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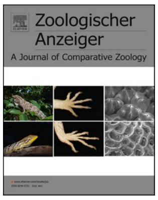

Phylogenetic position of Setopus (Gastrotricha, Paucitubulatina) among planktonic Gastrotricha, with the description of a new species

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ABSTRACT

The understanding of phylogenetic relationships within the phylum Gastrotricha is currently evolving, revealing significant discrepancies between the evolutionary interpretations based on morphology and molecular data. This inconsistency also applies to the families Dasydytidae and Neogosseidae, which include the remarkable planktonic gastrotrich species of the order Chaetonotida. The inter- and intra-relationships within these families remain unclear, mainly due to incomplete taxonomic sampling. In this study, we describe a new species of *Setopus* and provide its 18S, 28S, and COI genetic sequences. We also provide the sequences of *Setopus tongiorgii* (Balsamo, 1983), offering the first available molecular data for the genus. The new sequences were used in a phylogenetic analysis that encompassed 53 terminals, covering a broad taxonomic range. The results indicate that the two *Setopus* species are closely related to *Ornamentula* Kisielewski, 1991 and highlight a possible non-monophyletic nature of the family Dasydytidae, with members of the family Neogosseidae falling within it.

1. Introduction

Gastrotricha represents a fascinating phylum of tiny aquatic creatures, boasting over 900 known species. Remarkably, approximately 520 of these species belong to the order Chaetonotida, demonstrating its significant diversity. In fact, this order is home to an impressive 378 out of the 382 gastrotrich species that have been identified in freshwater habitats. Freshwater chaetonotidans may be considered ubiquitous (Balsamo et al., 2020; Saponi & Todaro, 2024; Gammuto et al., 2024 Saponi et al., 2024; Minowa et al., 2025a; Minowa et al., 2025b; Minowa et al., 2025c; Sochiarelli et al., 2025). This ubiquity is likely due to their rapid development and ability to reproduce parthenogenetically; these characteristics allow these animals to potentially establish a stable population in any water body, provided their ecological needs are met. This can occur starting from just one or a few eggs or specimens that are accidently or naturally introduced (Hummon, 1986; Balsamo & Todaro, 1988). Indeed, these gastrotrichs can be found in virtually every natural aquatic biotope, including rivers, streams, swamps, moors, lakes, ponds and limno-terrestrial environments (Todaro et al., 2019; Majdi et al., 2024; Minowa et al., 2025a), but also in human-made water reservoir such as fishing lakes (Balsamo, 1977; Leasi et al., 2006; Rataj Križanová & Vďačný, 2021), quarry lakes (Balsamo, 1977; Saponi et al., 2024), outfall drains (Maysoon, 2022), ornamental ponds (Balsamo, 1980; Kånneby, 2013), explosion craters filled with rainwater (Balsamo & Todaro, 1995), roadside ditches (Mola, 1932; Balsamo, 1980), palm houses (Kolicka, 2014; Kolicka et al., 2016), greenhouses (Kolicka, 2019) and even gardens' fountains (Todaro M.A., unpublished).

Despite their ubiquity and abundance, many questions about the biology and evolution of the freshwater gastrotrichs remain unanswered. Currently, the freshwater chaetonotidans are classified in 31 genera and six families (Saponi & Todaro, 2024; Gammuto et al., 2024; Saponi et al., 2024; Minowa et al., 2025a; Minowa et al., 2025b; Minowa et al., 2025c; Sochiarelli et al., 2025). However, the existing classification, traditionally based mostly on the cuticular ornamentations, is debated (Kånneby et al., 2012; Rataj Križanová & Vďačný, 2024).

The growing application of molecular data in phylogenetic studies has revealed the non-monophyly of several genera and families within the order Chaetonotida, highlighting the lack of homology in the

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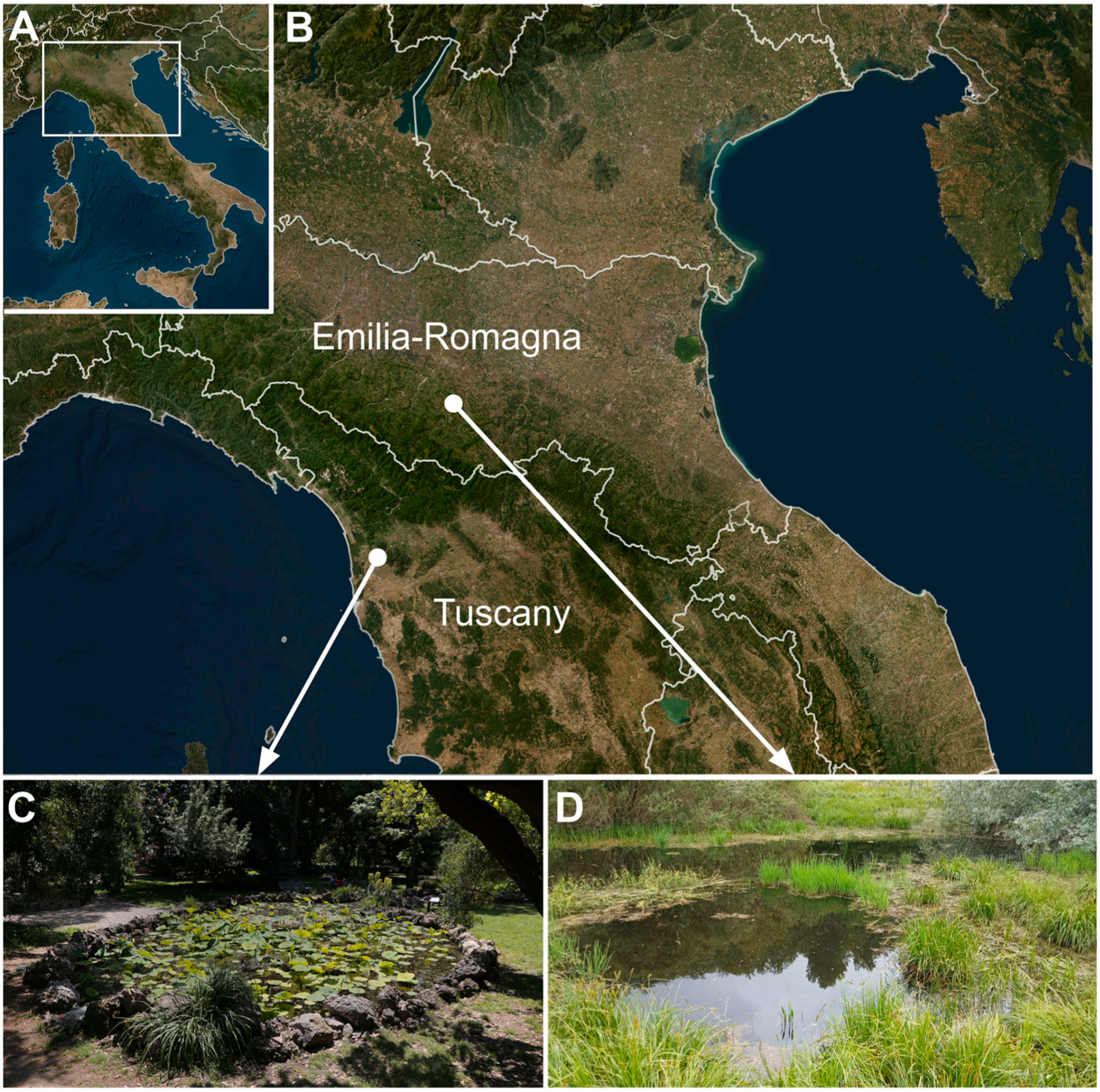

Fig. 1. **A.** Comprehensive map of Italy, with a white square highlighting the area shown in Figure B. **B** Satellite view of Northern Italy, displaying the administrative regional boundaries; the white dots indicate the locations of the sampled biotopes. **C.** Photo of the pond sampled at the Pisa Botanical Garden. **D.** Photo depicting the sampling site Sassomassiccio Pond.

morphological traits used for traditional classification (Kånneby et al., 2013; Garraffoni et al., 2017; Rataj Križanová & Vďačný, 2022; Gammuto et al., 2024; Saponi et al., 2024). The results of these studies emphasize the importance of an integrated approach based on both morphological and molecular data in order to obtain a classification that accurately reflects the evolutionary relationships between species (Cesaretti et al., 2025). Currently, there is a lack of molecular data for many species, and in some cases, for entire genera, particularly for the rarer ones. One such genus is *Setopus* Grünspan, 1908, which includes nine species and is classified within the family Dasydytidae. Like all members of this family, *Setopus* species exhibit typical adaptations of planktonic gastrotrichs. These adaptations include locomotory ciliation organized in transverse bands around the head and along the trunk, the presence of paired groups of long moveable spines, and the absence of adhesive tubes (Kieneke et al., 2008a; Kieneke and Ostmann, 2012; Todaro et al., 2013; Sochiarelli et al., 2025). A key feature of the genus *Setopus* is the presence of one pair of long spines on the bilobed caudum. These spines may be of equal or unequal length.

Setopus was first described as a separate genus by Grünspan (1908). However, a few years later, Remane (1936) revised its taxonomic position, reclassifying it as a subgenus of *Dasydytes*. This classification remained for 54 years until Schwank (in Schwank & Bartsch, 1990) erected the subgenus *Setodytes* to host the species with spines provided with accessory denticle and the terminal spines of unequal length. Kisielewski (1991) elevated all species back to genus level. Nevertheless, in the comprehensive phylogenetic study by Kieneke et al. (2008b), the two Setopus species were identified within subgenera:*Dasydytes* (*Setopus*) *bisetosus* and *Dasydytes* (*Setodytes*) *tongiorgii*. Balsamo et al. (2009) formally synonymised *Setodytes* with *Setopus*, affirming that spines with an accessory point and the unequal caudal spines are traits featured by other species of Dasydytidae and consequently do not appear to be diagnostic at subgeneric level.

The taxonomic instability and the relatively low support for the placement of *Setopus* species within the Dasydytidae-Neogosseidae cluster, as highlighted by the phylogenetic analysis of morphological traits conducted by Kieneke et al. (2008b), warrant further investigation using alternative approaches, specifically molecular genetic methods. Unfortunately, no genetic data had been available for the genus prior to this study.

The recent discovery of several specimens from a new species of *Setopus* allowed us to describe its morphology and obtain genetic sequences for three genes: 18S rDNA, 28S rDNA, and COI mtDNA. This novel information opens the door to a phylogenetic study aimed at uncovering the genus's origins and its position within the Oiorpata gastrotrichs (Gammuto et al., 2024). During the planning phase of this study, we also obtained sequences from an additional species, *Setopus tongiorgii* Balsamo, 1983, which have been included in the analysis.

This study is part of a large Italian biodiversity project (NBFC – National Biodiversity Future Center) and falls under the mission of Spoke 3, focusing on terrestrial and freshwater biodiversity, representing the fourth contribution dealing with freshwater gastrotrichs (Saponi & Todaro, 2024; Gammuto et al., 2024; Saponi et al., 2024).

2. Material and methods

2.1. Study sites

Specimens of the new species were collected in November 2022, in a freshwater pond situated in the newest section of the botanical garden of Pisa (Tuscany, Italy; Fig. 1B and C). This area, known as “Orto del Gratta”, is located in the garden's northernmost part (43°43′16.60″ N, 10°23′45.75″ E) (Cardiello and Peruzzi, 2018). This artificial pond, built at the beginning of the past century, has a water depth of 60–70 cm in the whole area, measuring approximately 150 m^2, most of which is represented by a circular part, whose diameter spans 10–15 m. The edges and the bottom of the pond are cemented, covered with a thin layer of organic detritus. It hosts several aquatic plant species such as *Nelumbo nucifera* (Gaertner, 1788), *Lemna minuta* K., *Ceratophyllum demersum* L. and *Nymphaea* sp. and occasionally some amphibian species such as *Pelophylax* species. From this biotope a new genus and species of gastrotrich, *Litigonotus ghinii* Gammuto, Serra, Petroni & Todaro 2024, was recently described (Gammuto et al., 2024). Specimens of *S. tongiorgii* were collected in October 2023 from Sassomassiccio Pond (44°18′49″ N, 10°52′40″ E; Fig. 1B and D). This small water body is located at the bottom of a dolina in the Modena Apennines (Emilia Romagna, Italy), approximately 640 m above sea level. The pond shrinks significantly during the summer months and may eventually dry up completely. However, in the fall, it fills with rainwater and becomes a breeding site for many species of amphibians: *Pelophylax* sp., *Rana dalmatina* Fitzinger, 1839, *Hyla intermedia* Boulenger, 1882, *Triturus cristatus* Laurenti, 1768, *Lissotriton vulgaris* (Linnaeus, 1758) and *Ichthyosaura alpestris* (Laurenti, 1768) (Benassi et al., 2020). The pond hosts also a variety of aquatic plants including *Hottonia palustris* L., *Ranunculus acquaticus* L. and *Veronica scutellata* L. (Alessandrini and Bonafede, 1996). The gastrotrich fauna reported in print so far from Sassomassiccio pond consists of three species: *Chaetonotus heterospinosus* Balsamo, 1977, *Chaetonotus maximus* Ehrenberg, 1838 and *Chaetonotus robustus* Davison, 1938; Balsamo & Todaro, 1988; Balsamo & Tongiorgi, 1995; Saponi & Todaro, 2024).

2.2. Sampling

Sampling was conducted in both biotopes by scooping sediment from the bottom and edges of the pond, as well as around the submerged vegetation, using a plankton net with a mesh size of 29 μm (Todaro et al., 2019). The material collected from each pond, which consisted of detritus, water, and a small amount of plant material, was stored in four 500 ml plastic jars. These samples were transported to our lab in Modena, where they were kept under controlled conditions (18 °C temperature and a 12-h light/dark cycle) and analyzed for gastrotrichs within ten days of collection.

2.3. Sample processing and morphological analysis

Small subsamples (approximately 15 mL) of water and sediment mix were transferred to Petri dishes (9 cm in diameter) and observed under a Wild M8 stereomicroscope. Gastrotrichs were picked out through a hand-made glass micropipette and transferred on a microscope slide, in a drop of 1 % $MgCl_2$ solution. Morphological analysis and photographic vouchering were conducted on living, relaxed animals using a Nikon Eclipse 90i microscope equipped with differential interference contrast Nomarski optics (DIC) and a Nikon DS-Fi3 digital camera, controlled through the Nikon NIS-Elements D software (v.4.6). Following the observation and documentation, one specimen of each species was successfully retrieved from the slides and transferred to absolute ethanol in an individual 1.5-mL Eppendorf tube for subsequent molecular genetic analyses. The description of the new species adheres to the convention established by Hummon et al. (1992), where the positioning of key morphological traits along the longitudinal axis is indicated in percentage units (U) relative to the total length, measured from the anterior to the posterior end.

2.4. DNA amplification and sequencing

For each species, the single specimen stored in ethanol was first washed in clean absolute ethanol and then transferred into a sterile 200 μl Eppendorf tube using a clean glass micropipette. The tubes containing the specimens were left overnight at 25 °C in a cleaned ISCO Micra 18 incubator to allow for the evaporation of any residual ethanol. The dried animals were processed for DNA extraction and WGA (Whole-Genome Amplification), employing REPLI-g Single Cell Kit (QIAGEN®), following the manufacturer's guidelines. The extracted and amplified DNA material was sent to Macrogen Europe (https://www.macrogen-europe.com/), where it was processed with a TrueSeq DNA PCR Free Library kit and whole genome *de novo* sequencing at NovaSeq 6000 Illumina Platform to generate a total of 40 million reads (paired-ends 2 x 150 bp).

2.5. Genes assembly

The obtained reads were assembled through the bioinformatic pipeline described in Serra et al. (2021) for protozoans and slightly modified for Gastrotricha by Gammuto et al. (2024), Saponi et al. (2024) and Cesaretti et al. (2024, 2025). In brief, reads were preliminarily assembled with SPAdes v3.13.1 software (Bankevich et al., 2012). The assembled sequences (contigs) matching our queries were identified using Blastn and tBlastn analysis using ribosomal and mitochondrial genes sequences of several Chaetonotida species available on GenBank as queries. The contigs of interest were extracted and assembled manually using the Bioedit software (Hall, 1999) in order to obtain the complete 18S, 28S and COI gene sequences, which were subsequently used for the phylogenetic analysis.

2.6. Phylogenetic analysis

In our phylogenetic analysis, we incorporated newly obtained sequences from two *Setopus* species alongside the dataset utilized in the recent study by Sochiarelli et al. (2025). This dataset provides a fair representation of the very diverse and mainly freshwater, parthenogenetic species forming the clade Oiorpata within Chaetonotida Paucitubulatina (Gammuto et al., 2024), specifically focusing on species that exhibit adaptations for a planktonic lifestyle. It includes at least one representative from the freshwater genera within the family Chaetonotidae, as well as species of Dasydytidae and Neogosseidae for which sequences of at least the two ribosomal genes (18S, and 28S) are available. We excluded one terminal of *Ornamentula sanctipetri* Sochiarelli et al., 2025 from the dataset because it was redundant (> three terminals). The final matrix comprised 53 terminals: 38 from the

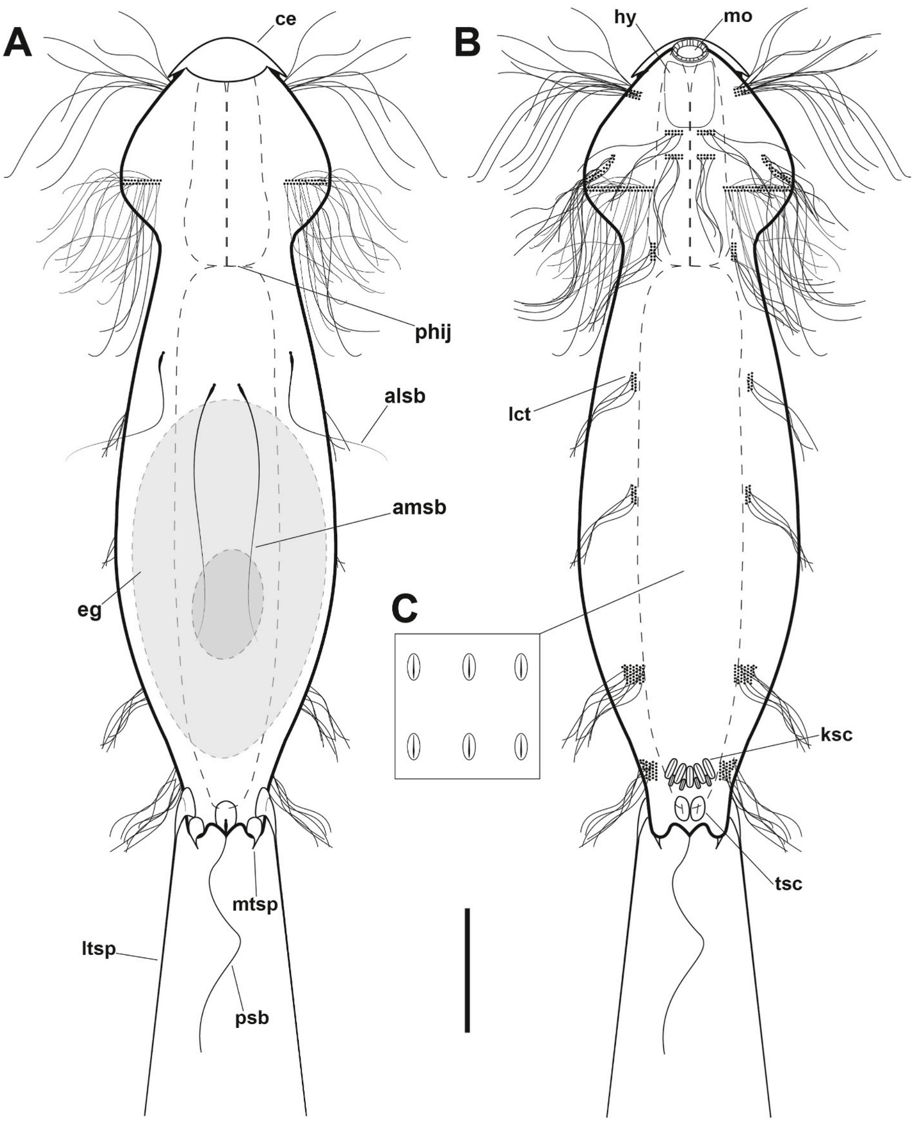

Fig. 2. Schematic illustration of *Setopus pentasetosus* n. sp. **A.** Dorsal view. **B.** Ventral view. **C.** Detail of the keeled scales visible on the ventral side of some specimens. The drawing is mainly based on the holotype. Scale bar = 20 μm. Abbreviations: alsb = anterior lateral sensory bristles; amsb = anterior medial sensory bristles; ce = cephalion; eg = egg; hy = hypostomion; ksc = keeled scales; lct = locomotory cilia tuft; ltsp = lateral terminal spines; mo = mouth opening; mtsp = medial terminal spines; phij = pharyngo-intestinal junction; psb = posterior sensory bristle; tsc = terminal plates.

family Chaetonotidae, 14 from the family Dasydytidae, and three from the family Neogosseidae (Supplementary Table 1).

The phylogenetic analysis was based on the concatenated sequences of three genes (18S, 28S and COI). Each gene was aligned separately using the MUSCLE algorithm embedded in the MEGA X software (Kumar et al., 2018), applying a codon-aware alignment to COI due to its protein-coding nature. Individual alignments were trimmed to the length of the majority of the sequences, resulting in 1665, 2568 and 660 nucleotides for 18S, 28S and COI respectively. Finally, all three alignments were concatenated in a single matrix resulting in 4893 sites through the concatenation tool available in MEGA X. The matrix has been used to build the phylogenetic trees through Maximum likelihood (ML) and Bayesian Inference (BI) algorithms. The ML analysis was performed in IQ-TREE v.3.0.1 (Nguyen et al., 2015), with 1000 standard bootstrap replicates, while the BI analysis was performed through MrBayes v.3.2.7 software (Ronquist et al., 2012) with 5 million Markov Chain Monte Carlo (MCMC). In both analyses, the best substitution model obtained with the MEGA X dedicated tool (GTR + G + I for each partition) was used for each partition. Both ML and BI tree were computed as unrooted and subsequently rooted with the dedicated tool in FigTree v.1.4.4 (https://tree.bio.ed.ac.uk/software/figtree) using the marine *Aspidiophorus* clade (*Aspidiophorus paramediterraneus* Hummon, 1974; *Aspidiophorus polystictos* Balsamo & Todaro 1987; *Aspidiophorus tentaculatus* Wilke, 1954) as the outgroup. The final tree was edited for better readability using Adobe Illustrator CS6 software (www.adobe.com/products/illustrator).

3. Results

3.1. Taxonomic accounts

Order Chaetonotida Remane 1925; Rao & Clausen, 1970]
Suborder Paucitubulatina d'Hondt, 1971
Family Dasyditidae Daday, 1905.
Genus *Setopus* Grünspan, 1908.

3.1.1. *Setopus pentasetosus n. sp.* (Figs. 2–7)

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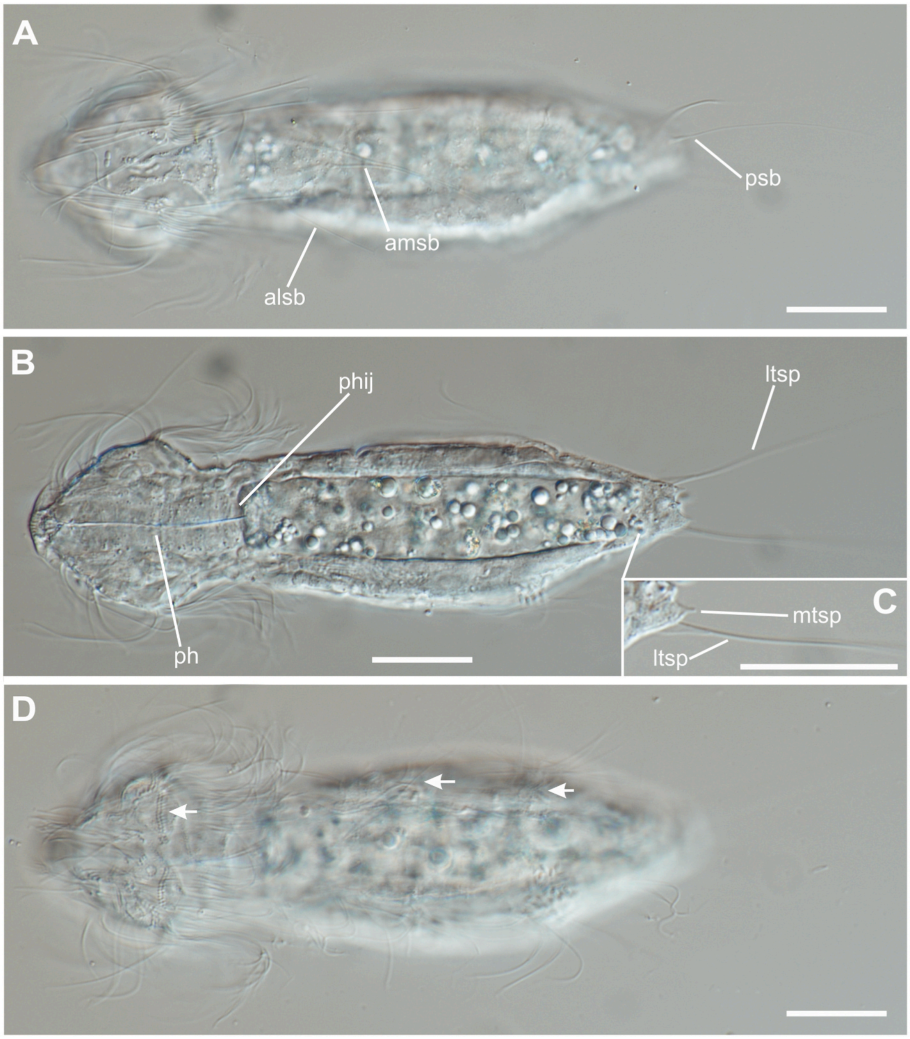

Fig. 3. *Setopus pentasetosus* n. sp., holotype habitus. **A.** Dorsal view. **B.** Internal view. **C.** Close-up of the caudal lobe, showing the two types of terminal spines. **D.** Ventral view, showing the insertion of the locomotor ciliary tufts (arrows). Differential Interference Contrast microscopy (Nomarski). Scale bars = 20 μm. Abbreviations: alsb = anterior lateral sensory bristles; amsb = anterior medial sensory bristles; ltsp = lateral terminal spines; mtsp = medial terminal spines; ph = pharynx; phij = pharyngo-intestinal junction; psb = posterior sensory bristle.

3.1.1.1. Diagnosis. Setopus having slender body, 135.3 ± 5.5 μm long (mean ± SD), with short paired caudal lobes measuring 5.4 ± 0.6 μm. The head is roughly triangular, with a large subtriangular cephalion and a flat, rectangular, barely visible hypostomion. The cuticle is almost completely naked, except for few scales and spines in the posterior trunk region. Two very long (50.4 ± 2.2 μm) thin, simple dorsolateral terminal spines, originating from a pair of small trilobed scales located on the sides of the caudal lobes base. On the dorsal side of the caudal lobes base a pair of small hexagonal scales, each bearing a small simple medial terminal spine. On the ventral side, a group of nine small, elongated, keeled scales on the posterior part of the trunk region, followed by a pair of elliptical, keeled terminal scales. Two pairs of dorsal sensory bristles on the anterior part of the trunk, one of these very long (49.8 ± 4.6 μm). One single, long (45.3 ± 3.3 μm) posterior bristle emerging from a medial, elliptical terminal dorsal scale. Mouth small (3.8 ± 0.6 μm in diameter) and subterminal, leading to a stout pharynx, slightly enlarged in the posterior part, which ends in a pharyngo-intestinal junction. The straight intestine ends in a ventral anus. Parthenogenetic, showing a large egg dorsal to the intestine.

3.1.1.2. Etymology. The new species name derives from the combination of the Greek term "pente" (πέντε), meaning five, and the Latin word "setosus", which means bristly, and alludes to the five long bristles on the dorsal side of the animal.

3.1.1.3. Material examined

3.1.1.3.1. Holotype. ITALY • an adult specimen with a mature egg inside (Figs. 3–4), no longer extant, collected in November 2022 (International Code of Zoological Nomenclature, Articles 73.1.1 and 73.1.4; see also recommendations 73G-J – Addition of recommendation to Article 73) (ICZN International Commission on Zoological Nomenclature, 1999; 2017). After conducting the morphological survey and photographic recording, the specimen was successfully retrieved from the slide and processed for DNA analysis.

3.1.1.3.2. Additional studied specimens. Four adult specimens and a subadult; same sampling data as for holotype. All five specimens were lost during the morphological survey.

3.1.1.3.3. Type locality. Italy, Pisa, Pond of the Botanical Garden (43°43′16.60″ N; 10°23′45.75″ E). The specimens were collected from the water column and around the submerged vegetation. More details about the studied biotope are provided in the Materials and Methods section.

3.1.1.3.4. Gene sequences. The 18S rDNA, 28S rDNA and COI mtDNA sequences of the holotype specimen have been deposited in GenBank under the following accession numbers: PX674476, PX674477 and PX672993 respectively (see also Supplementary Table 1).

Fig. 4. *Setopus pentasetosus* n. sp., holotype. **A.** Habitus, dorsal view showing the origin of anterior sensory bristles (arrows). **B.** Close-up of the dorsal, anterior trunk region showing the sensory bristles (arrows). Differential Interference Contrast microscopy (Nomarski). Scale bars = 20 μm.

3.1.1.3.5. Description. General morphology: The description is mainly based on the holotype: an adult specimen of 143 μm in total length, terminal spines excluded (Figs. 2–4, Table 1). The body is slender, tenpin shaped, with a clear neck constriction that separates the head from the trunk; the posterior end consists of two caudal lobes of 6 μm in length. The head is roughly triangular in shape, a typical feature of most of the Dasydytidae species, with a pair of tufts of long sensory cilia on the antero-ventral side. The cephalic plates consist of a large subtriangular cephalion (17 μm wide, 8 μm long), giving the head a loosely trilobed shape, and a flat, rectangular, barely visible hypostomion (9 μm wide, 10 μm long) located ventrally after the mouth opening (Fig. 5A). The body widths at head/neck/trunk/caudal lobes base, measured at U30/U32/U64/U95, are respectively 38 μm, 24 μm, 41 μm and 10 μm.

Body ornamentations: The cuticle is almost completely naked. Dorsally, two pairs of sensory bristles originate from papillae located in the anterior area of the trunk: the first pair, shorter (approximatively 23 μm), originates more laterally at U50, while the second pair, significantly longer (approximatively 55 μm) is located near the midline at U52. On the rearmost part of the trunk, a single elliptical scale (Fig. 5B) bears a 48 μm long bristle. On the dorsal side of the caudal lobes base are two small hexagonal scales, each bearing a small simple medial terminal spine measuring 2 μm in length (Fig. 5B). Dorsolaterally there is a pair of trilobed scales, located on the sides of the caudal lobes base, each bearing a 50 μm long simple lateral terminal spines, very thin except for the thick base. On the ventral side a group of nine small, keeled, elongated scales are located in the posterior part of the trunk at U92 (Fig. 5D), between the last tuft of cilia; these scales are organized in two alternating rows, the first consists in 5–7 scales measuring 4 μm in length, the second row consists in four smaller scales (2 μm in length). On the rearmost part of the ventral side, there is a pair of terminal elliptical keeled scales (4 μm in length and 3 μm in width), protecting the anal opening. In some specimens, a barely visible group of six small keeled scales was observed on the ventral side of the mid-trunk region, approximately at U65 (see below Variability and Remarks).

Locomotory ciliation: The locomotory ciliation consists of ten pairs of separated groups of long cilia (23–34 μm long), most of which are located in the ventral side of the body. The anteriormost tufts originate just posterior to the hypostomion (U19). One pair of long transversal bands surround the widest part of the head (U30), interrupted on the ventral and dorsal medial region. These ciliary bands, together with a curved ventrolateral band just anterior to them, are the cilia mainly involved in the locomotion (Fig. 5C). On the neck and trunk region five small, separated tufts of cilia run along the ventral side of the body.

Digestive tract: The subterminal mouth ring is small (4 μm in diameter) and reinforced with rod-like cuticular structures. The pharynx is stout (39 μm long, 10 μm wide) with a slight posterior enlargement (14 μm wide). The pharyngo-intestinal junction is at U37; the intestine is straight, narrowing posteriorly, and ends at U96 with the ventral anus.

Reproductive structures: All observed adult specimens were in the parthenogenetic phase, showing a large egg dorsal to the intestine.

3.1.1.3.6. Variability and remarks. Out of the five additional specimens found, four displayed a large egg located above the intestine, indicating that they can be categorised as adults. The shape and general characteristics of these adult specimens are similar to those observed in the holotype. However, there are some appreciable differences in size and measurements of certain traits: i) their total length, which varied between 130 and 134 μm, ii) the length of the second pair of dorsal sensory bristles, varying between 45 and 55 μm, and iii) the trunk width, which spanned from 35 to 48 μm (Table 1). Additionally, in two adult specimens, two alternating rows of three small scales with barely visible keels were observed on the ventral side of the mid-trunk region (Fig. 6A and B). These metric differences can be considered part of normal intraspecific variability and are generally related to the size of the observed animal. The small size of the ventral scales and their weak cuticularization may have caused them to be overlooked in other specimens; alternatively, they likely reflect intraspecific variability. The fifth additional individual examined did not show a mature egg; instead, it displayed two small oocytes (18 × 10 μm and 15 × 10 μm) that were at an early stage of development. Therefore, this individual can be classified as a subadult. Its total length is 112 μm, which is approximately 20 μm shorter than the adults (Table 1). This information may serve as a useful indicator in the specific identification process, which, in our view, should primarily focus on adult specimens.

3.1.1.3.7. Taxonomic affinities. The genus *Setopus* currently

Table 1

Setopus pentasetosus n. sp. main taxonomic characters and measurements (in µm) of the studied specimens. Specimen n.1 is the holotype; mean and standard deviation refer to the adults.

Trait	Adults						Subadult
	1	2	3	4	5	Mean ± SD	
Total length	143	–	134	134	130	135.28 ± 5.5	112
Pharynx length	39	35	38	38	40	37.9 ± 1.7	38
Caudal lobes length	6	5	6	5	5	5.4 ± 0.6	7
Head width	38	36	36	33	33	35.3 ± 1.9	30
Neck width	24	20	24	18	21	21.4 ± 2.5	20
Trunk width	41	48	35	37	36	39.3 ± 5.5	33
Caudal lobes base width	10	7	10	10	10	9.4 ± 1.1	9
Pharynx width	10	10	11	9	10	9.9 ± 0.5	10
Posterior pharynx width	14	13	13	12	12	12.7 ± 0.9	–
1st pair bristle	23	–	17	–	23	21 ± 3.4	21
2nd pair bristle	55	45	–	–	50	49.8 ± 4.6	42
Posterior bristle	48	–	47	–	42	45.3 ± 3.3	45
Posterior bristle scale length	4	6	6	6	5	5.4 ± 0.6	6
Posterior bristle scale width	5	4	5	4	4	4.6 ± 0.4	4
Spines length	50	49	51	48	54	50.4 ± 2.2	49
Terminal spine's scale length	5	5	5	4	5	4.8 ± 0.4	6
Terminal spine's scale width	3	3	–	3	3	3 ± 0	3
Ventral keeled scales 1st row length	4	–	–	4	–	4 ± 0	5
Ventral keeled scales 2nd row length	2	–	3	2	–	2.3 ± 0.5	2
Terminal ventral scale length	–	–	–	4	–	4 ± 0	4
Terminal ventral scale width	–	–	–	2	–	2 ± 0	3
Cephalion length	8	–	8	7	6	7.2 ± 0.8	–
Cephalion width	17	–	18	17	17	17.6 ± 0.6	18
Mouth diameter	4	–	3	4	4	3.8 ± 0.6	3
Egg length	–	69	–	–	60	64.5 ± 6.4	–
Egg width	–	40	–	–	36	37.8 ± 3.1	–

comprises nine species, most of which have ventrolateral spines along their bodies. The shape and arrangement of these spines serve as the key characteristics for distinguishing between the different species (Balsamo et al., 2014). The exception is *Setopus abarbitus* (Visvesvara, 1963), which does not possess lateral spines. *S. abarbitus* was found in India in 1963 (Visvesvara, 1963) and originally ascribed to the genus *Stylochaeta* Hlava, 1904. The lack of lateral spines makes the Pisa species similar to the Indian one. However, the specimens from Pisa can be easily distinguished from the Indian species by several morphological differences: i) the presence in *S. abarbitus* of a pair of dorsal spines located on the trunk, absent in our specimens, ii) the total length, which is significantly shorter in the Indian species than in the herein described species, iii) the trunk shape, which is large and roundish in the species described by Visvesvara, while it is slender in ours, iv) the cilia arrangement along the trunk, which consists of a single, paired tuft in the Indian species, while in the species from Pisa cilia are organized in five distinct pairs of tufts, v) the pharynx shape, which is straight in *S. abarbitus* while in our specimens it shows a slight but clear posterior enlargement. Another difference regards the number of spines emerging from the caudal lobes. *S. abarbitus* presents three pairs of spines arranged around the terminal lobes: one pair of long spines on the lateral side and two pairs of shorter spines more medially. In our specimens there are only two pairs of spines around the caudal lobes: a pair of very long spines laterally and a single pair of tiny spines located medially. Based on the differences reported above, it seems reasonable to affiliate the specimens from Pisa to a new species for which the name *S. pentasetosus* n. sp. is proposed.

3.1.2. Setopus tongiorgii (Balsamo, 1982) (Fig. 8, Table 2)

3.1.2.1. Material recorded. Two adult specimens and one subadult; two specimens were lost during the microscopy survey, while one was recovered from the slide for molecular data processing.

3.1.2.2. Gene sequences. The 18S rDNA, 28S rDNA, and COI mtDNA sequences of the recovered specimen have been submitted to GenBank with the following accession numbers: PX664611, PX664613 and PX676545, respectively.

3.1.2.3. Morphometric features. Slender body, measuring 122–136 µm in total length. Head with a roughly triangular shape bearing a small cephalion, four tufts of sensory cilia and two transversal bands of locomotory cilia. Dorsal cuticle completely naked except for small scales located on the head and on the neck, each bearing a thick spine with an accessory denticle (Fig. 7B). Laterally and ventrolaterally are five series of two or three small scales, each bearing a long bifid spine. The ventral ciliation consists of five tufts of locomotory cilia. The ventral interciliary field is covered with small round keeled scales and, on the rearmost part, with a pair of large terminal roughly triangular keeled scales (Fig. 7C). A pair of conspicuous asymmetrical terminal spines of unequal length (the shorter one measures 26 µm in length, while the longer one measures 47 µm in length) originates from the rear extremity of the body. The measures reported above refer exclusively to the adult specimens.

3.1.2.4. Remarks. S. tongiorgii was described from lake Greppo, a bog tarn located in the Tuscan Apennine, and it has been reported so far only in Italy, Poland and Germany (Balsamo, 1982; Balsamo & Tongiorgi, 1995; Nesteruk, 1996; Kieneke & Riemann, 2008). The first record outside the type locality comes from the curated checklist by Balsamo and Tongiorgi (1995), who reported it in an alpine lake in the Dolomites (northern Italy; Saponi and Todaro, 2024). The following year, Nesteruk (1996) reported high density of the species in a shallow peatbog in eastern Poland. Several years later, Kieneke and Riemann (2008) reported the presence of four adult specimens, two of which in post-parthenogenetic phase, in two ditches located in the northern part of Germany. Only Kieneke and Riemann provided morphometric data in addition to the original species description.

The overall shape of the body, along with the shape and arrangement of the cuticular ornamentation in our specimens, aligns with the original description and with the dichotomous key provided by Balsamo et al. (2019). The measurements are slightly higher than those reported in the original description by Balsamo (1982), but they fall within the range observed in German specimens (Table 2). Considering the few records, the intraspecific variability may be broader than currently recognized. The smaller size and lack of a mature egg in the drawings and photos that enrich the original description suggest that the species may have been described based on subadult specimens. Regardless of size, the presence of small, ovoid, keeled scales in the interciliary field, the two prominent terminal interciliary plates, the arrangement of the lateral spines, and the presence of short, barbed spines on the head strongly indicate that our specimen belongs to the species *S. tongiorgii*. The geographic proximity of the type locality to the Sassoguidano pond further supports our hypothesis.

3.2. Molecular phylogeny

Our phylogenetic analyses of the concatenated dataset produced maximum likelihood (ML) and Bayesian inference (BI) trees with highly congruent topologies. In general, the results align with the evolutionary scenarios proposed by recent studies, particularly regarding the

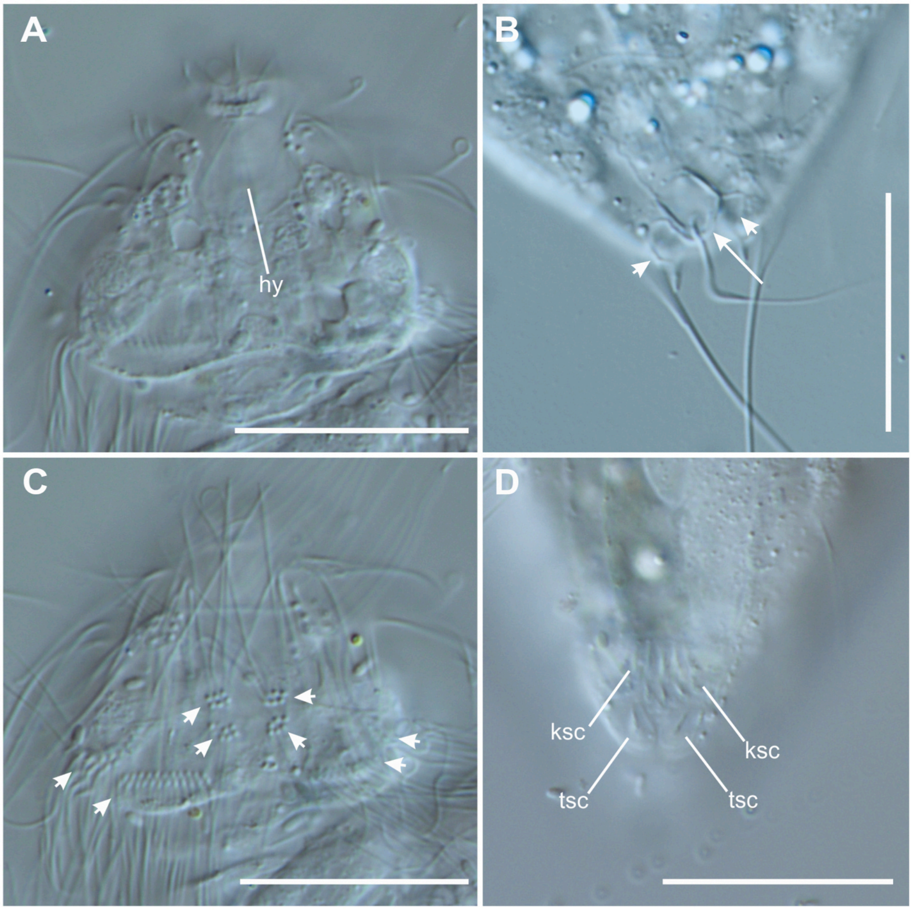

Fig. 5. *Setopus pentasetosus* n. sp., another adult specimen close-ups showing different details. **A.** Barely visible hypostomion (hy). **B.** Dorsal side of the posterior end, with the single scale bearing a long bristle (arrow) and a pair of small hexagonal scales, each bearing a short simple spine (arrowhead). **C.** Ventral side of the head, where the cilia arrangement is clearly visible; arrowheads indicate the insertion points of ciliary tufts. **D.** Ventral side of the posterior end, where the keeled scales rows (ksc) and the terminal plates (tsc) are visible. Differential Interference Contrast microscopy (Nomarski). Scale bars = 20 μm.

paraphyletic nature of the family Chaetonotidae Gosse, 1864 and the phylogenetic placements previously inferred for the recently established genera: *Carianotus* Minowa, Kieneke, Balsamo, Guidi & Garraffoni, 2025, *Dendroichthydium* Minowa, Kieneke, Campos, Balsamo, Plewka, Guidi, Araújo & Garraffoni, 2025, and *Halichaetoderma* Križanová and Vďačný, 2023; Minowa et al., 2025a, b; Rataj Križanová & Vďačný, 2024).

To narrow the information to the specific focus of this study, our analyses show that all examined planktonic species are part of a well-supported grouping (89 % bootstrap, BB and 100 % posteriori probability, PP). The sister taxon of this planktonic clade consists invariably of *Chaetonotus heteracanthus* Remane, 1927 and *L. ghinii* (90 % BB and 100 % PP). In detail, both the ML and the BI analyses found, with high support, the two analyzed *Setopus* species clustering together (89 % BB and 100 % PP), and nested within a paraphyletic Dasydytidae. More specifically, the *Setopus* clade appears, with high support (98 % BB and 100 % PP), in a sister position to the genus *Ornamentula* Kisielewski, 1991. The sister taxon of the *Setopus* + *Ornamentula* group is *Haltidytes squamosus* Kisielewski, 1991, which is shown, with strong support (96 % BB ad 100 % PP), as an early divergent lineage.

The analyses did not clearly indicate the sister taxon to the aforementioned group. In fact, the clade that includes *H. squamosus* and the combined group of *Setopus* and *Ornamentula* is shown to be related to a relatively weakly supported cluster (53 % BB and 98 % PP) comprising the other planktonic taxa i.e., *Dasydytes* Gosse, 1851; *Stylochaeta* Hlava, 1904, and Neogosseidae species (Fig. 9). More specifically, the other planktonic species appear to be divided into two distinct and well-supported branches (>75 % BB and 100 % PP). One branch includes representatives of Neogosseidae, while the other consists of *Stylochaeta* Hlava, 1904 and *Dasydytes* Gosse, 1851 species. As expected, in the former branch, *Neogossea acanthocolla* Kisielewski, 1991 and *N. antennigera* (Gosse, 1851) cluster together forming a sister–taxon relationship with *Kijanebalola devestiva* Todaro, Perissinotto & Bownes, 2013. In contrast, in the latter branch, *Dasydytes carvalhoae* Kisielewski, 1991 is recovered as sister to a clade comprising *D. elongatus* Kisielewski, 1991 + *D. papaveroi* Kisielewski, 1991 and *Setopus fusiformis* (Spencer, 1890) + *Stylochaeta scirtetica* Brunson, 1950 (Fig. 9).

4. Discussion

Our analyses involved 53 terminals; although the species used offer a good coverage of the taxonomic spectrum of the Oiorpata, this number may not be sufficient to provide robust indications about the deeper phylogenetic relationships within the group. Consequently, we prefer not to discuss every single grouping that may have received statistical support by our study, especially if the results of our analyses are in contrast with previous finding. Notwithstanding, we highlight that our analyses confirm once again the paraphyletic nature of the family Chaetonotidae, with Dasydytidae + Neogosseidae nested in it, as repeatedly demonstrated by the phylogenetic studies conducted so far (Kånneby et al., 2013; Kånneby & Todaro, 2015; Garraffoni et al., 2017; Rataj Križanová & Vďačný, 2024; Gammuto et al., 2024; Saponi et al., 2024). Likewise, our results support previous studies regarding the phylogenetic alliances of the most recently established genera of

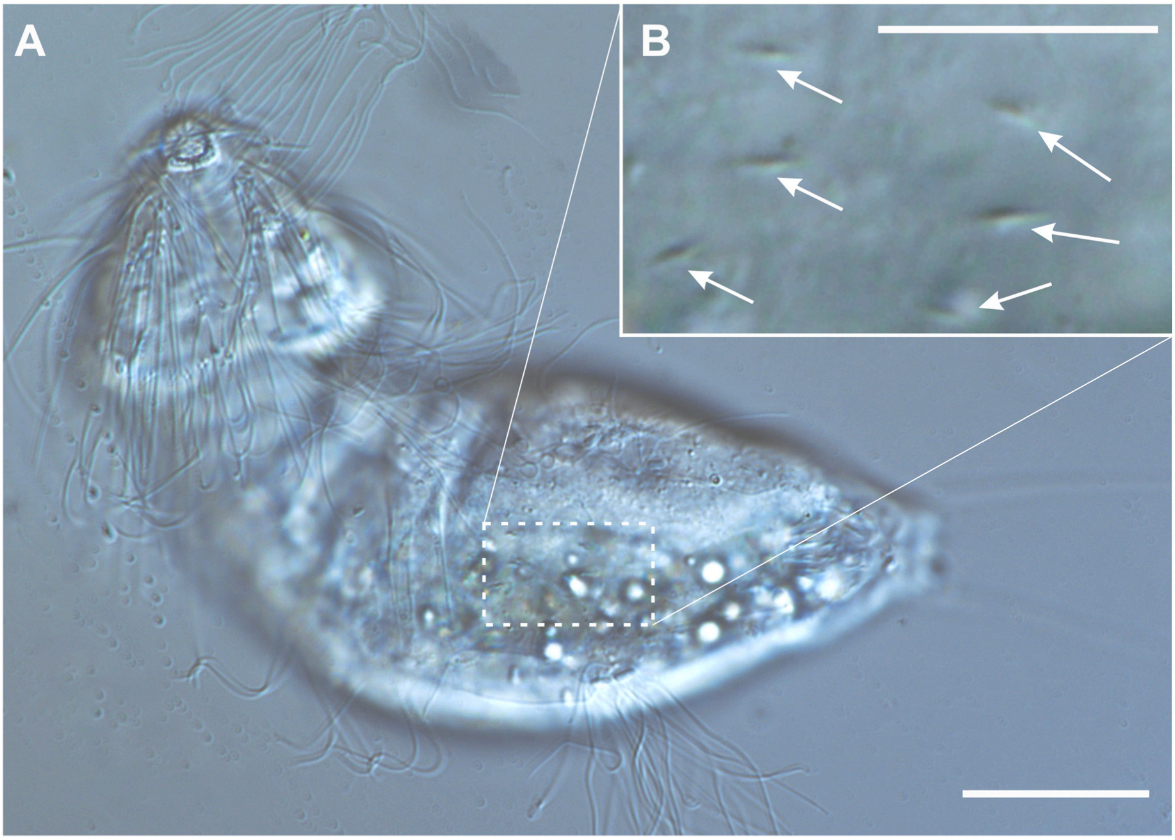

Fig. 6. *Setopus pentasetosus* n. sp. another specimen. **A.** Ventral view **B.** Close up of the ventral mid-trunk region; keeled scales are indicated with arrowheads. Differential Interference Contrast microscopy (Nomarski). Scale bars A = 20 μm; B = 10 μm.

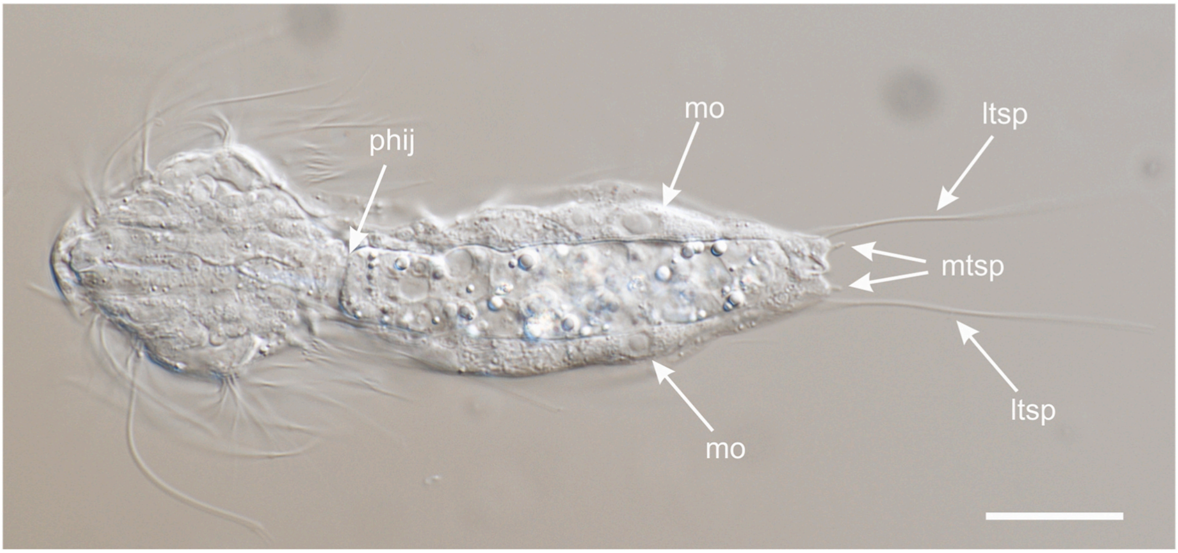

Fig. 7. *Setopus pentasetosus* n. sp. subadult specimen. Differential Interference Contrast microscopy (Nomarski). Scale bar = 20 μm. Abbreviations: ltsp = lateral terminal spines; mo = maturing oocytes; mtsp = medial terminal spines; phij = pharyngo-intestinal junction.

freshwater gastrotrichs (Minowa et al., 2025a, b; Rataj Križanová & Vďačný, 2024).

While our dataset may not be particularly ample, it contains sequences from the genus *Setopus*, providing the most comprehensive taxonomic coverage of planktonic species ever included in a study focused on the origins and phylogenetic relationships of these fascinating gastrotrichs (e.g., Kånneby & Todaro, 2015; Kolicka et al., 2020; Minowa & Garraffoni, 2020; Sochiarelli et al., 2025). Moreover, the inclusion of representatives of all freshwater genera for which the sequences of two-three genes are available offer a sound taxonomic framework for the focus of our research. Therefore, the topologies with high support in our study are unlikely to change in future, more extensive studies on planktonic gastrotrichs.

In our phylogenetic analysis, we found that all planktonic species

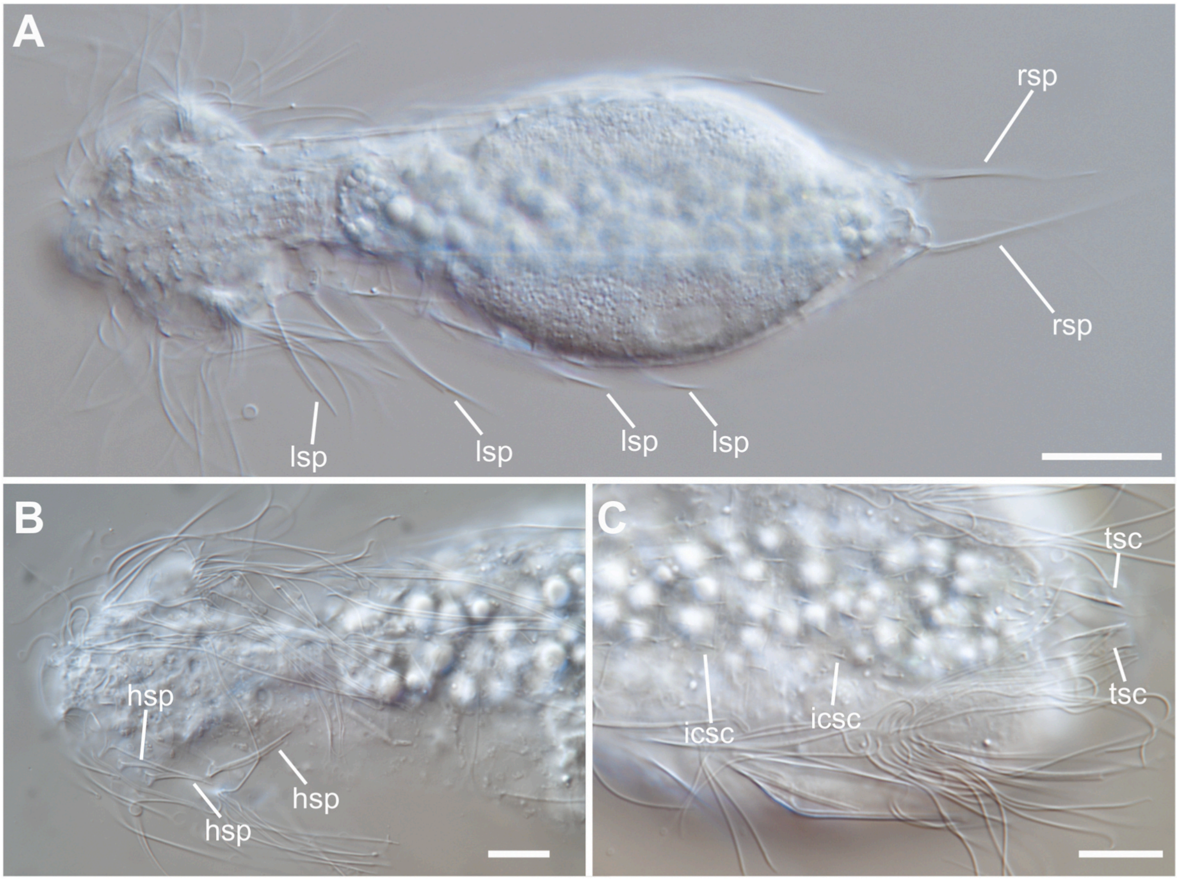

Fig. 8. *Setopus tongiorgii*, adult specimens from Sassomassiccio pond **A.** Habitus showing lateral spines (lsp) and the unequal rearmost spines (rsp). **B.** Closeup of the dorsal side of the head region, showing some of the small scales bearing the spines (hsp). **C.** Close up of the ventral trunk region, showing the interciliary field covered with small, rounded, keeled scales (isc), and the roughly triangular keeled terminal scales (tsc) are visible. Differential Interference Contrast microscopy (Nomarski). Scale bars A = 20 μm; B, C = 10 μm.

Table 2
Measurements (in μm) of selected features of *Setopus tongiorgii* from Sassomassiccio pond along with data from the literature.

Trait	Current specimens			Balsamo, 1983	Kieneke & Riemann (2008)
	Adult 1	Adult 2	Subadult		
Total length	136	122	86	84–101	105–140
Head width	38	30	31	25–32	NA
Neck width	22	15	21	14–17	NA
Trunk width	45	31	33	26–34	NA
Pharynx length	38	39	28	27–28	NA
1st terminal spine length	NA	26	35	27–30	NA
2nd terminal spine length	NA	47	48	43–55	NA

clustered together in a highly supported clade, which includes members from both the Dasydytidae and Neogosseidae families. This grouping suggests a common origin for all gastrotrichs exhibiting a semipelagic lifestyle. Previous phylogenetic studies, whether based on morphological characteristics (Hochberg & Litvaitis, 2000; Kieneke et al., 2008b) or molecular traits (e.g., Kånneby & Todaro, 2015; Sochiarelli et al., 2025), have also suggested a common origin for planktonic gastrotrichs. Additionally, a study based on total evidence (Minowa & Garraffoni, 2020) supports this finding.

Our results reveal that planktonic gastrotrichs are distributed across three highly supported branches: one branch consists of the *Dasydytes* and *Stylochaeta* species, another includes the Neogosseidae taxa, and the third encompasses species from the genera *Haltidytes*, *Setopus*, and *Ornamentula*. Apart from the nesting Neogosseidae, the phylogenetic scenarios of two branches sharing a common ancestor, *Stylochaeta* + *Dasydytes* on one side and *Haltidytes*, *Setopus*, and *Ornamentula* on the other, was also proposed by Kisielewski (1991) based on the traits of cuticular ornamentations. More specifically, the *Stylochaeta* + *Dasydytes* lineage is characterised by taxa possessing lateral spines bearing two or more accessory denticle and numerous, small scales, whereas the *Stylochaeta* + *Dasydytes* evolutionary line is characterised by species possessing lateral spines with at most one lateral denticle and a reduced number of scales (Kisielewski, 1991).

The topologies derived from our analyses show the first two branches (*Dasydytes* + *Stylochaeta* and Neogosseidae) in a sister group relationship, suggesting that the family Dasydytidae may be paraphyletic. This scenario, Dasydytidae being paraphyletic, has also been proposed in some previous studies e.g., Minowa & Garraffoni (2020), and Sochiarelli et al. (2025).

It is important to note that the statistical support for the node linking these two branches is relatively low in all these studies, including ours, which presents a bootstrap value of 53 % and a posterior probability (PP) of 0.98. The low support indicates that we should be cautious in drawing strong conclusions regarding a shared ancestry between the two groups.

The Dasydytidae being paraphyletic contrasts with the current classification and the phylogenetic scenarios proposed by Kisielewski (1991). His research, based on the characteristics of cuticular ornamentations, shares some similarities with our findings. Specifically, Kisielewski (1991) divided the genera involved in our study into two

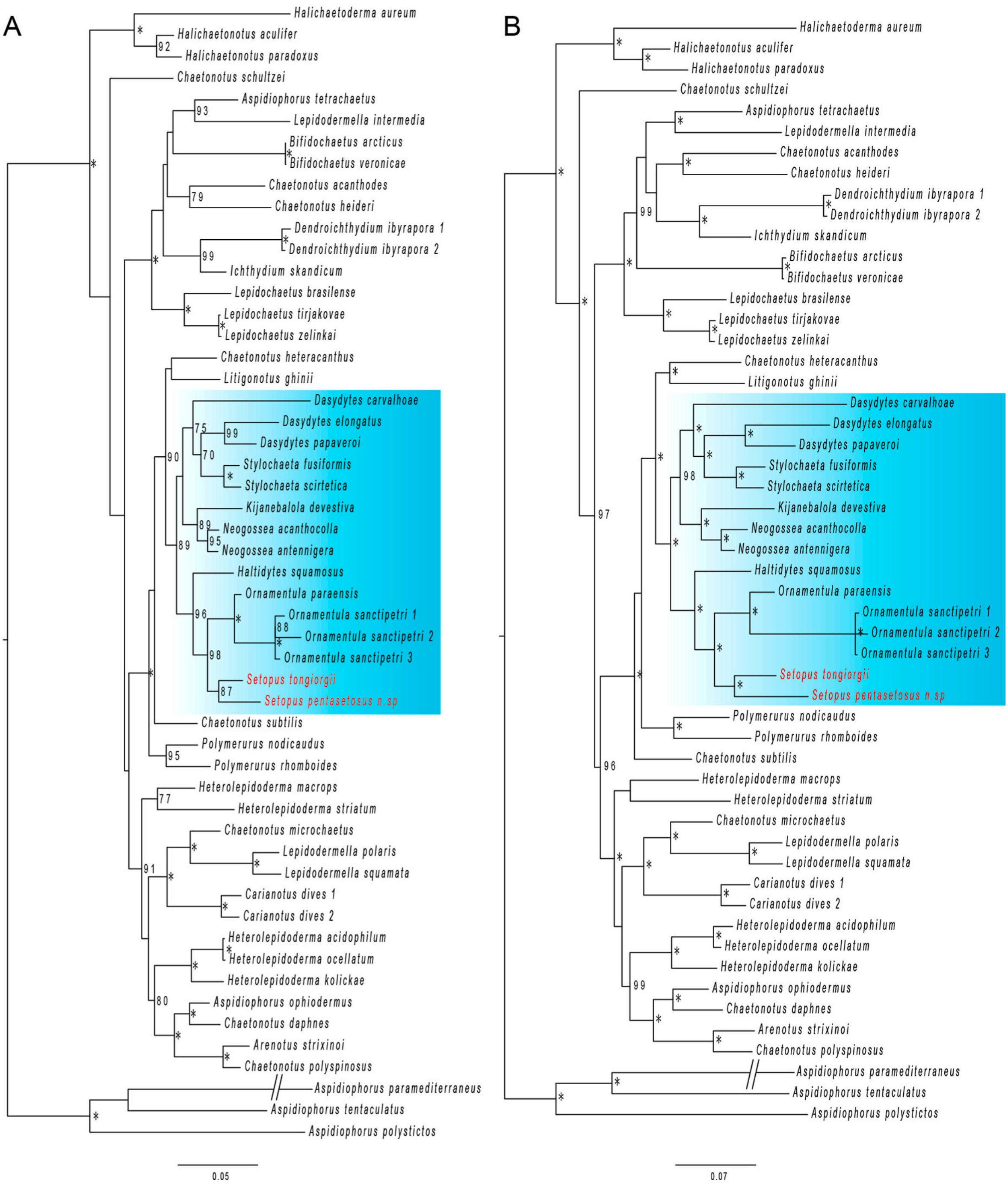

Fig. 9. Phylogenetic relationships of 53 chaetonotidans inferred from the maximum likelihood analysis (A) and Bayesian analysis (B) based on 18S rDNA, 28S rDNA and COI mtDNA concatenated alignment. The clade composed by the Dasydytidae and Neogosseidae species is highlighted in blue. The sequences obtained in the present work are highlighted in red. The lower support values (BB<70 %, PP<95 %) have not been reported to improve the readability of the tree. The asterisk indicates full support for bootstrap (1000 replicates) or posterior probability (BB/PP = 100/100). The scalebars indicate the number of substitutions per site. In both trees the branch of *Aspidiophorus paramediterraneus* is cropped and therefore not drawn to scale to improve the visualization. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

branches that share a common ancestor. One lineage is characterized by taxa with lateral spines that have two or more accessory denticles and numerous small scales, represented by the *Stylochaeta* + *Dasydytes* lineage. In contrast, the other evolutionary line includes species with lateral spines featuring at most one lateral denticle and a reduced number of large scales, such as *Haltidytes*, *Setopus*, and *Ornamentula* (Kisielewski, 1991).

Similarly, our finding that the Neogosseidae were found to be nested within Dasydytidae was also reported by Kieneke et al. (2008b). However, in that study, based on morphological traits, the sister taxon of Neogosseidae was identified as *Ornamentula*. The grouping of *Ornamentula* and Neogosseidae had *Dasydytes* (*Prodasydytes*) *papaveroi* as its sister taxon. The evolutionary scenario proposed in Kieneke et al.*ii* contrasts with results from phylogenetic studies based on molecular traits, which indicate that *Ornamentula* is closely associated with *Haltydytes* (Sochiarelli et al. 2025) or *Setopus* (this study). Additionally, their finding contradicts the empirical evolutionary scenario suggested by Kisielewski (1991), which also indicated that, based on features of the cuticular ornamentation, *Ornamentula* is strictly related to *Haltydytes* and *Setopus*.

In summary, additional research is needed to establish whether the Dasydytidae family is non-monophyletic. Including in the analysis additional species of *Dasydytes* and *Stylochaeta* could also clarify the status of *Dasydytes*, which in our analysis appears to be paraphyletic due to the nested position of *Stylochaeta*. In detail, our trees show *D. papaveroi* and *D. elongatus* clustering together and in sister position to a clade formed by *S. scirtetica* and *S. fusiformis*. The placement of these species into separate evolutionary lineages supports their classification into two distinct genera. More broadly, this placement validates the reasons that led to the formation of these two genera based on their distinct morphologies (see Kisielewski, 1991). In other words, the diagnostic characteristics of each genus should be regarded as homologies. However, this hypothesis is contradicted by the position of *Dadydytes carvalhoae*, which in our analyses emerges as an early evolutionary line along the *Dasydytes* + *Stylochaeta* branch. The separation of *D. carvalhoae* from the other two species of *Dasydytes* is puzzling, especially considering that, according to Kisielewski (1991), all of them belong to the subgenus *Prodasydytes*. It should be highlighted that within the *Prodasydytes* group, *D. carvalhoae* exhibits unique morphological characteristics, including: i) a complete reduction of the dorsal covering scales and a partial reduction of the ventral scales, ii) significant variation in spine length among the lateral spines (with some being at least three times longer than others), and iii) the presence of a third denticle on the lateral spines (Kisielewski, 1991). However, presently it is difficult to assess the phylogenetic significance of the anatomical traits that have assisted the current classification. Future studies should include other species of *Dasydytes*, particularly those from the subgenus *Dasydytes*, to confirm or disprove the monophyletic status of this genus. This would help provide a framework for assessing the evolutionary connotation of the morphological traits that currently underpin its classification.

The focal point of our research was to elucidate the phylogenetic relationships of the genus *Setopus*. Our phylogenetic analyses indicate a strong alliance between the species of *Setopus* and those of the genus *Ornamentula*, with support values of 98 % Bayesian posterior probability (BB) and 100 % posterior probabilities (PP). Additionally, the clade formed by *Setopus* and *Ornamentula* has *Haltydytes* as its sister taxon, demonstrating strong support at the relevant nodes (96–98 % BB and 100 % PP). This finding aligns with previous studies based on molecular data; however, those earlier works did not include *Setopus*, which resulted in *Ornamentula* being identified as the sister taxon to *Haltydytes* (e.g., Kånneby et al., 2013; Kånneby & Todaro, 2015; Minowa & Garraffoni, 2022; Sochiarelli et al., 2025). In phylogenetic analyses based on morphological traits, *Setopus* was not included in the study by Hochberg and Litvaitis (2000), while Kieneke et al. (2008b) found that the species of *Setopus* and *Ornamentula* evolved along separate paths. Overall, our findings regarding *Setopus*, *Ornamentula*, and *Haltydytes* are closely aligned with the proposals made by Kisielewski (1991). However, a key difference is that Kisielewski's work placed *Setopus* as an early divergent lineage within the same branch as *Ornamentula* and *Haltydytes*.

Morphologically, the representatives of *Ornamentula* and *Haltydytes* are very similar. The main distinction between these two genera lies in the large, elaborate scales found in *Ornamentula* species. In contrast, *Setopus* species exhibit an almost bare cuticle, featuring only spines and/or small, simple scales.

In our phylogenetic trees, the clade composed by *Setopus* and *Ornamentula* result to be the sister taxon of *H. squamosus*, the only representative of this genus included in the analysis. On a morphological level, species of the three genera exhibit several similarities, such as a triangular-shaped head and a short and almost rounded trunk. However, members of *Haltidytes* differs from *Setopus* and *Ornamentula* primarily in the presence and arrangement of the body spines. Species of *Haltidytes* lack rear spines, and their remaining spines show clear differentiation. The frontmost spines are bent and extend obliquely on the dorsal side, while other lateral spines have been modified into saltatorial spines. In contrast, members of *Setopus* and *Ornamentula* possess rear spines but do not have the long bent spine or the saltatorial spines. The early divergence of *Haltidytes*, as revealed by our molecular phylogeny, indicates the need to reevaluate the morphological traits that may be useful for tracing ancestry within this group.

The present study not only introduces a new species but also clarifies the phylogenetic position of the genus *Setopus* and elucidates the evolutionary relationships within the family Dasydytidae. Using an integrated approach that combines both morphological and molecular methods is crucial. This strategy greatly improves our ability to accurately identify species and position them within a larger phylogenetic context. By adopting this comprehensive perspective, we enhance our understanding of each species, which is vital for advancing biological knowledge.

CRediT authorship contribution statement

Francesco Saponi: Writing – review & editing, Writing – original draft, Visualization, Validation, Investigation, Formal analysis, Data curation, Conceptualization. **Agata Cesaretti:** Writing – review & editing, Validation. **Anush Kosakyan:** Writing – review & editing, Validation, Investigation, Data curation. **Valentina Serra:** Writing – review & editing, Validation. **M. Antonio Todaro:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jcz.2026.01.012.

Data availability

Data will be made available on request.

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Update

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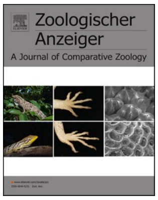

Corrigendum to “Phylogenetic position of Setopus (Gastrotricha, Paucitubulatina) among planktonic Gastrotricha, with the description of a new species” [Zool. Anz. (2026) 248-261]

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In the original publication of the article titled “Phylogenetic position of Setopus (Gastrotricha, Paucitubulatina) among planktonic Gastrotricha, with the description of a new species” (DOI: https://doi.org/10.1016/j.jcz.2026.01.012), the ZooBank LSID associated with the publication was inadvertently omitted. The correct ZooBank LSID is: *urn:lsid:zoobank.org:pub:D862EE05-C890-4169-9D8A-59C594C4F6C8*. Additionally, the final sentence of the manuscript was unintentionally left out. The complete sentence should read as follows: “During the review process of the present manuscript, Kosakyan et al. (2026) published a study reporting the mitochondrial genome of the herein described species, provisionally referred to as *Setopus* sp.”

The authors would like to apologise for any inconvenience caused.

Reference

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