and in the paired leaf-like organs (lo). Leaf-like organs appeared to be connected by a dorsal commissure, which comprises several perikarya (Figs 10B and 11A, 11B). The anterior sensory structures (cilia and leaf-like organs) are likely linked to the posteriorly located cerebral ganglion by neurites, even though this connection was not visualised in our stainings (Fig 9A and 9B).
Serotonin and FMRFamide immunoreactive structures are generally detectable in the same regions of tubulin, namely in the cerebral ganglion and ventral nerve cords, with some differences (Figs 8–11). Neither serotonin nor the FMRFamide immunoreactive staining was identified anteriorly in the region of the sensory structures. Scattered and inconsistent positive signal along the body was detected at the level of the cuticle, and interpreted as autofluorescence.

The serotonergic nervous system consists of three anterior pairs of somata in the region of the cerebral ganglion. One pair is located dorsally and is connected by fibres to form a dorsal cerebral commissure (26.5 μm length, 1 μm width; Figs 8B, 8C and 9A, 9B). From the dorsal commissure extends two paired longitudinal neurites (Figs 8B and 9A, 9B) that run slightly anteriorly and ventrally until they join two paired serotonin-IR somata, located ventral to the pharynx (Figs 8B and 9A, 9B). These latter neurons extend paired parallel ventrolateral cords (Figs 8A, 8B and 9) that run until joining at the posterior end to form a posterior ventral commissure (Figs 8A and 9C). Several serotonin IR perykarya (1.3–2 μm each) are seen along the ventrolateral cord (e.g. Fig 8B). A weak immunoreactive signal is also detected in a neurite.
located in the stomatogastric compartment, namely in the central region of the pharynx, from the cerebral ganglion to the pharyngo-intestinal junction (Fig 9A).

FMRFamide immunoreactive staining is present in the cerebral ganglion, along the ventrolateral cords, and in the stomatogastric system (Figs 10 and 11). The cerebral ganglion has paired clusters of 5–7 IR cells (Figs 10C and 11A, 11B) connected by three consecutive cerebral dorsal commissures, the central of which appear broader. (Figs 10C and 11A). The lateral ends of the broad commissure extend neurites to paired ventral, slightly anterior somata (Figs10C and 11A, 11B), which extend a short posterior neurite to connect additional paired ventral somata (Figs 10C and 11A, 11B). These latter FMRFamide-IR cells are connected with a cerebral ventral commissure and are the origin of the longitudinal ventrolateral nerve cords (Figs 10 and 11). The ventrolateral cords merge in a loop in the rear end and are connected by two FMRFamide-IR peripheral commissures in the anterior region of the body: 1) just past the head, at the level of the first pair of adhesive tubes and, 2) at the base of the pharynx (Figs 10A, 10B and 11A). There are also four very thin FMRF-IR neurites in the splanchnic compartment. Three neurites are visible in the anterior region of the body and one is located in the posterior region. Of the anterior neurites, one is longer and extends medially from the cerebral ventral
commissure to the pharyngo-intestinal junction (anterior ventromedial neurite), the other two (anterior ventrolateral neurites) are shorter and extend paired from the cerebral ventral commissure to the first commissure of the peripheral system (Figs 10A, 10B and 11A). The posterior neurite (posterior ventromedial neurite) is visible in between the end of the caudal organ and the ventral posterior commissure (Figs 10A and 11C).

Variability and remarks on general morphology. The total body length of the measured adult specimens (i.e. showing a large egg and/or a fully structured accessory sexual organ) ranged from 710 μm to 806 μm (mean = 806 μm ± 33.8 SD, n = 8); maximum body width varied from 55 μm to 68 μm (mean = 61 μm ± 5 SD, n = 8). The number and, to a lesser extent, the arrangement of the adhesive tubes belonging to the different series varied among the observed
DNA-based phylogenetic analysis

The final rDNA dataset included 1895 alignable positions, 977 of which are constant, and 707 parsimony-informative. The three phylogenetic analyses, carried out with ML, MP and
Bayesian approaches, yielded topologies that were congruent with each other, with most of the groups that are in common bearing high nodal support: i.e. bootstrap and Bayesian posterior probability values >70 and 98%, respectively (Figs 12–14). The robustly supported groups include: 1) the densely sampled families Thaumastodermatidae and Turbanellidae and their recognized subgroupings; 2) the recently highlighted alliance between Redudasys fornerise Kisielewski, 1987 and Anandrodasys agadasys Hochberg, 2003 (Fam. Redudasyidae, see [24]) and their sister-group relationship with a clade composed of Cephalodasys and Dolichodasys (Fam. Cephalodasyidae); and 3) the sister-group relationship between Crasiella Clausen, 1968.
and Megadasys Schmidt, 1974, recently united in the family Planodasyidae [33]. In contrast, the currently recognised Macrodasyidae and Cephalodasyidae never appear as monophyletic due to the scattering along the evolutionary tree of their respective species and/or the alliances between members of different families. Genera represented by two or more species were also recovered as monophyletic in the analyses (except Ptychostomella Remane, 1926 and Tetranchyrodema Remane, 1926). With regard to the new species from Thailand, all of the three analyses strongly indicate (bootstrap ≥ 87, Bayesian posterior probability = 1) a sister-group relationship with the clade containing the two species of Macrodasys Remane, 1924 (Fam. Macrodasyidae) involved in the study (Figs 12–14). Finally, the inclusion of the Thaidasys tongiorgii gen. et sp. nov., did not produce a consistent find a steady position for the recently described Hummondasys jamaicensis Todaro, Leasi & Hochberg, 2014 (Fam. Hummondasyidae, see [21]) in the Macrodasys phylogenetic tree.

Discussion

Diagnostic features and morphology

The metric and meristic information reported for the examined specimens (see above) testify to the variability of some traits (e.g., number and distribution of the adhesive tubes) and highlight the fact that variations are only in part related to the specimen size. With regard to the reproductive system, it seems that the muscular organ begins to form first, at a total length of about 550 μm, while an ovary with distinct oocytes becomes visible when an individual reaches a total length of about 700 μm.

Allowing for the high number of individuals examined, of different sizes/ages, the absence of sperm appears to be the normal condition of T. tongiorgii gen. et sp. nov. Within Macrodasyida, the absence of spermatozoa was a phenomenon unreported before in specimens provided with accessory reproductive structures. Sperm are absent in parthenogenetic taxa that lack such structures i.e., Anandrodasys agadasys Todaro, Dal Zotto, Jondelius, Hochberg, Hummon, Kånneby & Rocha, 2012, Redudasys fornerise Kisielewski, 1987 and Urodasys viviparus Wilke, 1954.

It could be argued that perhaps the muscular organ of T. tongiorgii gen. et sp. nov. is not a sexual accessory structure. However, the morpho-functional anatomy of the Gastrotrocha, and common sense, suggest otherwise. Therefore, we believe that the massive muscular organ found in T. tongiorgii gen. and sp. nov. is most likely an accessory reproductive organ because 1) no other structure have been reported in Macrodasyida except for the accessory sexual organs [1], 2) the absence of any alternative hypotheses regarding its function.

The position, in the posterior trunk and ventral to the intestine, shape, and especially the structure (thick muscular wall) lead us to consider the muscular organ found in the new species comparable to the caudal organ present in other Macrodasyida. For instance, a caudal organ similar in many respects to the one present in T. tongiorgii gen. et sp. nov. has been recently described by Hochberg et al. [8] for the Brazilian species Lepidodasys ligni Hochberg, Atherton & Gross, 2013. Possible uses of a copulatory organ in animals that lack spermatozoa remains unknown.

The vermiform appearance, along with the cuticular covering made up of only smooth cuticle (i.e., absence of spines and/or scales) and the adhesive tubes of the anterior series (TbA)
originating singly and directly from the body surface, make the gastrotrichs from Thailand most similar to some members of the families Cephalodasyidae (i.e., *Dolichodasys* Gagne, 1977 and *Mesodasys* Remane, 1951), Planodasyidae (i.e., *Megadasys* Schmidt, 1974), and to some extent to the recently described *Hummondasys jamaicensis*. In contrast with the new species and members of the taxa mentioned above, *H. jamaicensis* possess a posterior body region that is shaped in the form of two caudal pedicles, a trait that makes it clearly distinct from the others [21]. At a level of external anatomy, several differences also emerge between the new species and members of *Dolichodasys*, *Mesodasys* and *Megadasys*, making it difficult to affiliate the Thai species to one of these taxa. *Dolichodasys*, for example, is characterized by a single TbA per side (vs. 3) and lateral adhesive structures in form of papillae (vs. tubes); *Mesodasys* possesses many TbA regularly arranged over the post oral region (vs. 3 tubes per side, arranged in columns) while *Megadasys* possess TbAs that appear as the anterior continuation of the adhesive tubes of the TBVL series and, most importantly, shows a posterior end in the form of a large lobe surrounded by many adhesive tubes arranged in a fan-like fashion (vs. 5–6 tubes at the posterior edge shown by the new species).

Clear-cut differences also exist between the new species and taxa mentioned with regard to the reproductive apparatus. These differences go well beyond the simple absence of testicles and sperm in the new species. For instance, *Dolichodasys* shows a single ovary but the eggs develop in an antero-posterior direction, opposite to that in the new species; *Megadasys* possesses a pair of ovaries while the new species has a single ovary. Furthermore, both *Dolichodasys* and *Megadasys* bear two accessory reproductive organs (i.e., frontal- and caudal organ) vs. a single organ noted in the new species. *Mesodasys*, similar to the new species, has a single ovary and a single accessory organ; however, the sexual accessory organ present in *Mesodasys* (i.e., caudal organ) is directly connected to the sperm ducts and its wall does not appear to be muscular, which is in contrast with the heavily muscularised wall of the caudal organ present in the new species.

The leaf-like sensorial organs at the posterior lateral sides of the anterior region of the head, and the pharyngeal pores located far off from the pharyngeal base are additional characters that further differentiate the Thai gastrotrichs from all the species belonging to the genera mentioned above, and, when combined, to all the other gastrotrich species known to date.

In conclusion, traits of the external morphology along with the simplified reproductive system call for both the erection of a new species and a new genus for the specimens from Phuket island; consequently, the name *Thaidasys tongiorgii* gen. et sp. nov. is proposed for the new taxon.

Unfortunately, the morpho-functional traits, inclusive of the muscular and nervous systems, of *Thaidasys tongiorgii* gen. et sp. nov. do not permit the formulation of a preferred hypothesis concerning the affiliation of the new genus to any of the currently recognised macrodasyidan families (e.g., [27]).

Guidi et al. [33] and Todaro et al. [21] highlighted the special relevance that may assume the layout of the reproductive system in the systematisation of the Gastrotricha at a rank of family. In this framework, the simplified reproductive system of the new taxon does not offer robust clues about its potential phylogenetic alliances. In fact, the simplified system of *T. tongiorgii* gen. et sp. nov. may be equally interpreted as the result of an evolutionary reduction from one or the other of the different systems found for example in Cephalodasyidae and

---

**Fig 13.** Phylogenetic relationships of *Thaidasys tongiorgii* gen. et sp. nov. inferred from Maximum likelihood analysis of 18S rRNA. The analysis includes 45 Gastrotricha Macrodyasida and the outgroup is represented by *Xenotrichula intermedia* (Chaetonotida, Xenotrichulidae). The tree with the highest log likelihood (-19946.9339) is shown. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Number at nodes represents bootstrap values (1000 replicates).

doi:10.1371/journal.pone.0130278.g013
Planodasyidae, but also in Macrodasyidae, whose members share with the new species the presence of muscular caudal organ (for a review see [1]).

On the other hand, based solely on morphology, it cannot be completely ruled out that such a simplified system may have an alternative origin, including a derivation from an as yet unknown phylogenetic line; a circumstance that could justify the erection of a new family for the new species.

Muscular and nervous systems

The muscular system of *Thaidasys tongiorgii* gen. et sp. nov. is organised in longitudinal, circular and helicoidal orientation, and it resembles the general arrangement of the musculature so far described for other gastrotrich species (reviewed in [1]).

According to Hochberg [53], the character pattern that was probably present in the last common ancestor of Gastrotricha consists of muscle strands in three different orientations: circular, helicoidal and longitudinal in a splanchnic position, and circular and longitudinal in a somatic compartment. Each arrangement might be present, absent, or differently quantified according to the species. Like other gastrotrichs investigated so far, the new species possesses visceral (= splanchnic) musculature organised as inner circular and external (both ventral and dorsal) longitudinal muscles along the pharynx, and inner longitudinal muscles surrounded by circular rings along the intestine. Helicoidal muscles are present along the pharynx, whereas they are absent posteriorly to the pharyngo-intestinal junction, a condition shared with *Macrodasys caudatus* and *Turbanella ambronensis* [54].

In the somatic compartment, *T. tongiorgii* gen. et sp. nov. displays paired longitudinal muscles in a ventrolateral position (*musculus principalis* according to Remane [55]). They insert anteriorly in the mouth rim and run posteriorly past the anus. The insertion in the mouth rim is a condition shared with other taxa that bear TbAs very close to the mouth e.g., *Lepidodasys*, *Macrodasys*, and Thaumastodermatidae. In contrast, species that possess TbAs inserted at some distance from the mouth rim e.g., *Dactylopodola*, *Dolichodasys*, *Paradasys* and and *Turbanella*, possess ventrolateral muscle bands that insert near to where the tubes originate (see [1] for a review).

Somatic circular fibres were seen along the intestinal region but not in the pharyngeal region. As somatic muscles arranged in rings along the entire digestive tube have been described for all macrodasydians except the Thaumastodermatidae (but see [56]), it is possible that in the new species the somatic circular muscles of the pharyngeal region were not visible due to their reduced thickness and to the strong signal of the pharynx musculature. In Macrodasysidae somatic circular musculature along the intestinal region are reported to enclose the ventrolateral muscle bands on either side of the midgut (e.g., [54]). This view has recently been challenged by Kienke and Schmidt-Rhaesa [1] according to whom the somatic circular muscles surround the somatic longitudinal muscles and probably all the other muscular component. Our finding supports Kienke and Schmidt-Rhaesa’s hypothesis and call for additional studies to clarify the issue.

*T. tongiorgii* gen. et sp. nov. possesses a distinct caudal organ, which is supplied and surrounded by slightly spirally arranged musculature that define an internal canal. Such muscular
arrangement in the caudal organ is known to be present in several other species e.g., in *Lepidodasys ligni*, *Macrodasys* sp. and in *Tetranchyroderma papii* [8, 54, 57]. Muscle contractions of the caudal organ are used to support the release of spermatozoa from the caudal organ lumen or, as in *Macrodasys* spp., to evert the copulatory tube (reviewed in [1]). The caudal organ of *T. tongiorgii* gen. et sp. nov. appears to be a combination of both the caudal organ of *L. ligni* and *Macrodasys* spp. For instance, with the caudal organ of *L. ligni*, it shares a lumen that is open at both ends, while like the caudal organ of *Macrodasys* spp. contains a putative copulatory tube (see above).

The nervous system of *T. tongiorgii* gen. et sp. nov. resembles what has been described for other gastrotrichs, and namely it consists of a bilateral brain (cerebral ganglion) that encircles the dorsal and lateral anterior regions of the pharynx, and which is connected to a pair of peripheral ventrolateral cords and anterior sensory structures (reviewed in [1] and [58]).

The serotonergic system in macrodasyidan gastrotrichs is known in species of the genera *Dactylopodola, Dolichodasys, Macrodasys* and *Turbanella*, [30, 59, 60]. In all species, as in *T. tongiorgii* gen. et sp. nov., the serotonergic system is generally present as a pair of cell clusters in the cerebral ganglion connected by a dorsal commissure. These cells project neurites to ventral cells, which project off a paired ventral serotonergic nerve running along the entire body until the posterior end. An exception is *Macrodasys*, which apparently does not show a Serotonin-IR signal in the dorsal commissure [30]. However, as information of *Macrodasys* are based on epifluorescence microscopy, investigation using the more powerful confocal microscopy are needed to confirm the absence of serotonin in the cerebral dorsal commissure of this taxon.

In *T. tongiorgii* gen. et sp. nov., a paired serotonin-IR cell was present in the dorsal region of the cerebral ganglion (Fig 9A). These two cells were connected by a dorsal commissure and extended serotonergic fibres to two paired somata located in the ventral side. These ventral cells extended neurites posteriorly to establish a paired ventrolateral serotonin-IR nerve cord, which coalesced posteriorly. In *Dactylopodola baltica, D. typhle* and *Turbanella cornuta* there are two paired serotonergic cells connected by a commissure in the dorsal region of the cerebral ganglion, whereas *Dolichodasys elongatus*, *Macrodasys caudatus*, *T. ambronesis* and *T. hyalina* possess, similarly to the new species, only one paired serotonin immunoreactive cell in the same region [30, 60, 61]. Connection of the dorsal somata to ventral perikaria from which neurites extend as the paired ventrolateral nerve cord is a condition that the new species shares solely with *Dactylopodola* [60].

However, as the number of perykaria that are serotonin-IR might 1) depend on the microscopy technique (epifluorescence vs. clsm; cfr. [30] vs. [60] for *Dactylopodola baltica*), 2) vary across species, and 3) also among individuals of the same species [60], it is difficult to ascertain potential homologies.

*T. tongiorgii* gen. et sp. nov. bears the putative ancestral character state of the absence of a ventral serotonin-IR commissure in correspondence of the dorsal one and, as such, it is in particular contrast with *Turbanella* species that do have this ventral commissure [61].

In gastrotrichs, the FMRFamide immunoreactivity has, in general, a wider distribution along the nervous system compared to the serotoninergic component. This is also true for *T. tongiorgii*, gen. et sp. nov. In the cerebral ganglion of the new species there are three dorsal commissures that are FMRFamide immunoreactive, a condition shared with *Lepidodasys* and *Turbanella* while e.g., *Dactylopodola* and *Xenodasys* possess only two dorsal commissures [60, 62, 63].

The presence of FMRFamide immune reactivity in a commissure ventral to cerebral ganglion is a characteristic common to all gastrotrichs including the new species. On the other hand, *T. tongiorgii* gen. et sp. nov. appears unique in that it shows two additional ventral commissures posterior to the brain: one is located just past the head at about U10, and the other at
the level of the pharyngo-intestinal junction at U26. It should be emphasized that a second ventral commissure, not too far from the first one, is also present in Dactylopodola [60], but it is difficult to say whether it is a homologous condition or not.

The paired nerve cord of T. tongiorgii gen. et sp. nov. is FMRFamide imunoreactive like all other gastrotrichs investigated so far. However, some differences appear evident. The new species displays a single pair of ventral neurites that project off the brain and run posteriorly the length of the body, whereas Lepidodasys and Turbanella species possess a pair of additional ventral fibres that project off the brain and reach the anterior end of the body. On the other hand, Dactylopodola possesses two paired ventral cords and several anterior projections while Xenodasys shows four pairs of nerve cords [60, 63].

In the splanchnic compartment, the new species displays three neurites located in the pharynx and a single neurite that extends ventrally from the end of the genital organ to the posterior end. FMRFamide immune reactivity is widely present in the splanchnic compartment of all macrodasyidans, both in the anterior and posterior body region; however, the general weak and scattered staining might also display different conditions among individuals of the same species, hence, homologies are difficult to determine.

Importantly, we did not perform simultaneous stainings with serotonin and FMRFamide antibodies, so we cannot determine if both neurotransmitters colocalised to the same cell bodies and/or neurites. Still, the new species does show FMRFamide and serotonin in perykarya along the nerve cords.

In short, T. tongiorgii gen. et sp. nov. shows a immunoreactive to FMRFamide system similar to all gastrotrichs, but with peculiarities unique of this species. Because of the presence of three dorsal commissures, the lower number of neurite projections, the lower number of IR perykarya in the cerebral ganglion, the FMRFamide-IR system of this species resembles more Lepidodasys and Turbanella instead of Dactylopodola and Xenodasys species.

To summarize, both the muscular- and nervous systems of the specimens from Thailand show some peculiarities (e.g., absence of somatic circular muscles along the pharynx, the presence of three serotonin-IR somata in the cerebral ganglion, and a third FMRFamide-IR commissure ventral to the pharyngo-intestinal junction) that may further justify the erection of new genus and species. However, the general arrangement of the muscles and nervous system of the new taxon, similar to that of many other gastrotrichs, makes it difficult to reliably establish potential phylogenetic relationships with any of the other taxa studied so far.

Phylogenetic remarks

Currently, Gastrotricha systematics is in a state of flux due to the discovery and/or the establishment of new high ranking taxa [21, 23–26] and the conflicts between cladistic studies and the classical systematisation (e.g., [28, 31, 33, 64]). Phylogenetic analysis of the Macrodasyida based on molecular traits (18S rDNA gene alone or in conjunction with the 28S rDNA and Cox 1 genes) have provided support for some of the traditional groupings based on morphological traits but have also unveiled surprising associations, e.g. congeneric species grouping with different families or morphologically disparate species grouping together (e.g., [24, 43, 44]).

Some of the phylogenetic novelties that emerged from molecular analyses have later been confirmed and are considered to be very likely based on re-examination of morphological traits of the taxa using an evolutionary perspective (e.g., [33]). Consequently, it may be said that phylogenetic hypotheses based on molecular markers appear to be credible and, hence, extremely useful in the on-going process of the natural systematisation of the Gastrotricha. This is especially true when the topology of the tree resulting from the analysis appears robust e.g., with
high statistical support at nodes and/or results are the same with different computational algorithms. The many statistically supported clades resulting from the current analyses appear, consequently, very robust and likely because 1) concordance among the three analyses, 2) agreement with results from comparable molecular analyses (e.g., [21, 24]) and 3) they are consistent, in general, with the current systematics of the group, which takes into account results from recent morphological investigations [21, 14, 26, 33].

Conclusion

In our phylogenetic analyses, T. tongiorgii gen. et sp. nov. always appears in a sister group relationship with a clade containing the two undetermined species of Macrodasyidae involved in the study; yet, the close phylogenetic alliance of Thaidasyidae and Macrodasyidae is supported by very high statistical values (bootstrap = 87 MP, 90 ML; Bayesian posterior probability = 1; Figs 12–14). Considering the reliability of the evolutionary scenarios based on molecular markers emerged from the previous studies (see above) we consider the position of Thaidasyidae along the Macrodasyidae phylogenetic branch suggested by the current study to be highly probable. From a morphological point of view, while there are not evidence against such a scenario, Thaidasyidae and Macrodasyidae share possible synapomorphies defined by the presence of a single ovary and the position of the pharyngeal pores far off the base. Consequently, we propose the affiliation of the new taxon to the Macrodasyidae and offer below an emended diagnosis of the family. In the current analysis, the only other genus affiliated with the Macrodasyidae, Urodasyidae, does not cluster with Macrodasyidae and Thaidasyidae; this presents a conflicting situation that should be addressed in future investigations.

Our knowledge of the biodiversity and phylogeny of the Gastrotricha are far from complete; however, Todaro in Appeltans et al. [65] estimated the number of morphological species yet to be discovered to range from 1310 to 1810. The recent find and description of high ranking taxa whose members bear traits that were unreported for Gastrotricha (i.e., H. jamaicensis and T. tongiorgii) suggests that among those there may be additional species highly significant from a phylogenetic point of view whose the anatomical peculiarities may shed light on the morphological ground pattern of these creatures and consequently may be helpful for the process of their natural systematisation.

Emended diagnosis

Macrodasyidae Remane, 1924

Elongate Macrodasyidae, up to 806 μm in length (tail excluded); body flattened ventrally and vaulted dorsally covered by naked cuticle. Mouth opening terminal, of medium size. Pharyngeal pores significantly anterior of the pharyngo-intestinal junction. Head indistinct or clearly distinct (Thaidasyidae) from body. Head sensorial structures in form of pestle organs, leaf-like organs (Thaidasyidae) or absent (some Urodasyidae). Posterior end in form of a short tail (Macrodasyidae), long tail (Urodasyidae) or ovoidal (Thaidasyidae). Locomotor ciliation covering the entire ventral side under the head and the pharyngeal region, thereafter forming two longitudinal rows or always forming two separated longitudinal bands (Thaidasyidae). Epidermal glands, inconspicuous or well discernable (Thaidasyidae). Anterior adhesive tubes singly, inserting directly on the cuticle; lateral and/or ventrolateral adhesive tubes present, occasionally numerous (Thaidasyidae); dorsal adhesive tubes absent or present (Thaidasyidae); posterior adhesive tubes on margin of the caudum or along the tail.

Usually hermaphroditic; one species parthenogenetic and viviparous; ovary unpaired or paired (Urodasyidae), egg maturing in caudo-cephalic direction. Testis, paired (Macrodasyidae and some Urodasyidae), unpaired (most Urodasyidae) or absent (Urodasyidae viviparous and Thaidasyidae).
Sperm duct(s) opening on the ventral side. Frontal organ, present or absent (some Urodasys, Thaidasys); caudal organ present as a muscular organ containing a copulatory tube (Macrodasys and Thaidasys) or a sclerotized stylet (most Urodasys), or absent (several Urodasys). Intertidal or subtidal in distribution; fine to medium sand, including dysoxic sediments. Type genus: Macrodasys Remane, 1924. Other genera: Urodasys Remane, 1926 and Thaidasys gen. nov.

Acknowledgments

Special thanks go to Saura Menabue who provided us with the sandy samples from Phuket Island; we are indebted with Francesca Bertacci for the line drawings of Fig 1 and with Herman Wirshing for the English revision. We thank R. Hochberg and an anonymous reviewer for their insightful comments on an early draft of the manuscript.

Author Contributions

Conceived and designed the experiments: MAT. Performed the experiments: MAT MDZ FL. Analyzed the data: MAT FL. Contributed reagents/materials/analysis tools: MAT. Wrote the paper: MAT FL.

References

1. Kieneke A, Schmidt-Rhaesa A (2014) Gastrotricha. In: Schmidt-Rhaesa A editor. Handbook of Zoology. Vol. 3 Gastrotricha and Gnathifera. De Gruyter, Berlin, Boston. pp 1–134.
2. Todaro MA (2015) Gastrotricha. In: World Register of Marine Species. Available from: http://www.marinespecies.org/aphia.php?p=taxdetails&id=2078 (accessed on 13 February 2015).
3. Känneby T (2013) New species and records of freshwater Chaetonotus (Gastrotricha, Chaetonotidae) from Sweden. Zootaxa 3701: 551–588.
4. Kolicka M, Kisielewski J, Nesteruk T, Zawierucha K (2013) Gastrotricha from the Poznan Palm House—one new subgenus and three new species of freshwater Chaetonotida (Gastrotricha). Zootaxa 3717: 231–279.
5. Suzuki TG, Maeda M, Furuya H (2013) Two new Japanese species of Gastrotricha (Chaetonotida, Chaetonotidae, Lepidodermella and Dichaeotididae, Dichaeotida), with comments on the diversity of gastrotrichs in rice paddies. Zootaxa 3691: 229–239.
6. Todaro MA, Perissinotto R, Bownes SJ (2013) Neogosseidae (Gastrotricha, Chaetonotida) from the iSimangaliso Wetland Park, KwaZulu-Natal, South Africa. Zookeys 315: 77–94. doi: 10.3897/zookeys.315.5593 PMID: 23878511
7. Schwank P, Känneby T (2014) Contribution to the freshwater gastrotrich fauna of wetland areas of southwestern Ontario (Canada) with redescriptions of seven species and a check-list for North America. Zootaxa 3811: 463–490. doi: 10.11646/zootaxa.3811.4.3 PMID: 24943182
8. Hochberg R, Atherton S, Gross V (2013) A new species of sublittoral marine gastrotrich, Lepidodasys ligni sp. n. (Macrodaesyida, Lepidodasyidae), from the Atlantic coast of Florida. Zookeys 289: 1–12. doi: 10.3897/zookeys.289.4764 PMID: 23794849
9. Kieneke A, Narkus S, Hochberg R, Schmidt-Rhaesa A (2013) Diplodasys rothei n. sp. (Gastrotricha, Macrodasyida), a new marine gastrotrich species from the Bahamas. Meiofauna Mar 20: 49–61.
10. Lee J M, Kim D, Chang CY (2013) Description of three new Tetranchyroderma gastrotrichs (Macrodaesyida, Thaumastodermatidae) from South Korea. Zootaxa 3709: 483–493.
11. Todaro MA (2013) A new non-naked species of Ptychostomella (Gastrotricha) from Brazil. ZooKeys 289: 13–24. doi: 10.3897/zookeys.289.4683 PMID: 23794850
12. Todaro MA, Leasi F (2013) A new eye-bearing Macrodasys (Gastrotricha, Macrodasyida) from Jamaica. Meiofauna Mar 20: 33–38.
13. Araujo TQ, Balsamo M, Garraffoni ARS (2014) A new species of Pseudostomodella (Gastrotricha, Thaumastodermatidae) from Brazil. Mar Biodiv 44: 243–248.
14. Atherton S (2014) Urodasys poculostylis sp. nov., a new stylet-bearing gastrotrich (Macrodasyida) from Capron Shoal, Florida. Mar Biol Res 5: 530–536.
15. Gilsa A, Kieneke A, Hochberg R, Schmidt-Rhaesa A (2014) Two new species of the genus *Dactylopo-
dola* (Gastrotricha: Macrodasyida) from the Bahamas, with an updated key to the genus. Cah Biol Mar
55: 333–345.
16. Hochberg R (2014) *Crasiella fonseci*, a new species of Gastrotricha (Macrodasyida, Planodasyidae)
from São Paulo, Brazil. Mar Biodiv 44: 237–242.
17. Hochberg R, Atherton S, Kieneke A (2014) Marine Gastrotricha of Little Cayman Island with the
description of one new species and an initial assessment of meiofaunal diversity. Mar Biodiv 44: 89–113.
18. Känneby T, Atherton S, Hochberg R (2014) Two new species of *Musellifer* (Gastrotricha: Chaetonotida)
from Florida and Tobago and the systematic placement of the genus within Paucitubulatina. Mar Biol
Res 10: 983–995.
19. Lee JM, Chang CY (2014) A new gastrotrich species of the genus *Tetranchyroderma* (Macrodasyida:
Thaumastodermalidae) from Korea. Proc Biol Soc Wash 127: 209–215.
20. Lee J, Chang CY, Kim D (2014) *Dendrodasyds duplus*, a new Gastrotrich Species (Macrodasyida: Dac-
tylopodolidae) from South Korea. Anim Syst Evol Diversity 30: 103–107.
21. Todaro MA, Leasi F, Hochberg R (2014) A new species, genus and family of marine Gastrotricha from
Jamaica, with a phylogenetic analysis of Macrodasyida based on molecular data. Syst Biodivers 12:
473–488.
22. Todaro MA, Perissinotto R, Bownes SJ (2015) Two new marine Gastrotricha from the Indian Ocean
coast of South Africa. Zootaxa 3905: 193–208. doi: 10.11646/zootaxa.3905.2.2 PMID: 25661205
23. Todaro MA, Guidi L, Leasi F, Tongiorgi P (2006) Morphology of *Xenos dys* (Gastrotricha), the first
species from the Mediterranean Sea and the establishment of *Chordodasiopsis* gen. nov and Xenodasyi-
dae fam. nov. J Mar Biol Assoc UK 86: 1005–1015.
24. Todaro MA, Dal Zotto M, Jonidelius U, Hochberg R, Hummon WD, Känneby T, et al. (2012) Gastrotri-
cha, a marine sister for a freshwater puzzle. PLoS One 7: e31740. doi:10.1371/journal.pone.0031740
PMID: 22381217
25. Leasi F, Todaro MA (2008) The muscular system of *Musellifer delamarei* (Renaud-Mornant, 1968) and
other chaetonotidans with implications for the phylogeny and systematization of the Paucitubulatina
(Gastrotricha). Biol J Linn Soc 94: 379–398.
26. Hummon WD, Todaro MA (2010) Analytic taxonomy and notes on marine, brackish-water and estua-
rine Gastrotricha. Zootaxa 2392: 1–32.
27. Todaro MA (2015) Systematics. In: Gastrotricha World Portal. Todaro, M.A. (ed.) (http://www.
gastrotricha.unimore.it/systematics.htm, accessed on 13 February 2015).
28. Känneby T, Todaro MA, Jonidelius U (2013) Phylogeny of Chaetonotidae and other Paucitubulatina
(Gastrotricha, Chaetonotida) and the colonization of aquatic ecosystems. Zootaxa 3772: 1–34.
29. Ruppert EE (1991) Gastrotricha. In: Harrison F.W., Ruppert E.E. Eds, Microscopic Anatomy of Inverte-
brates, 4, Aschelminthes. Wiley, New York, Pp 41–109.
30. Hochberg R, Litvaitis MK (2003) Ultrastructural and immunocytochemical observations of the nervous
systems of three macrodasyidan gastrotrichs. Acta Zool 84: 171–179.
31. Kieneke A, Riemann O, Ahiruchs WH (2008) Novel implications for the basal internal relationships of
Gastrotricha revealed by an analysis of morphological characters. Zootaxa 42: 89–105.
32. Rothe BH, Schmidt-Rhaesa A, Kieneke Å (2011) The nervous system of *Neodasys chaetonotoideus*
(Gastrotricha, *Neodasy*) revealed by combining confocal laser scanning and transmission electron
microscopy, evolutionary comparison of neuroanatomy within the Gastrotricha and basal Protostomia.
Zoomorphology 130: 51–84.
33. Guidi L, Todaro MA, Ferraguti M, Balsamo M (2014) Reproductive system and spermatozoa ultrastruc-
ture support the phylogenetic proximity of *Megadasys* and *Crasiella* (Gastrotricha, *Macrodasyida*).
Contr Zool 83: 121–133.
34. Todaro MA (2002) An interesting new gastrotrich from littoral meio-benthos (Long Beach Island, USA),
with a key to species of *Tetranchyroderma* (Gastrotricha: Macrodasyida). J Mar Biol Assoc UK 82:
555–563.
35. Todaro MA, Hummon WD (2008) An overview and a dichotomous key to genera of the phylum Gastro-
tricha. Meiofauna Mar 16: 3–20.
36. Todaro MA, Guidi L, Ferraguti M, Balsamo M (2012) A fresh look at *Dinodasys mirabilis* (Gastrotricha,
Macrodasyida), with focus on the reproductive apparatus and sperm ultrastructure. Zoomorphology
131: 115–125.
37. Hummon WD, Todaro MA, Tongiorgi P (1993) Italian marine Gastrotricha, II. One new genus and ten
new species of *Macrodasyida*. B Zool 60: 109–127.
38. Leasi F, Todaro MA (2009) Meiofaunal cryptic species revealed by confocal microscopy: the case of Xenotrichula intermedia (Gastrotricha). Mar Biol 156: 1335–1346.
39. Leasi F, Pennati R, Ricci C (2009) First description of the serotonergic nervous system in a bdelloid rotifer: Macrotrachela quadricornifera Milne, 1886 (Philodinidae). Zool Anz 248: 47–55.
40. Todaro MA, Leasi F, Bizzarri N, Tongiorgi P (2006). Meiofauna densities and gastrotrich community composition in a Mediterranean sea cave. Mar Biol 149: 1079–1091.
41. Seward-Thompson BL, Hails JR (1973) An appraisal of the computation of statistical parameters in grain size analysis. Sedimentology 20: 161–169.
42. Hummon WD, Balsamo M, Todaro MA (1992) Italian marine Gastrotricha, I. Six new and one re-described species of Chaetonotida. B Zool 59: 499–516.
43. Petrov NB, Pegova AN, Manylov OG, Mugue NS, Aleshin VV (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 41: 445–452.
44. Todaro MA, Telford MJ, Lockyer AE, Littlewood DTJ (2006). Interrelationships of the Gastrotricha and their place among the Metazoa inferred from 18S rRNA genes. Zool Scripta 35: 251–259.
45. Todaro MA, Kanneby T, Dal Zotto M, Jondelius U (2011) Phylogeny of Thaumastodermatidae (Gastrotricha, Macroaspidida) inferred from nuclear and mitochondrial sequence data. PLoS One 6: e17892. doi: 10.1371/journal.pone.0017892 PMID: 21455302
46. Giribet G, Sørensen MV, Funch P, Kristensen RM, Sterrer W (2004) Investigations into the phylogenetic position of Micrognathozoa using four molecular loci. Cladistics 20: 1–13.
47. Sørensen MV, Sterrer W, Giribet G (2006) Gnathostomulid phylogeny inferred from a combined approach of four molecular loci and morphology. Cladistics 22: 32–56.
48. Staden R (1996) The Staden sequence analysis package. Mol Biotechnol 5: 233–241. PMID: 8837029
49. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol 30: 2725–2729. doi: 10.1093/molbev/mst197 PMID: 24132122
50. Ronquist F, Huelsenbeck JP (2003) MRBAYES 3, Bayesian phylogenetic inference under mixed models. Bioinformatics 19: 1572–1574. PMID: 12912839
51. Nylander JA (2004) MrModeltest v2. Program distributed by the author. Evolutionary Biology Centre, Uppsala University.
52. Page RDM (1996) TREEVIEW, An application to display phylogenetic trees on personal computers. Comput Appl Biosci 12: 357–358. PMID: 9002363
53. Hochberg R (2005) Musculature of the primitive gastrotrich Neodasya (Chaetonotida): functional adaptations to the interstitial environment and phylogenetic significance. Mar Biol 146: 315–323.
54. Hochberg R, Litvaitis M (2001) The muscular system of Dactylopodola baltica and other macrodasidan gastrotrichs in a functional and phylogenetic perspective. Zool Scripta 30: 325–336.
55. Remane A (1936) Gastrotricha, In: Bronns H.G., ed. Klassen Ordnungen des Tierreichs, Band 4, Abteilung II, Buch I, Teil 2, Lieferungen 1–104. Akademie Verlagsgesellschaft, Berlin.
56. Rothe BH, Schmidt-Rhaesa A (2010) Oregnidosys cirratus, a new species of Gastrotricha (Macroaspidida) from Tenerife (Canary Islands), with a description of the muscular and nervous system. Meiofauna Mar 18: 49–66.
57. Ruppert EE (1978) The reproductive system of gastrotrichs. II. Insemination in Macrosyida. Zoomorphologie 89: 207–228. PMID: 16739161
58. Hochberg R, Atherton S (2011) A new species of Lepidodasys (Gastrotricha, Macroaspidida) from Panama with a description of its peptidergic nervous system using CLSM, anti-FMRFamide and anti-SCP. Zool Anz 250: 111–122.
63. Hochberg R (2007) Comparative immunohistochemistry of the cerebral ganglion in Gastrotricha: an analysis of FMRFamide-like immunoreactivity in Neodasys cirritus (Chaetonotida), Xenodasys riedli and Turbanella cf. hyalina (Macrodasyida). Zoomorphology 126: 245–264.

64. Hochberg R, Litvaitis MK (2000) Phylogeny of Gastrotricha: a morphology-based framework of gastrotrich relationships. Biol Bull 198: 299–305. PMID: 10786949

65. Appeltans W, Ahyong ST, Anderson G, Angel MV, Artois T, Bailly N, et al. (2012) The Magnitude of Global Marine Species Diversity. Curr Biol 22: 2189–2202. doi: 10.1016/j.cub.2012.09.036 PMID: 23159596