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A contribution to the taxonomy and phylogeny of the genus *Chaetonotus* (Gastrotricha, Paucitubulatina, Chaetonotidae), with the description of a new species from Italian inland waters

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Abstract

Gastrotrichs of the genus *Chaetonotus* Ehrenberg, 1830 (Chaetonotida, Chaetonotidae) are known to be a speciose and pervasive group in both the marine and freshwater ecosystems, and recent investigations indicate that there is a significant amount of species diversity yet to be discovered. In the present study, a new species of this genus is described from two quarry lakes in northern Italy and characterized using an integrative morphological and molecular approach. *Chaetonotus polites* n. sp. is recognized by a rather stocky body, a five-lobed head, and the cuticular covering resolved in 13 alternating columns of partially imbricated scales, trilobed on the head and pentagonal on the trunk; each scale bears a peculiar simple, very thick, curved spine with a truncated apex. The new species is also distinguished by four putative molecular apomorphies at the 18S rRNA gene and two at the 28S rRNA gene. The location and type of these unique traits in the predicted secondary structure of the ribosomal genes is provided. The phylogenetic analysis based on concatenated sequences of three genes (the nuclear 18S and 28S rRNA genes and the mitochondrial COI gene) derived from 123 selected chaetonotidans, including the novel species, confirms that the genus *Chaetonotus* is a polyphyletic group, with several of its members resolved together with species of other genera. The new species forms a cluster with species of the subgenus *Hystricochaetonotus* Schwank 1990, suggestive of its potential stem lineage.

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Keywords: *Benthos*, *biodiversity*, *fresh water*, *integrative taxonomy*, *meiofauna*

Introduction

Chaetonotus Ehrenberg, 1830 (Chaetonotida, Paucitubulatina, Chaetonotidae) is the most speciose genus of the whole phylum Gastrotricha. It currently counts 232 species, 25.9% of the total number of gastrotrich species known to date (895 spp, Saponi & Todaro 2024). Members of this taxon are pervasive in both the marine (47 spp.) and especially in freshwater ecosystems (191 spp), where they may be considered a ubiquitous component of the gastrotrich taxocoenoses; actually, inside the gastrotrich communities, *Chaetonotus* is often the dominant taxon in terms of number of species and abundance (e.g., Nesteruk 1986). Moreover, recent investigations indicate that the alfa diversity of this successful branch of Gastrotricha may be much higher than what was previously understood (Rataj & Vďačný 2022). The remarkable abundance and morphological diversity that characterizes the genus *Chaetonotus* has led several researchers to attempt to highlight the possible intra-group evolutionary paths. The merger of various species, first in species groups and more recently in subgenera, are examples of such efforts (e.g., Remane 1927, 1936;

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Schwank 1990; Kisielewski 1997). Unfortunately, most of the traditional subgeneric divisions, proposed on morphological grounds, have not been confirmed by phylogenetic analyses based on molecular markers whose results cast doubts even on the natural status of the genus itself (e.g., Kånneby et al. 2013; Kolicka et al. 2020; Rataj & Vďačný 2024.

In this scenario, we report on some gastrotrich specimens found near Modena (Italy), unique for morphology and molecular genetics, as indicated by the microscopical observation and a phylogenetic analysis involving over 120 species/terminals and three genes. The study is part of a large Italian national biodiversity project (NBFC-National Biodiversity Future Center) and falls under the mission of Spoke 3, focusing on terrestrial and inland water biodiversity. This is the third contribution dealing with freshwater Gastrotricha (see Gammuto et al. 2024; Saponi & Todaro 2024).

Material and methods

Sampling

The new species described herein was found in October 2022 in samples from two of the several quarry lakes for inert extraction (gravel and sands) that are located near the St. Anna village (Modena, Italy), on the right side of the Panaro River (Figure 1). The two investigated lakes are adjacent to each other, and the sampled material was obtained by scraping the sediment banks and the submerged vegetation using a planktonic net (29 μm mesh size; Todaro et al. 2019). The collected material, 25% muddy sand, and 75% overlaying water was stored in two 1-L plastic jars and transported to the laboratory soon after. At the time of sampling, information about the physical-chemical characteristics of water was obtained with the YSI-63 multiparametric probe (Xylem Analytics, Germany). At the same time, geographic coordinates were gathered with a smartphone using the Examobile GPS Data application (www.examobile.com). No special authorizations were required to collect the specimens under study.

Sample processing and morphological examination

At the laboratory, samples were transferred to plastic aquaria, oxygenated through air stones, and examined within 10 days. To search for gastrotrichs, subsamples of sediment mixed with water were sucked out with a pipette, transferred to Petri dishes (9 cm in diameter), and observed under a stereomicroscope (Wild M8). The recognized gastrotrichs were picked out singly with a home-made glass micropipette, transferred in a drop of 1% $MgCl_2$ solution on a microscope slide, and observed alive. Morphological analysis was conducted using a Nikon Eclipse Ni-U microscope fitted with differential interference contrast optics (DIC). Measurements were derived from high-resolution photomicrographs obtained with a Nikon F3i camera operated by NIS-Elements F software (v.5.21). After documentation, two specimens were retrieved from the slides and transferred to absolute EtOH for molecular genetic analysis (see below). The description of the new species follows the convention of Hummon et al. (1992); the position of key morphological characters along the longitudinal axis is given in percentage units (U) of total body length, measured from the anterior to the posterior end.

DNA amplification and sequencing

The two ethanol-preserved specimens were washed, singly, three times with clean water, Miliq water, and then PBS, respectively, and then processed for DNA extraction and whole genome amplification using REPLI-g Single Cell Kit (QIAGEN®), following the manufacturer's instructions. The positively amplified DNA of one of the two specimens was then sent to Macrogen Europe (https://www.macrogen-europe.com/) and processed with a TrueSeq DNA PCR Free Library kit and whole genome de novo sequencing at NoveSeq 6000 Illumina Platform to generate a total of 40 million reads (paired-ends 2 × 150 bp).

Genes assembly and molecular characterization of the new species

Primary genome assembly of the processed specimen was obtained using a whole genome amplification pipeline, as described in Serra et al. (2020) and Gammuto et al. (2024). In brief, the assembled contigs matching ribosomal and mitochondrial genes were confirmed with blastn and tblasn analysis using available GenBank *Chaetonotus* sequences as queries. The reads mapped to these contigs were subsequently extracted and assembled with SPAdes v. 3.13.1 software (Bankevich et al. 2012) to obtain the whole ribosomal operon and mitochondrion in a single separate contig each. Prediction of the ribosomal operon structure was performed using the StructRNAFinder web tool (Arias-Carrasco et al. 2018), while the prediction of the structure of mitochondrion was assessed using the MITOS web server with invertebrate genetic code option (Bernt et al. 2013). The obtained 18SrDNA, 28SrDNA, and mtCOI

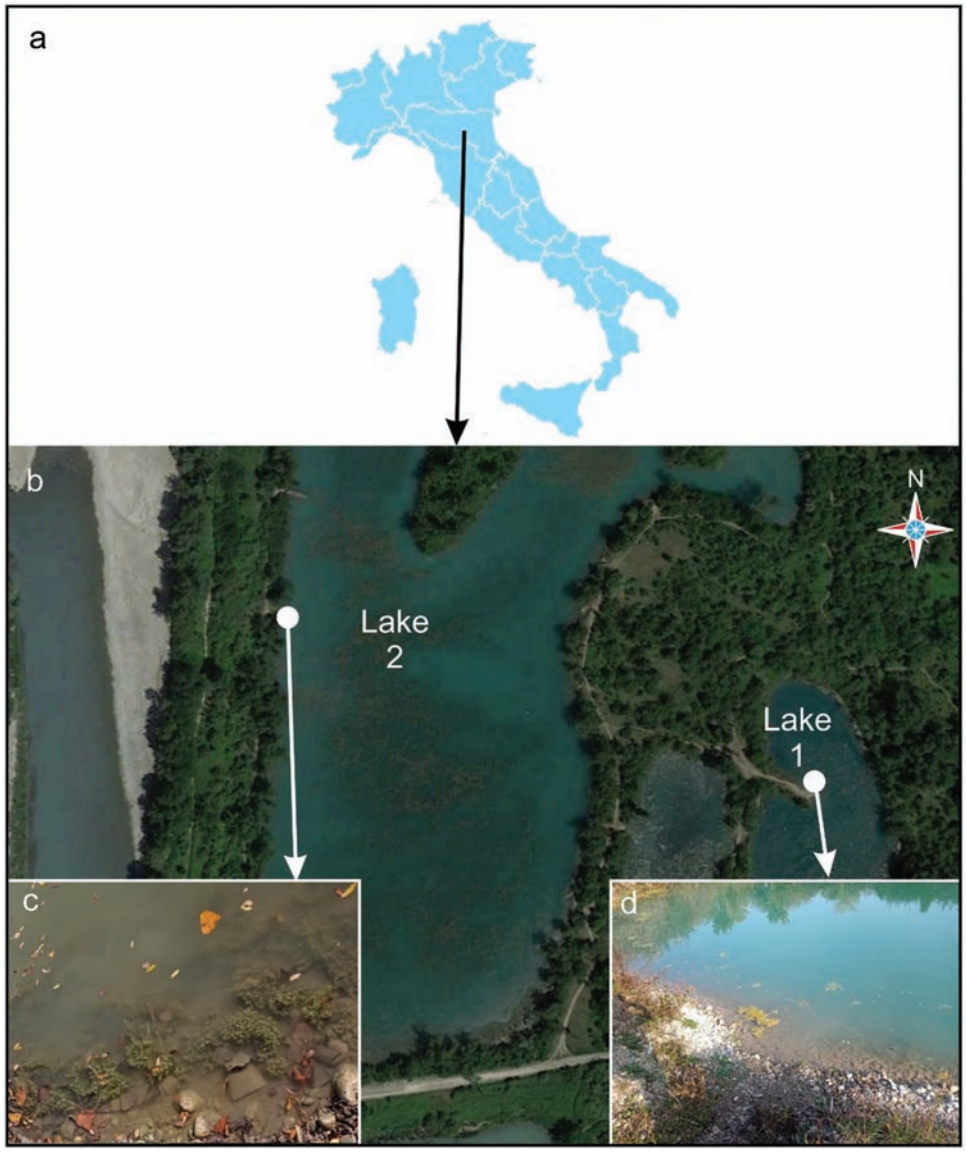


Figure 1. Sampling locations at St. Anna's quarry lakes. (a) Comprehensive map of Italy, highlighting the administrative regional boundaries and location of the investigated area in the Emilia Romagna region. (b) Aerial view of the two investigated lakes, showcasing the precise collecting sites. (c) Photo capturing the collecting site of lake 2; (d) Photo documenting the sampling site of Lake 1.

sequences were used for the phylogenetic analysis. The GC content of the assembled operon and mtCOI gene was calculated using GC content calculator tool on BiologicsCorps portal (https://www.biologicscorp.com/tools/GCContent/).

The secondary structures of the 18S and 28S molecules were predicted with R2DT with RNACentral portal https://rnacentral.org/r2dt (Sweeney et al. 2021), using the reference secondary structure map of *Saccharomyces cerevisiae* as a template with default parameters http://apollo.chemistry.gatech.edu/RibosomeGallery (Petrov et al. 2014).

Phylogenetic analysis

Selection of taxa. All freshwater Chaetonotida along with marine Chaetonotidae taxa having available sequences from 18S and 28S rDNA and COI genes were downloaded from GenBank and then filtered considering the following: i) taxa having 18S sequences shorter than 1500 bp, 28S shorter than 1200 bp and COI shorter than 500 bp were discarded, ii) low-quality sequences (e.g., having many N or ambiguous sites) were discarded, iii) to facilitate the tree reading, the number of multiple sequences belonging to the same species branching together as

100% on a preliminary tree (not shown) was reduced as two. Additionally, 14 sequences of eight *Chaetonotus* species described recently, for which sequences are available as supplementary material in Rataj and Vďačný (2022) were also added to the analysis according to the aforementioned criteria. A comprehensive list of the taxa and the accession numbers of the sequences used in the phylogenetic analysis of this study is presented in supplementary Table S1.

Alignments. Each gene was aligned separately. The 18S and 28S rDNA genes were aligned with MAFFT v.7 (https://mafft.cbrc.jp/alignment/server/), using default parameters. The mitochondrial protein-coding COI sequences were aligned with MEGA X using the invertebrate mitochondrial genetic code and the Muscle codon algorithm (Kumar et al. 2018). Before codon alignment, the COI sequences were individually examined for their correct reading frame using Geneious Prime software (v. 2019.2.3) (https://www.geneious.com/) to ensure the absence of any stop codon. Individual alignments were trimmed at the shortest sequence length, resulting in 1670, 1163, and 530 nucleotide columns for 18S, 28S, and COI genes, respectively. Next, all three alignments were concatenated into a single final matrix, resulting in 3364 sites.

Tree construction. The Maximum likelihood (ML) and Bayesian inference (BI) algorithms were used to build the phylogenetic trees. ML analyses were performed in IQ-TREE v.1.6.10 (Nguyen et al. 2015) with the following settings and considerations: i) the best-fit model according to BIC (Bayesian information criterion) for three partitions as such: SYM+I+G4 for 18S, GTR+F+I+G4 for 28S, and TVM+F+G4 for mtCOI; ii) edge-unlinked partition option, iii) 1000 ultrafast bootstrap pseudo-replicates with the SH-aLRT support activated to insure additional confirmation for Ultrafast bootsrap values (i.e., to consider clade confident with the values SH-aLRT ≥80% and UFbootstrap ≥95%); and iv) the rest of the parameters were left as default.

Bayesian analyses were performed in the program MrBayes v.3.2.7 (Ronquist et al. 2012) with the following settings and considerations: i) setting up all the parameters of evolutionary models as estimated with IQ-TREE (see above) except the TVM model for mtCOI partition. Since MrBayes does not have analogs to TVM model, we used GTR+F+G4 model because TVM is a model similar to GTR but with the A→G substitution rate equal to the C→T substitution rate and can be considered as an optimal option in this case as suggested by Yudina et al. (2021); ii) edge-unlinked partition option was considered, iii) five million Markov chain Monte Carlo (MCMC) simulations, with a sampling frequency of trees and parameters at 100, and with a relative burn-in fraction of 25%. Convergence of the MCMC analyses was confirmed with the in-built diagnostics of the program with the average standard deviation of split frequencies was 0.003791, the potential scale reduction factor converged to 1.00 for all parameters, the effective sample sizes (EES) of all parameters were >200 (i.e., min. ESS = 2251.764, av. ESS = 2356.053). ML and BI trees were computed as unrooted and then were rooted with the midpoint method in FigTree v.1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/), using the marine *Aspidiophorus* clade. The final tree was edited using CorelDraw X7 (Corel Corporation, Ottawa, Canada).

Results and discussion

Sampling sites

Although the two sampled lakes are located close to each other (Figure 1), there were some differences in the physical-chemical characteristics of their waters. Specifically, the water temperature was found to be two degrees higher in Lake 2, while water of Lake 1 had higher conductivity (Table I). While some water exchange between the two systems is possible because of their proximity and shared bed, the observed differences are likely due to the confinement of the two waterbodies and the depth from which the samples were collected, which was greater in Lake 1.

Gastrotrich fauna

Collection from the two sites yielded eight species for a total of nine records (species × sites/lake; Table II).

Table I. Characteristics of the investigated biotopes. Sampling sites, geographic coordinates, microhabitat characteristics and date of sampling.

Biotope	Lake	Geographic coordinates	Max depth of collection	Salinity	Temp.	Conductivity	Date of sampling
St. Anna lakes	1	44°35'06,88" N 10°59'46,77" E	−1.5 m	0.4 ppt	18.2 °C	877 µS	17/10/2022
	2	44°35'09,82" N 10°59'37,80" E	−0.5 m	0.4 ppt	20.5 °C	525 µS	17/10/2022

Table II. Gastrotrich species found at the two investigated St. Anna lakes in October 2022.

Species	Lake 1	Lake 2
Chaetonotus brevispinosus	X	
Chaetonotus heideri	X	-
Chaetonotus heterospinosus	X	-
Chaetonotus cfr. *hystrix*	X	-
Chaetonotus oculifer	-	X
Chaetonotus polites n. sp.	X	X
Chaetonotus sp.		X
*Heterolepidoderma lamellatum**	-	X

*Species new to the Emilia-Romagna region.

All species belong to the family Chaetonotidae, seven in the genus *Chaetonotus* and one in *Heterolepidoderma*. Five species were found in Lake 1 and four in Lake 2, with only a single species common to the two sites. In general, taxa were represented by few specimens and often by a single individual.

For the five positively identified species, the metric and meristic characteristics of the adult specimens are in substantial accordance with data reported in the literature (e.g., Schwank 1990). All five species were already known for the Italian fauna (e.g., Saponi & Todaro 2024). Four of them, i.e., *Chaetonotus brevispinosus* Zelinka, 1889, *C. heideri* Brehm, 1917, *C. heterospinosus* Balsamo, 1978 and *C. oculifer* Kisielewski, 1981 are reported to have a more comprehensive geographic range (e.g., Schwank 1990), with *C. heideri* even considered to be cosmopolitan (Krishnan et al. 2023). Conversely, *Heterolepidoderma lamellatum* Balsamo & Fregni 1995 is endemic to the Italian peninsula (Saponi & Todaro 2024). Initially described from the Nemi Lake (Central Italy), it was subsequently found in the Garda Lake (Northern Italy) Balsamo and Fregni (1995). The species is reported here for the first time in the Emilia-Romagna region, bridging the geographic gap between the two previous records.

Given the small number of specimens of the other two species reported in Table II, their complete identification was not possible. Consequently, they have been identified as *Chaetonotus* cfr. *hystrix* and *Chaetonotus* sp. On the other hand, the finding of several individuals belonging to an additional species allowed us to infer their phylogenetic position based on molecular traits besides an in-depth taxonomic examination, both of which suggest the establishment of a new species to allocate them (see below).

Molecular characterization of the new species

The ribosomal operon of the new species resulted of 5,708 nucleotides and was deposited in the NCBI GenBank database under the following accession number: PQ009211.

According to StructRNAFinder, the gene boundaries on the ribosomal operon are the following: 18SrRNA is situated on the nucleotide positions 1–1821, ITS1 from 1822 to 2134, 5.8S from 2135 to 2287, ITS2 from 2288 to 2470, and 28SrRNA from 2471 to 5708. While the GC content of the operon is 49%, for the individual regions it varies as as follows: 18S = 48%, ITS1 = 46%, 5.8S = 54%, ITS2 = 32%, and 28S = 50%. The whole mtCOI gene of the new species resulted of 1523 nucleotides and was deposited in NCBI GenBank database under the following accession number: PP996338.

The best BLAST hit identity for 18S gene on NCBI was with *Chaetonotus* aff. *euhystrix* MN496214 (97% identity; 3 gaps), and for 28S gene was with *Chaetonotus* aff. *euhystrix* MN496281 (95% identity; 11 gaps). The best BLAST hit identity for mtCOI gene on NCBI was with *Lepidodermella squamata* NC-026985 (82% identity; 2 gaps). NCBI Nucleotide blast software accessed on 20 January 2024 (results can vary depending on sequence length and taxa available in NCBI database).

The main diagnostic molecular autapomorphies of new species were identified from primary structure alignment, resulting in four for the 18S rRNA gene and two for the 28S rRNA gene, which is shown in the predicted secondary structures of these genes (see Figures 2, S1). Of the four autapomorphic traits present on 18S rRNA gene, three are situated on the 5'doman (i.e. nucleotide positions 126, 129, 514) and one on the C domain (i.e., nucleotide position 714) (Figure 2). The two autapomorphic traits present on the 28S rRNA gene are situated on the first and second domains (i.e. nucleotide positions 590, 874). Moreover, the new species in respect to the taxa of its sister clade (see below) shows an overall of 34 nucleotide differences in the 18S rRNA gene (Figure 2) and 50 in the 28S rRNA gene.

Molecular phylogeny

Phylogenetic analyses of the concatenated sequences show the genus *Chaetonotus* to be a non-monophyletic group, with several of its members resolved together with species of other genera along the paraphyletic Chaetonotidae branch (Figure 3). Notwithstanding, the new species appears as the sister taxon of a large, fully supported clade, including 30 specimens and 17 *Chaetonotus* species, most of which are currently affiliated to the subgenus *Hystricochaetonotus*

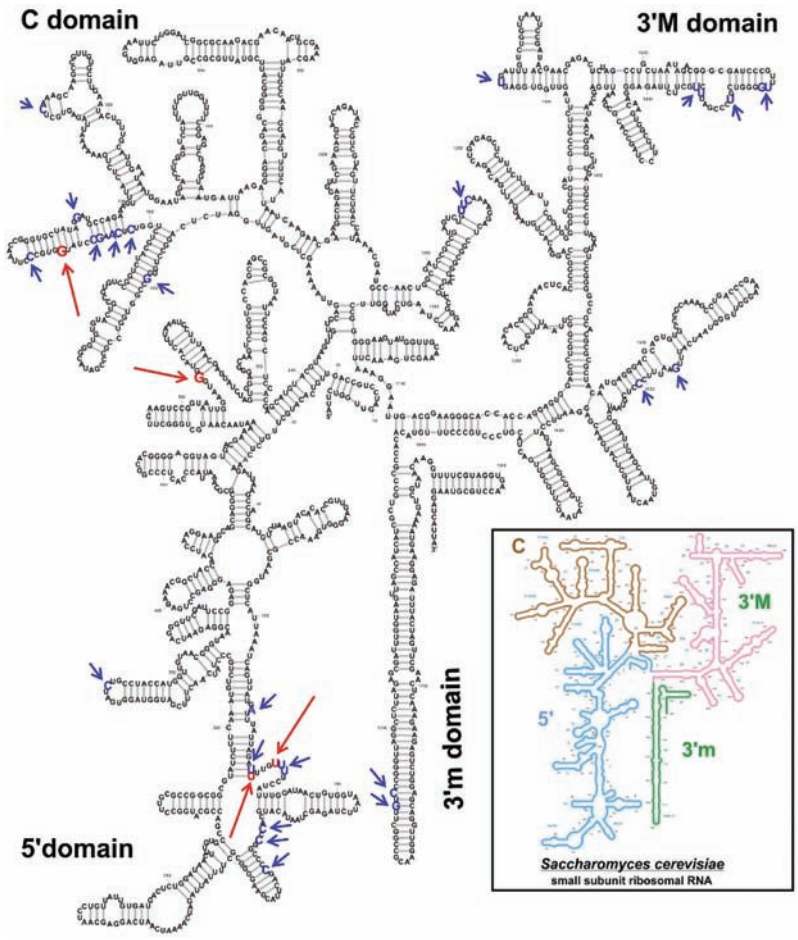


Figure 2. Secondary structure of 18S rRNA molecule of the new species. Diagnostic molecular autapomorphies are marked in red and indicated by long arrows. The nucleotide differences with taxa included in its sister clade (see text for details) are reported in blue and signposted by short arrows. The 18S secondary structure map of *Saccharomyces cerevisiae* (insert) was used as a reference.

Schwank 1990 (Figure 3). The exceptions are two species, *C. bombardus* Kolicka et al. 2018 and *C.* aff. *bombardus* (see Kolicka et al. 2020), currently affiliated with the subgenus *Chaetonotus sensu stricto* (Kolicka et al. 2018). However, based on their nested placement and specific alliances, the current multi-gene phylogenetic analysis indicates that the two latter species should be included in the subgenus *Hystricochaetonotus* instead. While the comparatively long branch separating taxa of this speciose clade from other *Chaetonotus* species is indicative of a distinct ancestry, and therefore supports the validity of the group (subgenus), the phylogenetic position of the new species and its anatomical uniqueness (see below) suggest a more conservative approach for its classification. Accordingly, a classification of the new species at a subgeneric level is not proposed here.

Taxonomic account

Order Chaetonotida Remane, 1925 [Rao & Clausen, 1970]

Suborder Paucitubulatina d'Hondt, 1971

Family Chaetonotidae Remane 1927

Subfamily Chaetonotinae Kisielewski, 1991

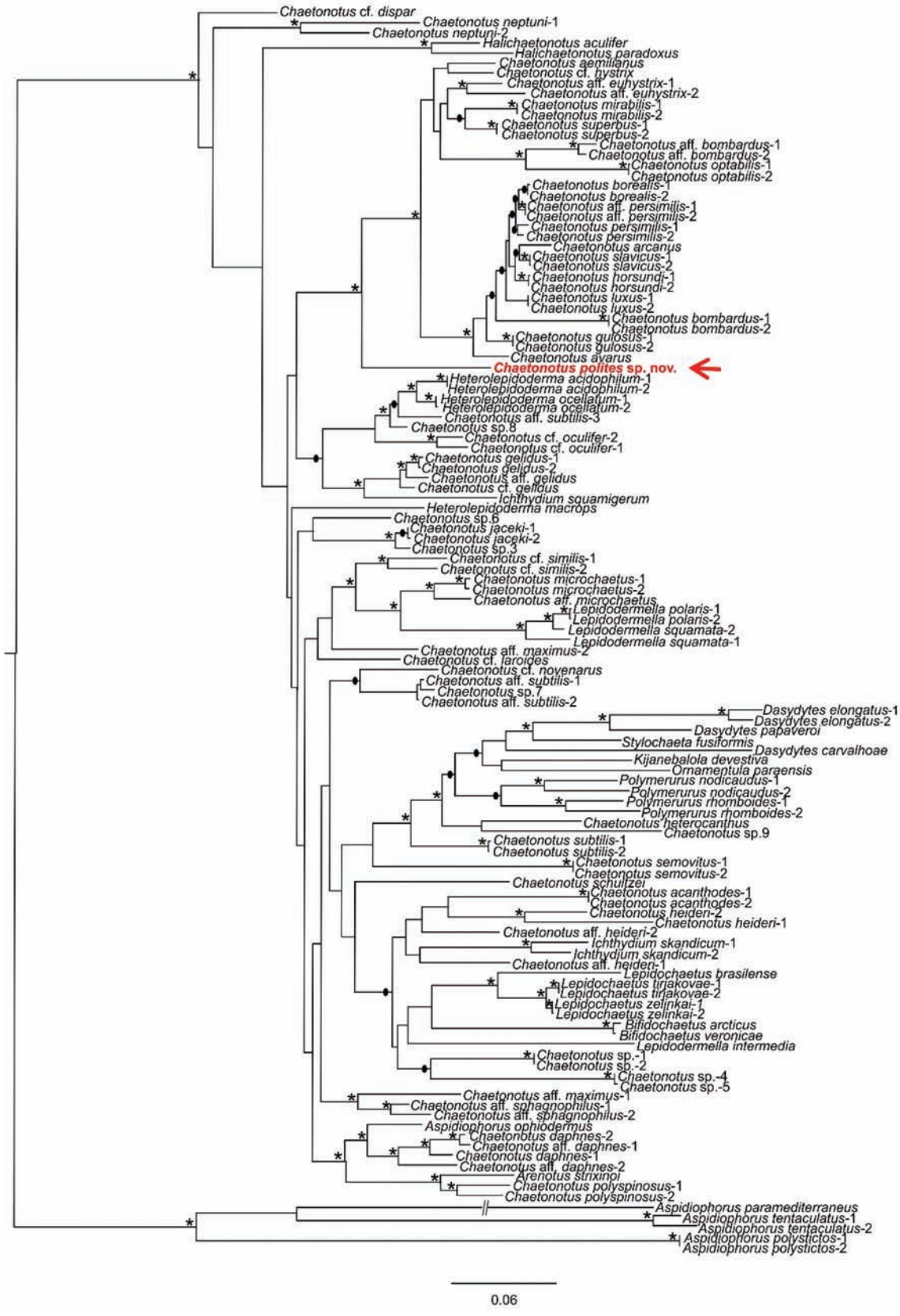
Genus *Chaetonotus* Ehrenberg, 1830

Chaetonotus polites n. sp.

(Figures 4–9)

Etymology

The specific name "*polites*" is an anagram of "*oplites*" to mirror the resemblance of the new species to *Chaetonotus oplites* Balsamo et al. 1994.


Chaetonotus cf. dispar
Chaetonotus neptuni-1
Chaetonotus neptuni-2
Halichaetonotus aculifer
Halichaetonotus paradoxus
Chaetonotus aemilianus
Chaetonotus cf. nystrix
Chaetonotus aff. euhystrix-1
Chaetonotus aff. euhystrix-2
Chaetonotus mirabilis-1
Chaetonotus mirabilis-2
Chaetonotus superbus-1
Chaetonotus superbus-2
Chaetonotus aff. bombardus-1
Chaetonotus aff. bombardus-2
Chaetonotus optabilis-1
Chaetonotus optabilis-2
Chaetonotus borealis-1
Chaetonotus borealis-2
Chaetonotus aff. persimilis-1
Chaetonotus aff. persimilis-2
Chaetonotus persimilis-1
Chaetonotus persimilis-2
Chaetonotus arcanus
Chaetonotus slavicus-1
Chaetonotus slavicus-2
Chaetonotus horsundi-1
Chaetonotus horsundi-2
Chaetonotus luxus-1
Chaetonotus luxus-2
Chaetonotus bombardus-1
Chaetonotus bombardus-2
Chaetonotus gulosus-1
Chaetonotus gulosus-2
Chaetonotus avarus
Chaetonotus polites sp. nov.
Heterolepidoderma acidophilum-1
Heterolepidoderma acidophilum-2
Heterolepidoderma ocellatum-1
Heterolepidoderma ocellatum-2
Chaetonotus aff. subtilis-3
Chaetonotus sp.8
Chaetonotus cf. oculifer-2
Chaetonotus cf. oculifer-1
Chaetonotus gelidus-1
Chaetonotus gelidus-2
Chaetonotus aff. gelidus
Chaetonotus cf. gelidus
Ichthydium squamigerum
Heterolepidoderma macrops
Chaetonotus sp.6
Chaetonotus jaceki-1
Chaetonotus jaceki-2
Chaetonotus sp.3
Chaetonotus cf. similis-1
Chaetonotus cf. similis-2
Chaetonotus microchaetus-1
Chaetonotus microchaetus-2
Chaetonotus aff. microchaetus
Lepidodermella polaris-1
Lepidodermella polaris-2
Lepidodermella squamata-2
Lepidodermella squamata-1
Chaetonotus aff. maximus-2
Chaetonotus cf. laroides
Chaetonotus cf. novenarus
Chaetonotus aff. subtilis-1
Chaetonotus sp.7
Chaetonotus aff. subtilis-2
Dasydytes elongatus-1
Dasydytes elongatus-2
Dasydytes papaveroi
Stylochaeta fusiformis
Dasydytes carvalhoae
Kijanebalola devestiva
Ornamentula paraensis
Polymerurus nodicaudus-1
Polymerurus nodicaudus-2
Polymerurus rhomboides-1
Polymerurus rhomboides-2
Chaetonotus heterocanthus
Chaetonotus sp.9
Chaetonotus subtilis-1
Chaetonotus subtilis-2
Chaetonotus semovitus-1
Chaetonotus semovitus-2
Chaetonotus schultzei
Chaetonotus acanthodes-1
Chaetonotus acanthodes-2
Chaetonotus heideri-2
Chaetonotus heideri-1
Chaetonotus aff. heideri-2
Ichthydium skandicum-1
Ichthydium skandicum-2
Chaetonotus aff. heideri-1
Lepidochaetus brasilense
Lepidochaetus tirjakovae-1
Lepidochaetus tirjakovae-2
Lepidochaetus zelinkai-1
Lepidochaetus zelinkai-2
Bifidochaetus arcticus
Bifidochaetus veronicae
Lepidodermella intermedia
Chaetonotus sp.-1
Chaetonotus sp.-2
Chaetonotus sp.-4
Chaetonotus sp.-5
Chaetonotus aff. maximus-1
Chaetonotus aff. sphagnophilus-1
Chaetonotus aff. sphagnophilus-2
Aspidiophorus ophiodermus
Chaetonotus daphnes-2
Chaetonotus aff. daphnes-1
Chaetonotus daphnes-1
Chaetonotus aff. daphnes-2
Arenotus strixinoi
Chaetonotus polyspinosus-1
Chaetonotus polyspinosus-2
Aspidiophorus paramediterraneus
Aspidiophorus tentaculatus-1
Aspidiophorus tentaculatus-2
Aspidiophorus polystictos-1
Aspidiophorus polystictos-2
0.06

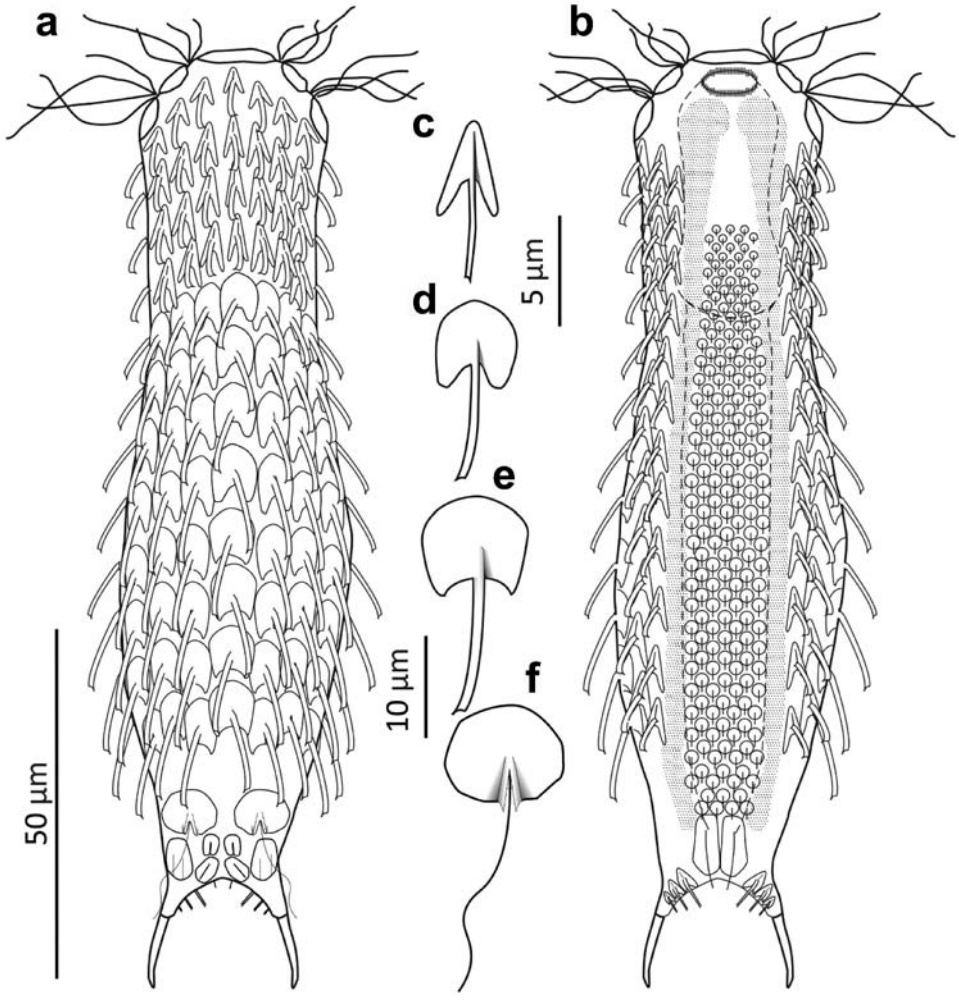


Figure 4. Schematic illustration of *Chaetonotus polites* n. sp. (a) Dorsal view; (b) Ventral view, showing also the pharynx and intestine (dotted lines); (c) Head scale; (d) Neck scale; (e) Trunk scale; (f) Bristle scale. The drawings are based mainly on the holotype.

Diagnosis

Rather stocky body; total length of 126.0 ± 10.8 µm, with furca measuring 17.2 ± 2.5 µm. Five-lobed head, with cephalic plates and four tufts of cilia. Body covered dorsally and dorsolaterally by nine alternating columns of partially imbricated scales, the median column is composed of 12 scales; scales trilobed in the cephalic part and pentagonal on the trunk, each bearing a simple, very thick, curved spine with a truncated apex, the length of which increases in the antero-posterior direction. Posterior to the spined scales, a pair of large, roundish, double-keeled scales carrying long tactile bristles, and three pairs of ovoidal keeled scales, the largest being more lateral. Ventrally there are four columns (two on each side) of small trilobed scales, bearing each a spine similar in shape but smaller in size than those born from the dorsal scales. Ventral interciliary field covered, from anterior to the pharyngo-intestinal junction up to the anal region, by eight alternating columns of round scales, each of which bears a short and thin spine; furcal base provided of three pairs of small, three lobed scales each bearing keel and short, simple spine. Mouth

←

Figure 3. Phylogenetic relationships of 123 chaetonotidans based on 18S rDNA, 28S rDNA and COI mtDNA concatenated alignment (merged maximum likelihood and Bayesian trees). An arrow points to the new species. The asterisk at the nodes indicates full support for both Bootstrap (1000 replicates) and posterior probability (BB/PP=100/1.00); the black ellipse indicates support >95% for both. The scale bar indicates the number of nucleotide substitutions per site.

relatively small (6.4 ± 0.5 µm in diameter) and sub apical; pharynx, 34.4 ± 3.2 µm in length, slightly swollen at both ends. Parthenogenetic.

Type locality

Italy, Modena, Sant'Anna (St. Anna lakes), Lake 1 (44°35'09.20"N, 10°59'54.60"E, Figure 1(b,d)). The specimens were found among the bottom sediment and, occasionally, in the water column. The site has the characteristics shown in Table I. The water was clear; the substrate made up of coarse sand mixed with mud and organic debris; the vegetation was mainly composed of species belonging to the genera *Phragmites* and *Leontodon*; moreover, algal masses are observed on the water's surface. Additional sites: A nearby lake (Lake 2, Figure 1(b,c)).

Type material

Holotype: the adult specimen shown in Figures 5–7 no-longer extant collected on 17/10/2022 (International Code of Zoological Nomenclature 19, Articles 73.1.1 and 73.1.4; see also recommendation 73 G–J of Declaration 45 – Addition of Recommendations to Article 73) (ICZN 1999, 2017). Additional studied specimens: five adults (showing a ripe egg inside) collected from the type locality and from the nearby Lake 2. All specimens were examined *in vivo* and went destroyed during the observation, except the holotype and another adult that were recovered from the slide, preserved in a 96% ethanol solution and subsequently used for molecular genetic analysis (see below).

Description

The description is mainly based on an adult individual of 135 µm in total length (Figures 4, 5; Table III). The body is rather stocky, with the neck region not clearly demarcated; the furca is short but marked. The head appears slightly five-lobed, with four tufts of cilia on the sides and the mouth in a subterminal position (diameter of 6 µm); the cephalic plates are represented by a large but slightly dorsally extended cephalion (7 µm long × 13 µm wide), two epipleuria and two hypopleuria; the hypostomion is apparently absent/not seen (Figures 4(a,b), 5(a,c), 9(a)). The width of the head/neck/trunk/furcal base is as follows: 29/25/31/17 µm at U09/U27/U54/U82 respectively. The furca has a length of 15 µm, of which the adhesive tubules represent 11 µm. The cuticular covering consists of 13 columns of spinated scales (7 dorsal, 1 + 1 dorsolateral and 2 + 2 ventrolateral), which extend over the entire body surface; the median column is composed of 12 scales (Figures 4(a), 5(a)). The scales gradually change morphology and increase in size, proceeding in an antero-posterior direction. In the head region, scales are clearly trilobed (6 µm long × 5 µm wide); at the same time, on the neck and trunk, they are pentagonal, with increasing dimensions in an antero-posterior direction (Figures 4(a, c-e), 7(a,c), 9(b)). The most anterior ones are 4 µm long × 5 µm wide, while the most caudal ones are 8 µm long × 10 µm wide. Each scale has a very thick, curved spine with a truncated apex, the length of which increases proceeding in an antero-posterior direction (from 8 µm on the head to 12 µm on the trunk). In the posterior dorsal region of the trunk, there is a pair of large polygonal scales (7 µm long × 8 µm wide), each characterized by two evident keels that converge frontally; a long tactile bristle emerges from the center of the two hulls (Figures 4(a,f), 7(d), 9). Posterior to the bristle' scales, there are three pairs of oval, keeled scales, the most lateral of which are larger and with two keels barely visible (Figures 4(a), 9).

On the ventral side, along the trunk region, there are four columns (two on each side) of small

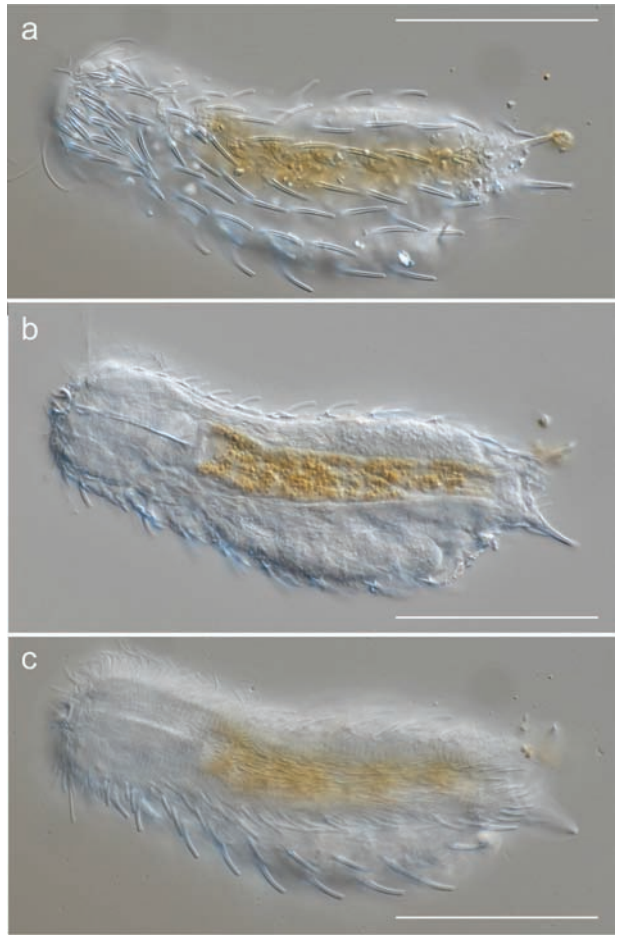


Figure 5. *Chaetonotus polites* n. sp., Holotype, habitus. (a) Dorsal view, (b) Internal view, and (c) Ventral view. Differential interference contrast microscopy (Nomarski). Scale bars, 50 µm.

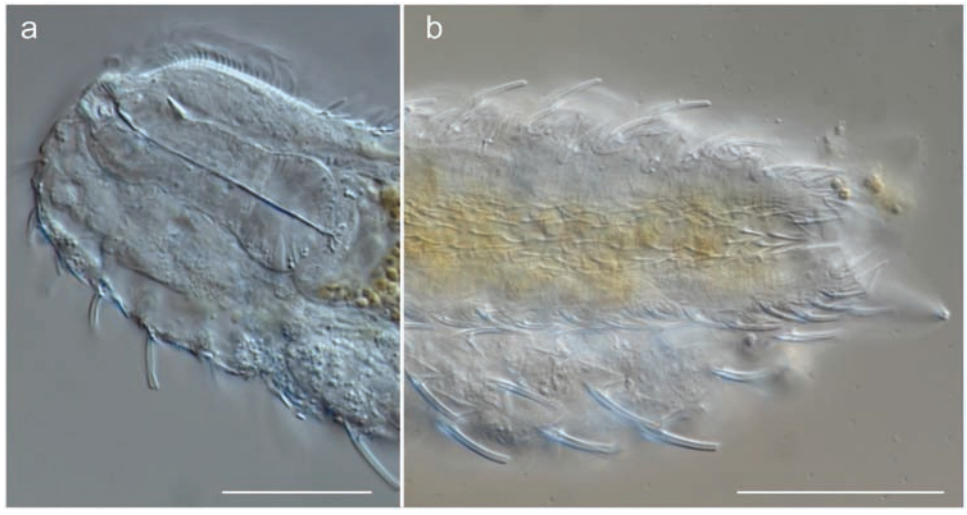


Figure 6. *Chaetonotus polites* n. sp. Holotype. (a) Anterior region, internal view, showing the pharynx; (b) Ventral view of the mid- and posterior trunk region. Differential interference contrast microscopy (Nomarski). Scale bars, (a), 20 μm; (b), 50 μm.

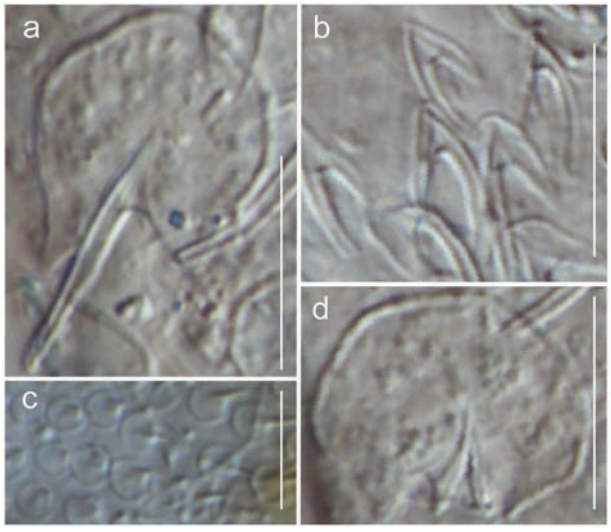


Figure 7. *Chaetonotus polites* n. sp. Holotype, details of the cuticular ornamentation. (a) Scale of the neck and trunk region; (b) Scale of the head; (c) Scales of the interciliary field; (d) Scale carrying the posterior bristle. Differential interference contrast microscopy (Nomarski). Scale bars, 10 μm.

trilobed scales, similar to the dorsal ones on the head (Figures 4(b), 5(c), 6(b), 8(b)); the number of column of these scales may be reduced to one per side in the anterior body region. The interciliary field is covered, from the region just anterior to the pharyngeal-intestinal junction (U18) down to the anal region (U80), by eight alternating columns of small, circular scales provided with a simple spine (Figures 4(b, 5(c), 6(b), 7(c), 8(b)). The scales' diameter and the spines' length progressively increase moving towards the caudal region. The most anterior scales have a diameter of 1 μm, the most caudal ones have a diameter of 3 μm; the spines vary from a length of less than 1 μm to about 3 μm. The ventral terminal scales are oval in shape (length = 5 μm, width = 3 μm), slightly keeled, and equipped with a short spine (4 μm). On the ventral side of the furcal base, there are three pairs of small (3.0–4.0 μm, trilobed, keeled scales, each carrying a simple, short spine (Figures 4(b), 8(b)).

The locomotor ciliation is organized into two longitudinal bands that extend from the posterior region of the mouth to the ventral terminal scales. The width of the two ciliary bands is wider in the anterior region and narrows posteriorly, but they remain always separated (Figures 4(b), 5(c), 8(b)).

The mouth is relatively small (diameter = 6 μm) and does not have particular internal cuticular reinforcements; the pharynx, 38 μm long, appears robust, slightly widened in the anterior region (width = 12 μm) and in the posterior region (width = 15 μm) (Figures 4(b), 5(b), 6(a)); in the mid portion, the pharynx reaches a width of 9 μm. The pharynx empties, via a pharyngo-intestinal junction (located at U 27), into a straight intestine, without particular differentiations, which ends with a ventral anus (U 80). All animals observed were in the parthenogenetic phase, often with a large egg (51 μm × 28 μm) in the central region of the trunk (U 51), dorsal to the intestine.

Variability and remarks

The shape and general characteristics of the other five specimens studied are similar to those of the holotype (Figures 8, 9; Table III).

Table III. *Chaetonotus polites* n. sp. Main taxonomic characters and measurements (in μm) of adult specimens. Specimen n. 1 is the holotype.

	Specimen						
Trait	1	2	3	4	5	6	Mean ± SD
Total length	135.0	-	111.0	132.0	118.0	134.0	126.0 ± 10.8
Pharynx length	38	34	37	-	30	33	34.4 ± 3.2
Furca length	15	21	18	14	18	17	17.2 ± 2.5
Adhesive tubes length	11	14	9	10	12	10	11.0 ± 1.8
Head width	29	-	30	-	26	30	28.8 ± 1.9
Neck width	25	-	23	-	23	28	24.8 ± 2.4
Trunk width	31	-	38	-	34	41	36 ± 4.4
Furcal base width	17	-	17	-	18	19	17.8 ± 1.0
Anterior pharynx width	12	-	12	-	12	13	12.3 ± 0.5
Mid pharynx width	9	-	9	-	9	11	9.5 ± 1.0
Posterior pharynx width	15	-	14	-	13	14	14 ± 0.8
Head scale length	6	5	6	-	-	8	6.3 ± 1.3
Head scale width	5	4	5	-	-	5	4.8 ± 0.5
Trunk scale length	8	10	7	8	9	8	8.3 ± 1.0
Trunk scale width	10	11	7	8	10	8	9.0 ± 1.5
Interciliary field scale length	3	-	3	-	-	5	3.7 ± 1.2
Interciliary field scale width	3	-	3	-	-	5	3.7 ± 1.2
Terminal interciliary scales length	5	-	5	-	-	-	5.0 ± 0.0
Terminal interciliary scales width	3	-	3	-	-	-	3.0 ± 0.0
Head spine length	8	9	9	8	9	8	8.5 ± 0.5
Trunk spine length	12	13	11	12	12	12	12.0 ± 0.6
Cephalion length	7	-	-	9	-	9	8.3 ± 1.2
Cephalion width	13	-	-	12	-	14	13 ± 1.0
Mouth diameter	6	6	6	-	7	7	6.4 ± 0.5
Bristle scale length	7	11	-	-	9	9	9.0 ± 1.6
Bristle scale width	8	13	-	-	9	9	9.8 ± 2.2
Egg length	51	65	70	-	57	66	61.8 ± 7.7
Egg width	28	-	35	-	29	40	33.0 ± 5.6

Differences among the studied specimens were found regarding i) the total length, varying between 111 μm and 135 μm, ii) the length of the pharynx ranging from 30 μm and 37 μm, iii) the length of the furca fluctuating between 30 μm and 38 μm. The length of the adhesive tubules was also variable among the individuals spanning 14–21 μm in length, and iv) the number of scales in the dorsal median column fluctuating from 11 to 13. The extents of these traits are generally related to the size of the individuals (Table III).

Taxonomic affinities

Chaetonotus currently includes 191 freshwater species, each displaying a range of cuticular ornamentations such as scales, spines, and plates. At first glance, the large number of species and the variety and the combinations of the morphological feature they possess may appear daunting when attempting to choose species for taxonomic comparisons. However, when it comes to the gastrotrichs from St. Anna, this task has been relatively straightforward for us. This is because they possess a distinctive trait rarely observed in other *Chaetonotus* species: very thick, curved dorsal spines with a trunked/concave apex.

Among *Chaetonotus* species, only three, namely *C. bifidispinosus* Tretjakova, 1991, *C. oplites* Balsamo et al. 1994, and *C. parafurcatus* Nesteruk 1991, possess this trait (see also couplet n. 66 in the dichotomous keys by Balsamo et al. 2019). The spines of the first species are simple, while those of the other two are barbed (show a lateral denticle). In *C. bifidospinosus*, the spines are simple, similar to the specimens from St. Anna, but with a swelling in the middle, which is absent in the St. Anna specimens. Furthermore, in *C. bifidospinosus*, spines originate from trilobate scales, whereas in the gastrotrichs from St. Anna, scales are polygonal in shape. These notable differences in the shape of the scales and spines allow for easy differentiation between specimens of the two taxa.

On the other hand, the specimens under study bear remarkable similarities with the Italian *Chaetonotus oplites* Balsamo et al. 1994, described from the island of Montecristo and also found in a small lake in the Tuscan-Emilian Apennines

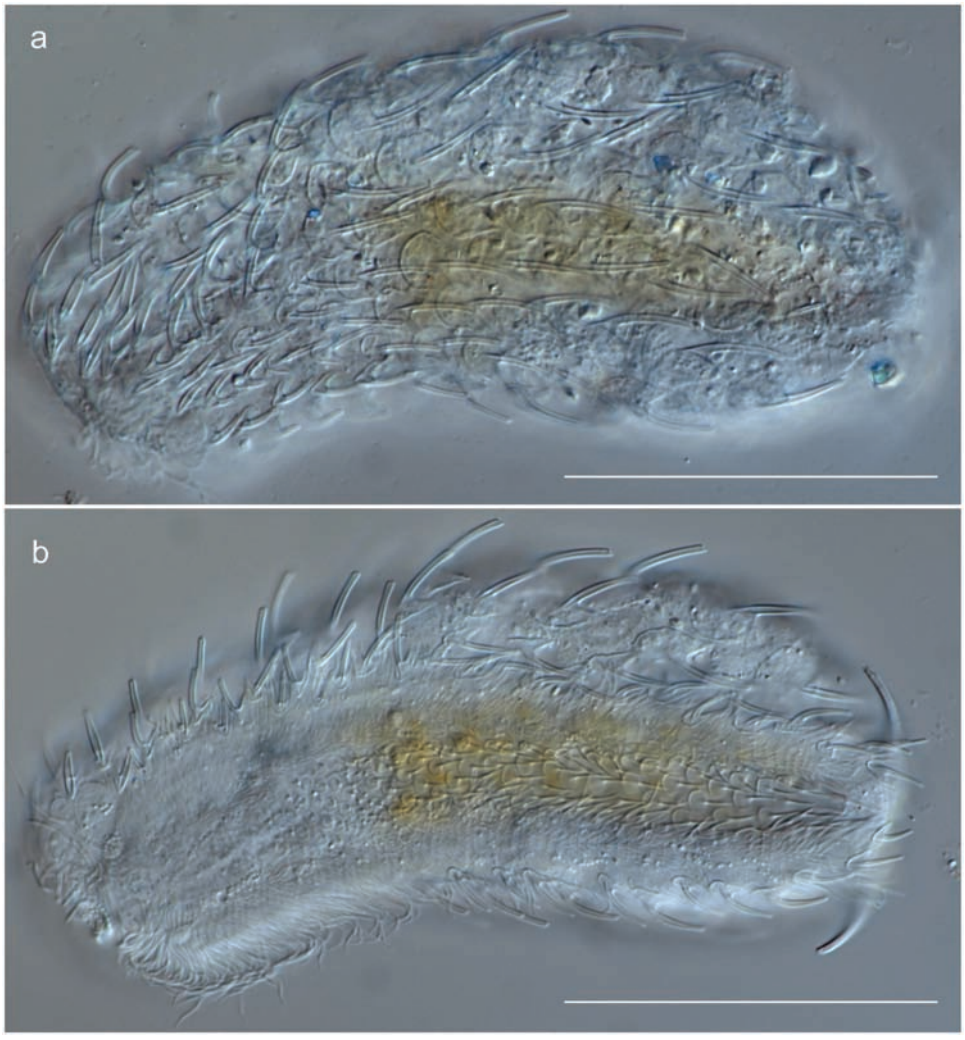


Figure 8. *Chaetonotus polites* n. sp. Another adult specimen. (a) Dorsal view; (b) Ventral view. Differential interference contrast microscopy (Nomarski). Scale, 50 μm.

(Balsamo et al. 1994; Saponi & Todaro 2024), and with *C. parafurcatus* Nesteruk 1991, reported from Lake Piaseczno in Poland (Nesteruk 1991).

Apart from the thick, curved dorsal spines, the resemblance between these species concerns the general appearance and the body size, but above all, the shape of the scales covering the trunk of the animals, which are somewhat pentagonal. The new species shares with *C. oplites* also the unusual presence of a pair of round, double keeled scales on the dorsal side of the furcal base (information not available for *C. parafurcatus*). Despite these similarities, the specimens under study are easily distinguished from the other two species because they carry simple spines rather than spines being equipped with an accessory denticle. Other differences, although more challenging to appreciate, distinguish the specimens inhabiting the St. Anna lakes from the other two species. In particular, from *C. parafurcatus* for the cephalion, which in the being described specimens adheres entirely to the head, while that of *C. parafurcatus* has a free posterior portion; from *C. oplites* for the pharynx shape, which in the specimens observed appears wider anteriorly and posteriorly, while in *C. oplites* it presents a widening only in the posterior part, and perhaps for the hypostomion, certainly reported in *C. oplites* but not seen in the individuals under study. Despite the shared similarities with the three species mentioned above, the specimens under study are unique and different enough to be classified as a new species, which we propose to name *Chaetonotus polites* n. sp.

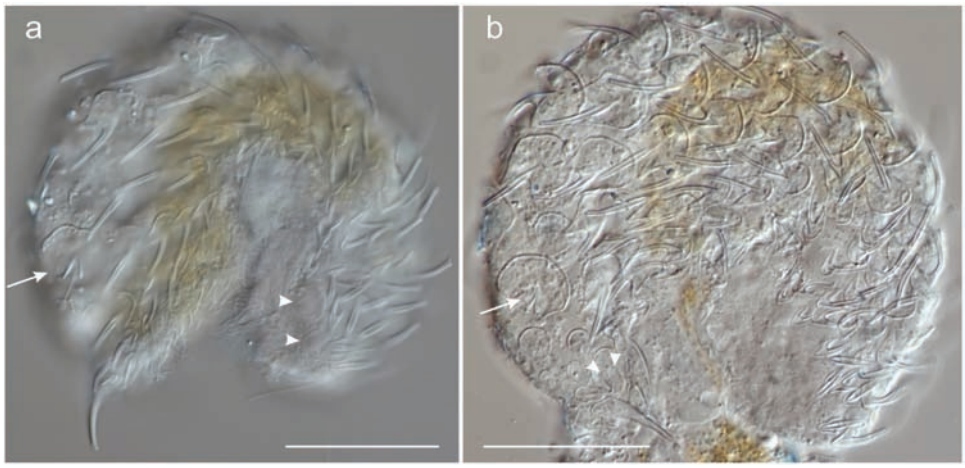


Figure 9. *Chaetonotus polites* n. sp. (a) Adult specimen in lateral view; the scale carrying the posterior bristle (arrow) and the epi- and hypopleuria (arrowhead) are visible; (b) Same specimen, more compressed, showing the dorsal and ventrolateral scales, the scale carrying the posterior bristle (arrow) and a posterior scale carrying two keels (arrowhead). Differential interference contrast microscopy (Nomarski). Scale bars, 50 μm.

Conclusions

The genus *Chaetonotus* has been found to be non-monophyletic based on molecular phylogenetic studies for quite some time now (e.g., Kånneby et al. 2013). The specific relationships among its members can vary depending on the taxa and the genes involved in the analyses. Our study confirms the results of recent studies with comparable taxonomic and gene sampling (e.g., Kolicka et al. 2020; Rataj & Vďačný 2022). As the main novelty, the present phylogenetic analysis (see Figure 3) includes *Chaetonotus polites* n. sp., which has been resolved to be the sister taxon of a large clade primarily consisting of *Chaetonotus* species belonging to the subgenus *Hystricochaetonotus*, except *C. bombardus* and *C.* aff *bombardus*, which are formally part of the *Chaetonotus* s.s. lineage (Kolicka et al. 2018; WoRMS 2024). Nevertheless, the current taxonomic systematization of these species is argued (Rataj & Vďačný 2022). For instance, the hypothesis that *C. bombardus* might be affiliated with the subgenus *Hystricochaetonotus* was discussed at the time of its description (Kolicka et al. 2018). However, since the concurrent phylogenetic analysis performed by Kolicka et al. (2018) had indicated the species only as the sister group of *Hystricochaetonotus*, the authors proposed its affiliation to the subgenus *Chaetonotus sensu stricto*. The decision was further guided by the fact that the spines of *C. bombardus* are simple, i.e., they lack the accessory denticle, a diagnostic trait of the *Hystricochaetonotus* species (Kolicka et al. 2018).

Recently, Rataj and Vďačný (2022) assigned to the subgenus *Hystricochaetonotus* three new species, even though their spines lack an accessory denticle (i.e. *C. avarus*, *C. hornsundi* and *C. optabilis*). The decision was made based on results from a molecular phylogenetic analysis showing these tree species clustering inside a large group of more "orthodox" *Hystricochaetonotus* species. This finding provided evidence that the presence/absence of a lateral denticle is a homoplastic feature and, consequently, of poor taxonomic value in the process of taxonomic classification above the species level (at least in this case). Accordingly, the authors also proposed the transfer of *Chaetonotus bombardus* and its kinds (e.g., *C.* aff. *bombardus*) to the subgenus *Hystricochaetonotus* (Rataj & Vďačný 2022).

Results of the current phylogenetic analysis support the resolutions proposed by Rataj and Vďačný (2022) on all the species mentioned above. In a larger framework, current molecular data analysis also corroborates their statement that *Hystricochaetonotus* is a valid/natural group (see Balsamo et al. 2009 for a different opinion) as testified by the strong statistical support at the node and the long evolutionary branch leading to it (Figure 3).

Regarding *C. polites* n. sp., the peculiar shape of the dorsal scales and of the scale carrying the posterior bristles suggest not to include the new species in the subgenus *Hystricochaetonotus* to avoid extending further the taxonomic/morphological boundaries of the subgenus. The relatively long phylogenetic distance leading to the *Hystricochaetonotus* cluster, the many nucleotide differences at the 18S and 28S rRNA genes, and the existence of two known species morphologically most similar to the new species further support this hypothesis. However, by

resolving the new species in a sister position to *Hystricochaetonotus*, the current phylogenetic study indicates a possible stem lineage of the latter group and the anatomical ground pattern from which the ample morphological disparity of this numerically significant clade has arisen.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

Author contributions

Conceptualization and resources: M.A.T.; methodology and data acquisition: all authors; writing: F. S. A.K., M.A.T.; funding acquisition: M.A.T. All authors have read and agreed to the published version of the manuscript.

Data availability statement

The data presented in this study are available in this published paper and supplementary materials.

Supplementary material

Supplemental data for this article can be accessed online at https://doi.org/10.1080/24750263.2024.2397473

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