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Invertebrate Systematics

Molecular phylogenetic position and description of a new genus and species of freshwater Chaetonotidae (Gastrotricha: Chaetonotida: Paucitubulatina), and the annotation of its mitochondrial genome

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ABSTRACT

Chaetonotidae is the most diversified family of the entire phylum Gastrotricha; it comprises ~430 species distributed across 16 genera. The current classification, established mainly on morphological traits, has been challenged in recent years by phylogenetic studies, indicating that the cuticular ornamentations used to discriminate among species may be misleading when used to identify groupings, which has been the practice until now. Therefore, a consensus is developing toward implementing novel approaches to better define species identity and affiliation at a higher taxonomic ranking. Using an integrative morphological and molecular approach, including annotation of the mitogenome, we report on some freshwater gastrotrichs characterised by a mixture of two types of cuticular scales diagnostic of the genera *Aspidiophorus* and *Heterolepidoderma*. Our specimens' overall anatomical characteristics find no correspondence in the taxa of these two genera, calling for their affiliation to a new species. Phylogenetic analyses based on the sequence of the ribosomal RNA genes of 96 taxa consistently found the new species unrelated to *Aspidiophorus* or *Heterolepidoderma* but allied with *Chaetonotus* aff. *subtilis*, as a subset of a larger clade, including mostly planktonic species. Morphological uniqueness and position along the non-monophyletic Chaetonotidae branch advocate erecting a new genus to accommodate the current specimens; consequently, the name *Litigonotus ghinii* gen. nov., sp. nov. is proposed. The complete mitochondrial genome of the new taxon resulted in a single circular molecule 14,384 bp long, including 13 protein-coding genes, 17 tRNA genes and 2 rRNAs genes, showing a perfect synteny and collinearity with the only other gastrotrich mitogenome available, a possible hint of a high level of conservation in the mitochondria of Chaetonotidae.

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Introduction

Gastrotricha Metschnikoff, 1865 is a phylum of microscopic invertebrates, ubiquitous and often abundant in various aquatic ecosystems of the world (Todaro *et al.* 2019). The phylum comprises ~895 species, in 2 orders: Macrodasyida Remane, 1925 (~380 species) and Chaetonotida Remane, 1925 (~515 species). Chaetonotidan gastrotrichs are unevenly split into 2 suborders: Multitubulatina d'Hondt, 1971 with only 3 species and Paucitubulatina d'Hondt, 1971 comprising ~517 species (Saponi and Todaro 2024). The latter taxon includes 2 families entirely marine or brackish (Muselliferidae Leasi & Todaro, 2008 and Xenotrichulidae Remane, 1927), 4 families entirely freshwater (Dasydytidae Daday, 1905, Dichaeturidae Remane, 1927, Neogosseidae Remane, 1927, and Proichthydidae Remane, 1927) and 1 family (Chaetonotidae Gosse, 1864) encompassing freshwater as well as marine forms (Balsamo *et al.* 2014; Kieneke and Schmidt-Rhaesa 2015). Chaetonotidae is also the most diversified family of the entire phylum; it

comprises ~430 species distributed across 16 genera; some genera exclusively inhabit either of freshwater or marine environments, whereas others may be found in both (Todaro *et al.* 2019). More specifically, 9 chaetonotid genera include exclusively freshwater representatives: *Arenotus* Kisielewski, 1987 (1 species), *Bifidochaetus* Kolicka & Kisielewski, 2016 (in Kolicka *et al.* 2016, 2 species), *Cephalionotus* Garraffoni, Araujo, Lourenço, Guidi & Balsamo, 2017 (1 species), *Fluxiderma* d'Hondt, 1974 (3 species), *Halichaetoderma* Rataj Križanová & Vďačný, 2023 (4 species), *Lepidochaetus* Kisielewski, 1991 (6 species), *Polymerurus* Remane, 1927 (16 species), *Rhomballichthys* Schwank, 1990 (1 species) and *Undula* Kisielewski, 1991 (1 species). Two chaetonotid genera are entirely marine: *Caudichthydium* Schwank, 1990 (3 species) and *Halichaetonotus* Remane, 1936 (31 species). The remaining 5 chaetonotid genera include both freshwater and marine species, with even some taxa recorded from both environments: *Aspidiophorus* Voigt, 1903 (24 freshwater species and 11 marine species), *Chaetonotus* Ehrenberg, 1830 (191 freshwater species and 47 marines, including 6 species reported mainly from freshwater habitats), *Ichthydium* Ehrenberg, 1830 (29 freshwater species and 3 marines), *Heterolepidoderma* Remane, 1927 (25 freshwater species and 15 marines) and *Lepidodermella* Blake, 1933 (15 freshwater species and 2 marines, including 1 species recorded mainly from freshwater habitat) (Todaro and d'Hondt 2023; Saponi and Todaro 2024).

The above-depicted classificatory scenario, established mainly on morphological traits, has recently been challenged by phylogenetic studies especially by those based on molecular sequence data (i.e. the *18S* rDNA alone or combined with the *28S* rDNA and the mitochondrial *COI* gene). Among the controversial aspects that bear relevance for the present work, it should be noted that molecular analyses have invariably indicated the family Chaetonotidae as paraphyletic since the members of the monophyletic freshwater families Neogosseidae and Dasydytidae have always been found nested within it (e.g. Kånneby *et al.* 2013; Kånneby and Todaro 2015; Garraffoni *et al.* 2017; Kolicka *et al.* 2020). This scenario is not much different from the one inferred by the morphological cladistics study of Hochberg and Litvaitis (2000), where Dasydytidae form a monophyletic group within a non-monophyletic Chaetonotidae, or from the results of Kieneke *et al.* (2008), in which Dasydytidae and Neogosseidae outline a clade within a non-monophyletic Chaetonotidae.

Moreover, the current classification of several genera requires further confirmation through molecular-based phylogenetic analyses. Interestingly, even if they share the same habitat, representatives of the same genus have been found not to cluster together, as seen in the case of *Aspidiophorus* and *Heterolepidoderma* species (e.g. Kånneby *et al.* 2013; Kånneby and Todaro 2015; Garraffoni *et al.* 2017). Even if the habitat is the same, representatives of the same genus have been found not phylogenetically allied, as in the case of *Ichthydium skandicum* Kånneby, Todaro & Jondelius, 2009 and *I. squamigerum* Balsamo & Fregni, 1995 plus *Ichthydium* sp. (Kolicka *et al.* 2020). Additionally, the genus *Chaetonotus*, which is the most speciose of the entire phylum, presents a taxonomically disruptive situation as its species are scattered along the evolutionary branch of Chaetonotidae and variously associated with representatives of other genera (e.g. Bekkouche and Worsaae 2016; Kolicka *et al.* 2020; Rataj Križanová and Vďačný 2024).

All in all, molecular phylogenetic studies suggest that the common understanding of cuticular ornamentations, such as scales and spines, may be misleading when used to identify groupings, which has been the practice until now. Therefore, a novel approach is needed to better define species identity, especially their affiliation to higher taxonomic ranking (e.g. at genus or family levels).

Recently, four new freshwater species similar to species of the genus *Heterolepidoderma* were discovered by Rataj Križanová and Vďačný (2024), who established the genus *Halichaetoderma* to allocate them. Although closely resembling *Heterolepidoderma*, molecular phylogenetic analyses revealed that *Halichaetoderma* is closely related to the marine *Halichaetonotus*. Stochastic mapping indicated that the characteristic oblong, keeled scales covering the bodies of the members of the three genera evolved through convergence at least four times (Rataj Križanová and Vďačný 2024), highlighting once again the inadequacy of cuticular characteristics in diagnosing groupings until their evolutionary origins are fully understood.

In this framework, we report on some gastrotrich specimens from an undescribed species found in summer 2021 in Tuscany (Italy). The phylogenetic analysis of the nucleotide sequence of the *18S* and *28S* ribosomal genes involving over 96 Paucitubulatina taxa supports the uniqueness of these gastrotrichs. Consequently, as repeatedly advocated, we employ a cutting-edge approach that integrates morphological and molecular data, along with mitochondrial genome information, to ensure the accurate taxonomic classification of these gastrotrichs.

Material and methods

Sampling site and sampling

Samples containing the gastrotrichs described herein were collected on 8 July 2021 from the larger freshwater pond of the botanical garden of the University of Pisa (43°43′16.60″N, 10°23′45.75″E). It is an artificial setting hosting many small and large invertebrates, vertebrates such as frogs (*Pelophylax* sp.), and protists. The pond is made up of two parts connected to each other. A circular shaped part is ~12 m in diameter. The narrowest part, digitiform, is ~13 m long and 3.10 m wide (4.10 m at the point where there is a small widening). The water level is

uniform and is ~60–70 cm deep. The pond, of artificial origin, was probably built at the beginning of the 20th century, in the part of the botanical garden called 'Orto del Gratta', located in the northernmost part of the complex. The pond hosts some aquatic plant species, such as the lotus, *Nelumbo nucifera* (Gaertn., 1788), *Nymphaea* sp., *Lemna minuta* Kunth, 1815 (which often forms a floating carpet above the water level), and *Ceratophyllum demersum* Linnaeus, 1753. The high metazoan biodiversity in the pond is likely due to repeated intentional introductions, possibly by local teachers who used the variety of fauna in zoology courses. Sampling was carried out by scooping up, with plastic jars and Falcon tubes, the thin layer of detritus covering the rocky and cemented pond edges down to a depth of ~50 cm. Two 0.5-L plastic jars and four 50-mL centrifuge tubes filled with water, some debris, and a little vegetation from the two long banks of the pond were collected. Samples were brought to the laboratory in Modena and analysed over 1 week. No special permission or permits were needed to collect the animals under study.

Sample processing and morphological analysis

Fauna was extracted by stirring the samples with a plastic pipette and aliquots of the sediment-water mixture were decanted into 5-cm diameter plastic Petri dishes and analysed under a Wild M8 stereo-microscope. Individual gastrotrichs were picked out with a hand-held micro-pipette and whole mounted on a slide in a drop of drop of 1% $MgCl_2$ solution. The morphological survey was carried out on living, relaxed specimens under a Nikon Eclipse 90i microscope provided with Nomarski optics (DIC) and fitted with a Nikon DSFi1 digital camera driven by the Nikon *NIS-Elements D* software (ver. 4.20, see https://www.microscope.healthcare.nikon.com/it_EU/products/software/nis-elements). Two identified specimens were retrieved from the slides, transferred to 0.5-mL centrifuge tubes filled with 96% ethanol, and used later in the laboratory at Pisa for molecular genetic analyses (see below). The description of the new species follows the convention of Hummon *et al.* (1992); the position of key morphological characters is given in percentage units (U) of total body length, measured from anterior to posterior end.

DNA amplification and sequencing

Starting from a single specimen, the total DNA material was amplified via the whole-genome amplification (WGA) method, using REPLI-g Single Cell Kit (QIAGEN, Hilden, Germany). In detail, the organism was washed in distilled water three times and the last time in Phosphate-Buffered Saline (PBS) solution (reagent provided in the kit). Then, it was transferred to a 0.2-mL Eppendorf tube together with 4 μL of PBS. The WGA protocol was completed following the manufacturer's instructions. The DNA material was processed with a Nextera XT library and sequenced at GENEWIZ Germany GmbH (Leipzig, Germany), using Illumina HiSeq X technology to generate 42,729,292 reads (paired-ends 2 × 150 bp).

Ribosomal operon and mitochondrial assembly and annotation

Preliminary assembly of resulting reads was performed using *SPAdes* software (ver. 3.6.0, see https://github.com/ablab/spades; Bankevich *et al.* 2012). Starting from this assembly, contigs (i.e. assembled sequences) belonging to either the rDNA operon and the mitochondrion were identified by blastn and tblastn analyses respectively. For the ribosomal operon, several *18S* and *28S* rDNA sequences were downloaded from the US National Center for Biotechnology Information (NCBI) to serve as queries for the blastn analysis, whereas the protein coding genes from the mitochondrion of *Lepidodermella squamata* (Dujardin, 1841) (ACC KP965862) were used as queries for the tblastn analyses. Reads mapped on those selected contigs were extracted from the original set and separately assembled using *SPAdes* in order to obtain the whole ribosomal operon and the whole mitochondrial genome in a single contig each. Prediction and annotation of Open Reading Frames (ORFs) was performed on the *MITOS* web server (see http://mitos.bioinf.uni-leipzig.de/; Bernt *et al.* 2013) setting the genetic code to 5, whereas prediction of rDNA genes on both the ribosomal operon and mitochondrial genome was performed with the *StructRNAfinder* web tool (see http://structrnafinder.integrativebioinformatics.me; Arias-Carrasco *et al.* 2018). Mitochondrial protein codon usage was calculated using a lab made script. The obtained *18S* rDNA and *28S* rDNA sequences were used for the phylogenetic analysis.

Phylogenetic analysis

The acquired *18S* rDNA sequence was aligned with the automatic aligner of the *ARB* software package (ver. 5.5, see http://www.arb-home.de; Westram *et al.* 2011) on the SSU ref NR99 SILVA database. To implement the *28S* rDNA sequence in our analyses, starting from the work of Kolicka *et al.* (2020), we selected a suitable set of Paucitubulatina representatives of the phylum Gastrotricha for which both *18S* and *28S* rDNA sequences were available (Supplementary Table S1). We decided to not include the *COI* genes in our phylogeny because for several organisms the gene is missing: using it only for some of them might have created artifacts in the branch lengths and stability of the nodes. After downloading the selected sequences, they were aligned using the *MAFFT* online tool (see https://mafft.cbrc.jp/alignment/server/; Katoh *et al.* 2019) together with the *28S* rDNA sequences of the new species. For the phylogenetic analysis, 92 concatenated *18S* and *28S* rDNA sequences belonging to representatives of the families Chaetonotidae, Dasydytidae, and Neogosseidae were selected, plus four sequences

belonging to the family Xenotrichulidae as the outgroup, for a total of 96 sequences. Both alignments were manually edited to optimise base pairing, and the *18S* nucleotide matrix was also trimmed at the shortest sequence length. The *18S* rDNA matrix and the *28S* rDNA matrix contained respectively 1697 and 3632 nucleotide columns and were concatenated to obtain a final matrix of 5329 columns (see Supplementary File S1). We also performed an additional analysis excluding outgroup sequences and rooting the resulting tree at midpoint, to assess the influence of the outgroup as a possible cause of the Long Branch Attraction phenomenum (see Rataj Križanová and Vďačný 2024).

For our dataset, the optimal substitution model was selected with *jModelTest* (ver. 2.1, see https://github.com/ddarriba/jmodeltest2; Darriba *et al.* 2012) according to the Akaike Information Criterion (AIC). The maximum likelihood (ML) tree was calculated with *PHYML* (ver. 5.3.2, see http://www.atgc-montpellier.fr/phyml/; Guindon and Gascuel 2003) software using the GTR + G + I substitution model, performing 1000 pseudoreplicates. Bayesian inference (BI) tree was inferred with *MrBayes* (ver. 3.2, see https://github.com/NBISweden/MrBayes/; Ronquist *et al.* 2012), using the GTR + G + I substitution model, three runs each with one cold and three heated Monte Carlo Markov chains, with a burn-in of 25%, iterating for 3,000,000 generations.

Results

Molecular phylogeny

The ribosomal operon of the new species resulted of 5682 nucleotides and was deposited in the NCBI GenBank database under Accession number OR915722.

The best BLAST hit identity on NCBI was with *Chaetonotus* aff. *subtilis* MN496228, (99.03%; 2 gaps, 17 mismatches). The topologies identified by the ML-based tree and the BI-based tree generally aligned with each other. However, it is important to note that they differed at some points, such as the placement of the marine *Aspidiophorus* clade (refer to Fig. 1 *v*. Supplementary Fig. S1). Despite these differences, both phylogenetic analyses placed our concatenated *18S–28S* rDNA sequence as sister of sequences belonging to *Chaetonotus* aff. *subtilis* (see Kolicka *et al.* 2020) with high statistical support and altogether inside a supported clade composed by *Dasydytes* spp., *Stylochaeta* spp., *Haltidytes squamosus* Kisielewski, 1991, *Neogossea antennigera* (Gosse, 1851), *Kijanebalola devestiva* Todaro, Perissinotto & Bownes, 2013, and *Chaetonotus heterocanthus* Remane, 1927 (Fig. 1). However, most internal nodes had weak support, casting doubt on the relationships within the supported clade.

A close phylogenetic relationship between *Litigonotus ghinii* gen. nov., sp. nov. and members of *Aspidiophorus* or *Heterolepidoderma* genera is not supported by our analysis. In the broader framework, both the latter two genera are shown to be polyphyletic. At the same time, the family Chaetonotidae appears paraphyletic due to the nested position of members of Dasydytidae and Neogosseidae (Fig. 1). These results are consistent with the outcome of the phylogenetic analyses that excluded Xenotrichulidae as an outgroup (Supplementary Fig. S2), providing robustness to the information obtained on these specific points.

Mitochondrial genome analysis

The complete mitochondrial genome of *Litigonotus ghinii* gen. nov., sp. nov. resulted in a single circular molecule 14,384 bp long, and has been deposited in NCBI under the Accession number PP105008. Its genome content includes 13 protein coding genes (mainly related to energy production), 17 tRNA genes and 2 rRNAs genes (Fig. 2*a*). Overall, its structure does not possess any striking features or unusual genes when compared to other metazoan mitochondrial genomes. The only other available mitochondrial genome of a gastrotrich belongs to *Lepidodermella squamata* (Golombek *et al.* 2015), and comparison of their structures showed a perfect synteny and collinearity between them (Fig. 2*b*). Also the analysis of codon usage in both of the mitochondria showed no outstanding variation in the two organisms (Supplementary Fig. S3).

The strict similarity in terms of both codon usage and gene order between the mitochondrial genome of the new species and *L. squamata* is indeed remarkable (Fig. 2, Supplementary Fig. S3), especially considering the phylogenetic distance between the two taxa (Fig. 1). Even if we understand that with just two mitochondrial sequences available it is premature to draw any conclusions, this could be a hint of a high level of conservation in the mitochondria of Chaetonotidae. The high conservation could probably be linked to the habitat and the reproductive biology of these animals, which includes ubiquitous parthenogenesis and clonal lineages.

Taxonomy

Order **CHAETONOTIDA** Remane, 1925 (Rao & Clausen, 1970)

Suborder **PAUCITUBULATINA** d'Hondt, 1971

Family **CHAETONOTIDAE** Remane, 1927

Litigonotus Gammuto, Serra, Petroni & Todaro, gen. nov.

ZooBank: urn:lsid:zoobank.org:act:ABE647F5-F58F-4CFC-B0D8-39B79F0FF7A

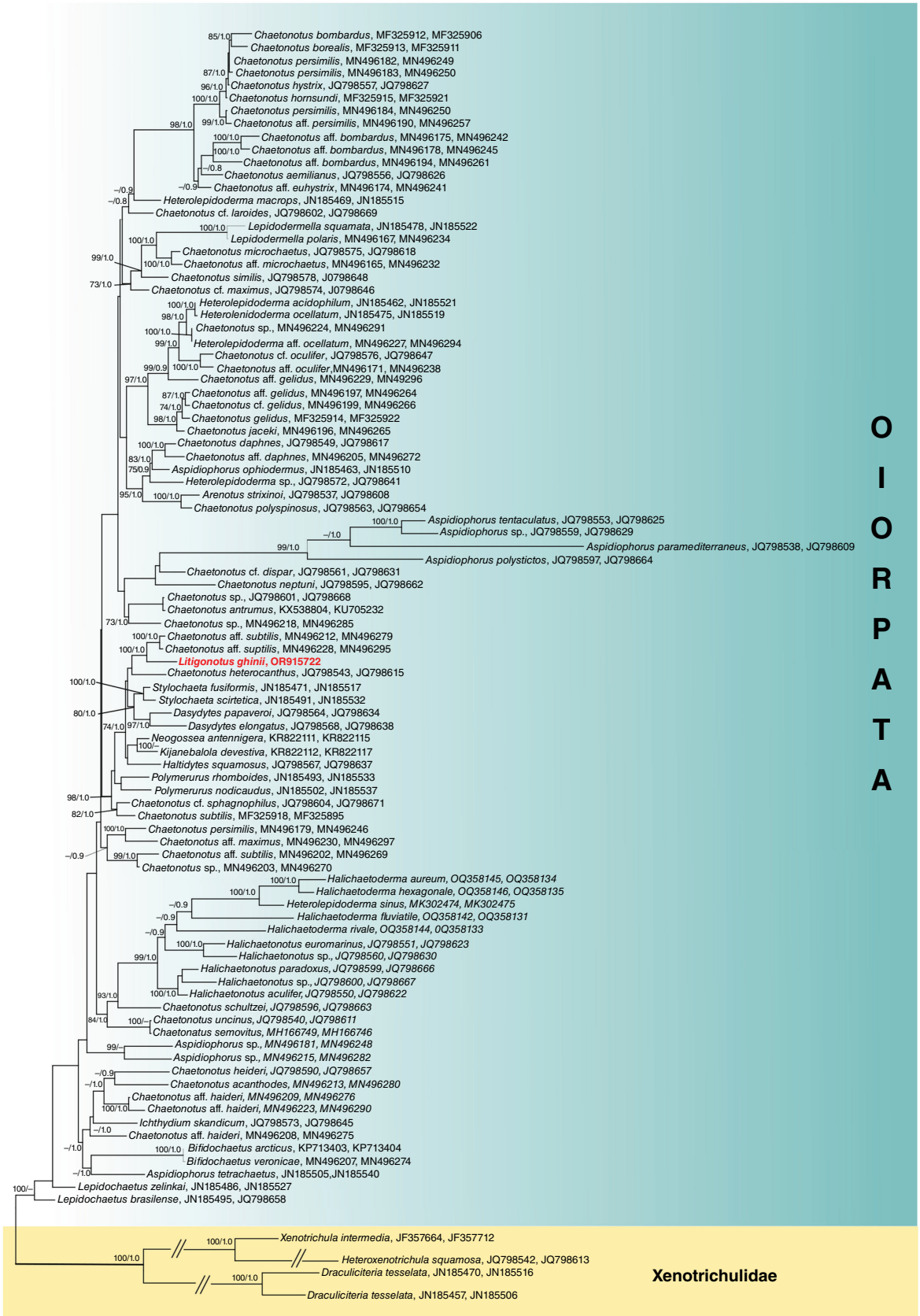

Fig. 1. (*Caption on next page*)

Fig. 1. Phylogenetic tree of suborder Paucitubulina. Phylogenetic tree based on the concatenation of *18S* and *28S* rDNA genes. The topology is based on the maximum-likelihood analysis. The two Accession numbers shown next to the names of the organisms are *18S* rDNA and 28rDNA respectively. Number pairs associated with nodes represent bootstrap values and posterior probabilities respectively (only values above 70 and 0.80 are shown). Values referring to posterior probability are reported only if the node is present in both the maximum-likelihood and the Bayesian inference based trees (see Supplementary Fig. S1). Sequence obtained in the present work is highlighted in red.

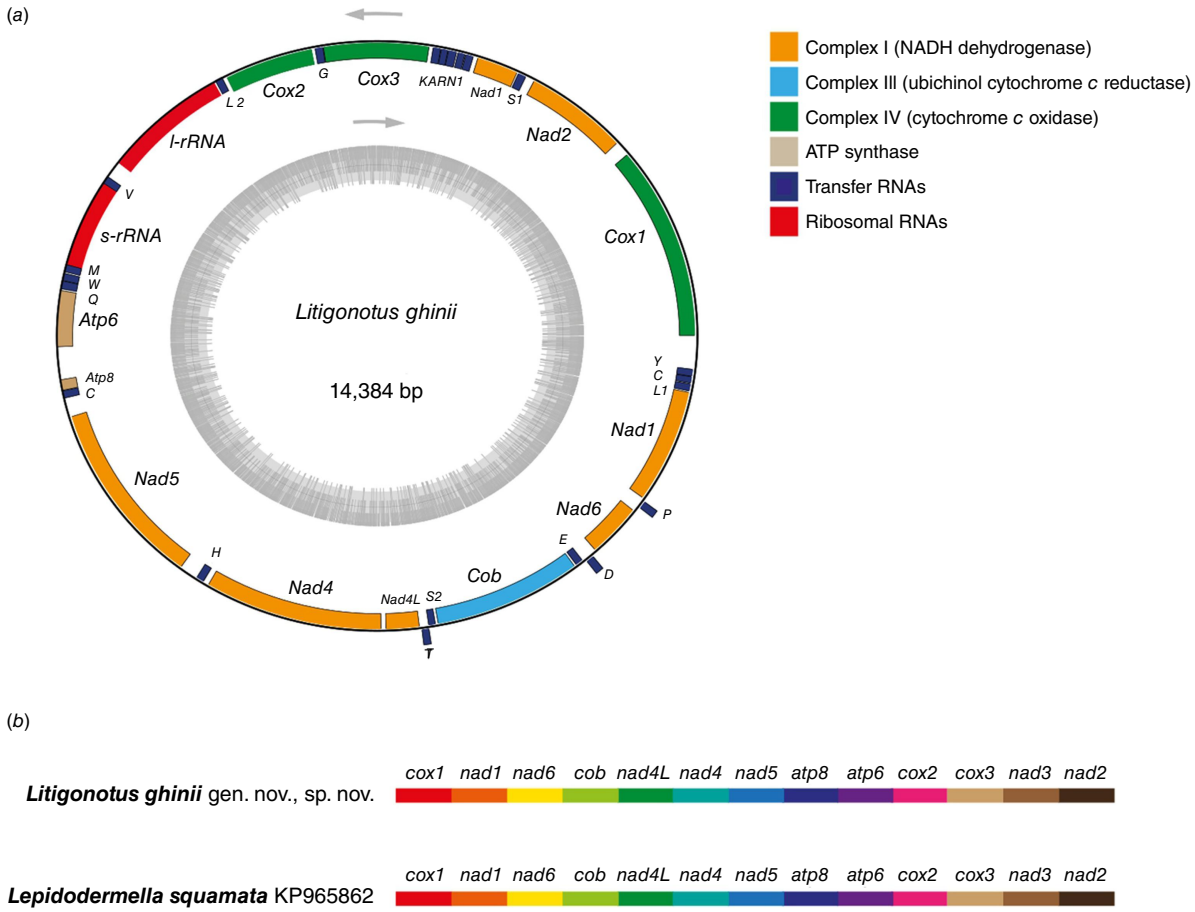

Fig. 2. Mitochondrial gene map of *Litigonotus ghinii* sp. nov. The gene map of the mitochondrion of *L. ghinii* (*a*) and a schematic representation of both *Litigonotus ghinii* and *Lepidodermella squamata* mitochondria, to show the complete synteny between them (*b*). Arrows indicate the direction of transcription, whereas the grey inner circle indicates the GC content of the corresponding region.

Etymology

From the Latin *litigo* (dispute) and *notus* (known), alluding to the pedunculated scales shared by several gastrotrich lineages.

Morphological diagnosis

Body stout, up to 148 μm long, tenpin-shaped, terminating in a furcate caudum; head slightly pentalobed, bearing cephalion, epi- and hypopleurae, and hypostomion; neck ill demarked; furca up to 18 μm long. Neck and trunk regions covered dorsally and lateroventrally by 34–45 alternating 34–36 pedunculated scales, up to 5 μm long and 3 μm wide; scales widen towards their rear end, just overlapping, and are strengthened by a weak keel extending for half their length; on dorsal side of the furcal base there are 8–10 pairs of non-pedunculated, keeled, elliptical scales; elliptic to oval keeled scales cover also the ventral proximal portion of the furcal branches; interciliary area fully covered by 9–10 alternating columns of pedunculated, keeled scales. One pair of round perianal keeled scales ~4–5 μm in size present. Sensorial ciliary elements distributed in two paired tufts of 4–8 cilia on the head and paired bristles on the neck, and on the posterior trunk region. Bristles of the neck region generally shorter, originating directly from the body; posterior bristles originating from round to triangular scales, each provided by two anteriorly converging keels. Ventral locomotor cilia distributed in two separate longitudinal bands extending from under the head to the furcal base; each band is approximately club-shaped anteriorly, but narrowing

considerably from the posterior pharyngeal region. Mouth ~5 µm in diameter, projecting slightly ventrally and leading into a 31 µm long pharynx; pharynx very muscular, showing a robust bulb at each end; two cuticular teeth are visible within the anterior bulb; pharyngeo-intestinal junction (PhIJ) at U22; intestine straight, wider anteriorly, anus ventral at U88. A pair of conspicuous, tubular protonephridia are present in trunk region, extending from past the PhIJ to approximately mid-body; parthenogenetic. Laid egg, 60 × 38 µm in size with egg shell smooth, without ornamentation.

Molecular diagnosis

18S rRNA gene: 246 T, 287 T, 521 T, 1402 A, 1411 C, 1421 A, 1438 C.

28S rRNA gene: 123 A, 460 A, 474 G, 525 T, 610 G, 639 T, 645 C, 647 A, 664 T, 667 A, 670 C, 736 G, 764 T, 772 A, 858 C, 859 A, 887 C, 946 G, 948 A, 963 T, 1803 A.

Analysis of molecular autapomorphies was carried out using the closest sequences in the phylogeny shown in Fig. 1, namely *Chaetonotus aff. subtilis* MN496212, MN496279; *Chaetonotus aff. subtilis* MN496228, MN496295; *Chaetonotus heterocanthus* JQ798543, JQ798615; *Dasydytes papaveroi* JQ798564, JQ798634; *Dasydytes elongates* JQ798568, JQ-798638; *Stylochaeta fusiformis* JN185471, JN185517; *Stylochaeta scirtetica* JN185491, JN185532. Nucleotide positions correspond to the reference alignments files, provided in Supplementary Files S2 and S3.

Litigonotus ghinii Gammuto, Serra, Petroni & Todaro, sp. nov.

(Fig. 3–8.)

ZooBank: urn:lsid:zoobank.org:act:EA943DD6-B6CC-43E8-AF69-7AF3ACDB73AE

Morphological and molecular diagnosis

As the genus.

Etymology

The new species is named after Prof. Luca Ghini (1490–1556) founder of the Botanical Garden where the species was first found.

Type locality

Italy, Pisa: the pond of the Botanical Garden (43°43′16.60″N; 10°23′45.75″E) epibenthic on organogenic detritus.

Type material

Holotype: the 146 µm long adult specimen shown in Fig. 4 and 5, no longer extant, collected on 8 July 2021 (International Code of Zoological Nomenclature, Articles 73.1.1 and 73.1.4; see also recommendation 73G–J of Declaration 45 – Addition of Recommendations to Article 73), (International Commission on Zoological Nomenclature 1999, 2017). Additional studied specimens: four adults (showing a ripe egg inside) and five subadults, collected from the type locality; all were examined *in vivo* and weredestroyed during the observation, except the holotype and another adult that were recovered from the slide, preserved in a 95% ethanol solution and subsequently used for molecular genetic analysis (see below). Another adult specimen was found in a sample collected from the same locality or pond on 1 December 2022.

Description

The description is mainly based on the holotypic specimen, 146 µm in total body length. Body medium sized, tenpin-shaped; head slightly pentalobed, neck faintly narrower than head, trunk sac-like, terminating in a furcate caudum. Body widths at the head/neck/trunk/caudum and their locations along the body are 26/25/48/18 µm, at U04/22.5/56/86.5 respectively. Caudum rather short (16.7 µm long), paired laterally divergent adhesive tubes (10.5 µm long) with a slightly enlarged base (6.2 µm), covered by scales (Fig. 3*a*, *d*, 4*a*–*c*).

Cuticular armature

Head bearing frontally and dorsally an obvious, approximately pentagonal, cephalion (12 × 9 µm), laterally small (7 × 4 µm) epi- and hypo-pleurae and ventrally a trapezoidal hypostomion (7 × 3 µm) (Fig. 3*b*, *c*, 4*a*–*c*); neck and trunk covered dorsally and lateroventrally by 35 alternating columns (19 dorsal and 8 + 8 lateroventral) of 34–36 pedunculated scales (Fig. 3*a*, *e*, 4*b*, *c*, 5*a*, *c*). The scales widen towards their rear end, just overlapping, and are strengthened by a weak keel extending for half their length (Fig. 3*e*, 5*a*, *c*, 6*e*, *f*). The median column of scales is straight, whereas the columns on either side slowly follow the lateral body outline (Fig. 4*b*). Scales increase slightly in size from the head to past mid-trunk, reducing again toward the rear end. Scales on the dorsal trunk measure and 5–4 µm long and 2–3 µm wide, the peduncle is ~0.5–1.0 µm high (Fig. 3*e*, 6*c*, *f*). The scales of the lateral and especially ventrolateral columns are smaller, down to half the size of the dorsal ones. On posterior trunk region, at U85, are two round to subtriangular, double keeled scales (5 × 4 µm) from each of which emerge a sensorial bristle (Fig. 3*a*, 6*a*, *d*). On the dorsal side of the furcal base 8–10 pairs of non-pedunculated, keeled, elliptical scales are present (Fig. 3*a*, *f*, 5*b*); elliptic to oval keeled scales cover also the proximal portion of the ventral side of the furcal branches (Fig. 3*d*). The interciliary area appears fully covered from under the head to the anal region (U09–U87) by 9–10 alternating columns of pedunculated, keeled scales (Fig. 3*c*, *d*, 4*c*, 5*c*, *d*). The interciliary field scales appear similar to the dorsal and ventrolateral ones but smaller (~2 µm) and with the keel that spans the entire length of the scale. At the furcal indentation, two oval to round keeled scales (~4–5 µm) are present (Fig. 3*d*, 5*d*).

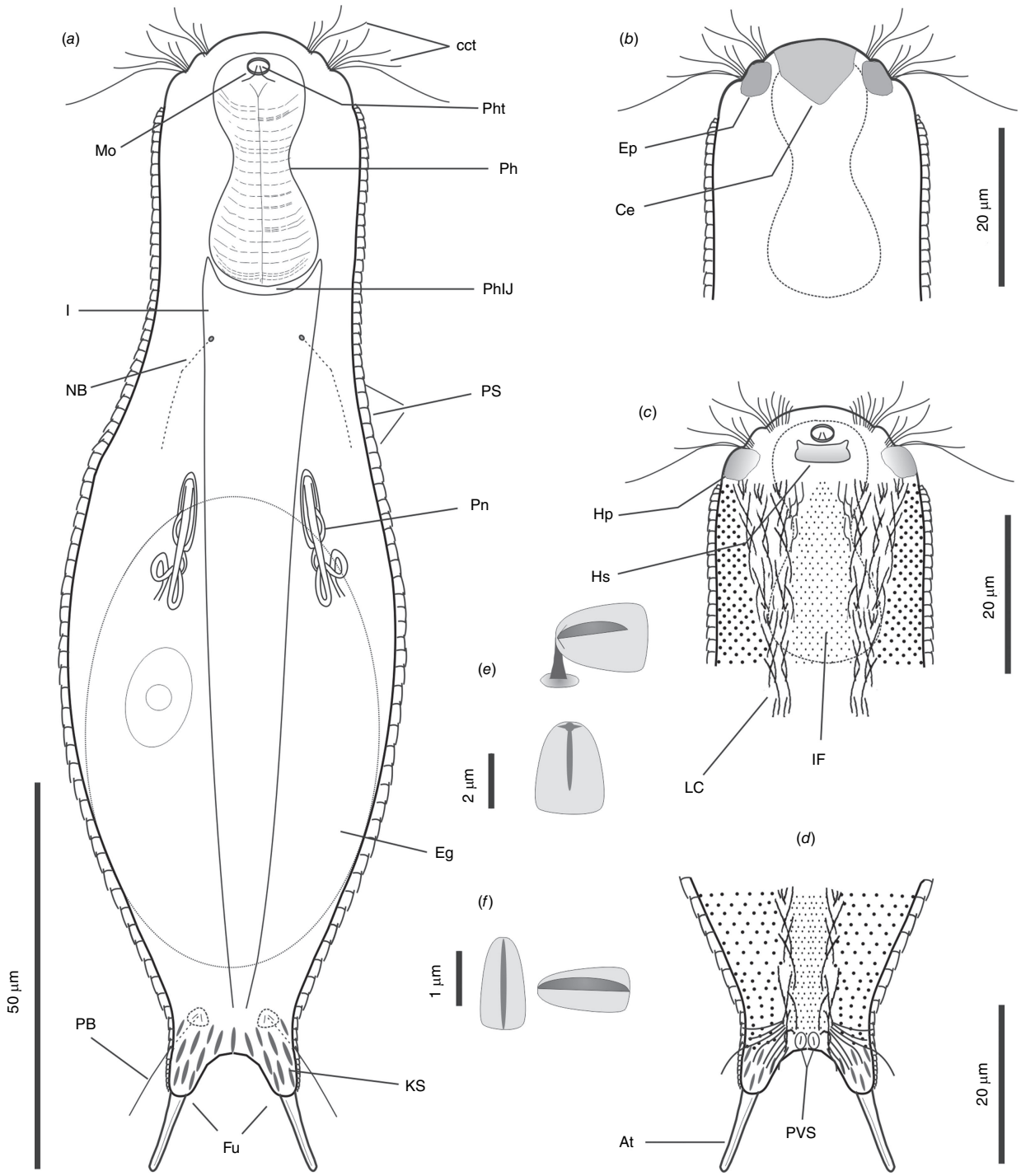

Fig. 3. Line art illustrations of *Litigonotus ghinii* gen. nov., sp. nov. (*a*) Habitus, ventral view, showing the internal organisation. (*b*) Anterior region, dorsal view. (*c*) Anterior region, ventral view. (*d*) Posterior region, ventral view. (*e*) Pedunculed scale. (*f*) Keeled scale. Drawings are made mostly from the holotypic specimen. At, adhesive tube; Cct, cephalic cilia tufts; Ce, cephalion; Eg, egg; Ep, epipleura; Fu, furca; I, intestine; IF, interciliary field; Hp, hypopleura; Hs, hypostomion; KS, keed scale; Lc, locomotor cilia; Mo, mouth; NB, neck bristle; PB, posterior bristle; Ph, pharynx; PhT, pharyngeal teeth; PhIJ, pharyngo–intestinal junction; Pn, protonephridium; PS, peduncled scales; PVS, posterior ventral scales.

Ciliation

Head sensorial cilia are distributed into two paired tufts of 4–8 elements, 5–16 µm long (Fig. 1*a–c*). Two pairs of sensory bristles (13–32 µm long) are present on the dorsal side on the neck and posterior trunk region, at U26 and U87 respectively (Fig. 3*a*, 6*a*, *c*). The bristles of the anterior pair

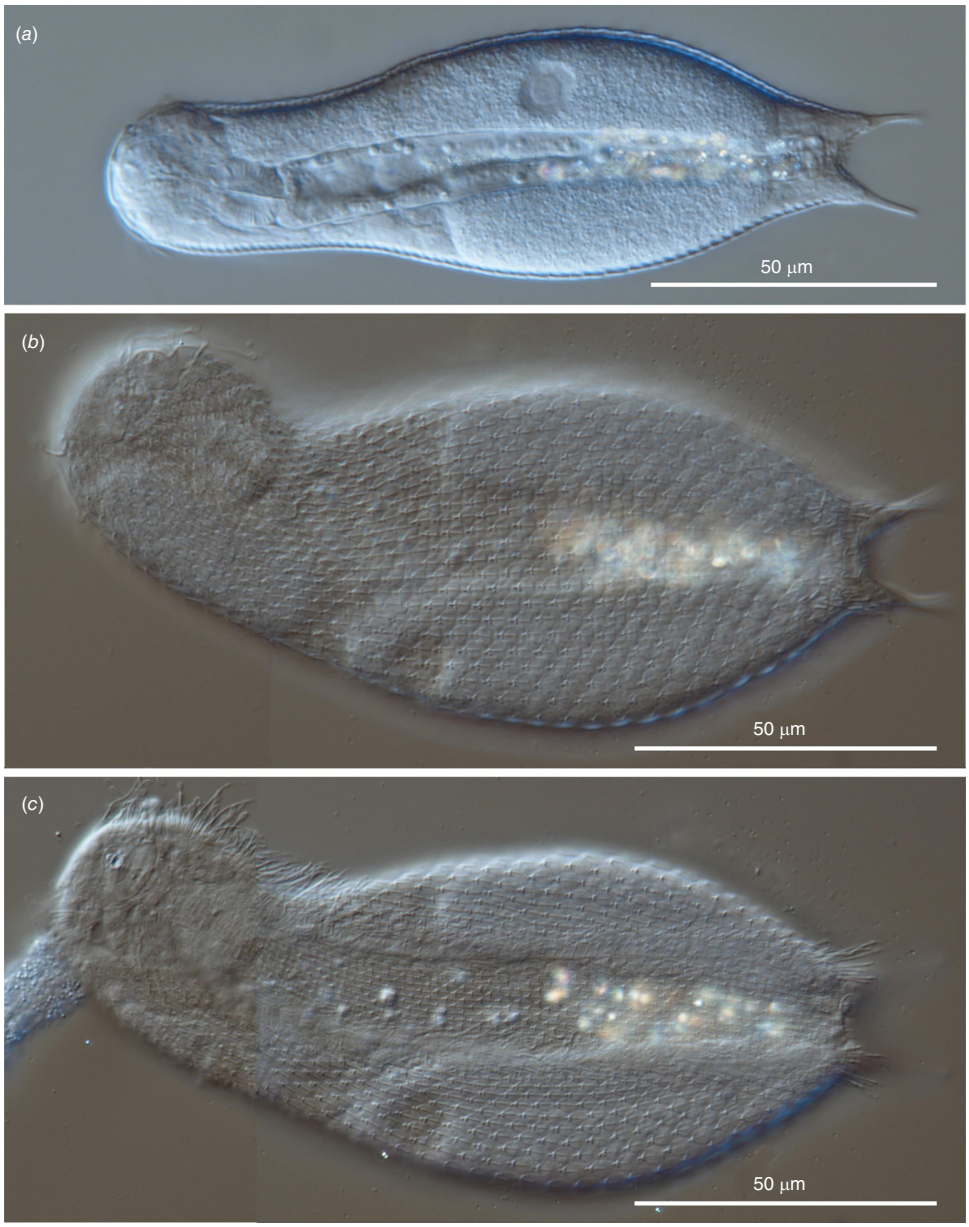

Fig. 4. Differential interference contrast photomicrographs showing the morphology of the holotypic specimen of *Litigonotus ghinii* gen. nov., sp. nov. (*a*) Habitus outlines. (*b*) Habitus, dorsal view showing the cuticular covering. (*c*) Habitus, ventral view showing the cuticular covering including the scales of the interciliary field.

generally are shorter and apparently originate directly from the cuticle, whereas posterior bristles originate from scales each provided by two anteriorly converging keels. Presence of additional sensory bristles hidden among the cephalic ciliary tufts cannot be excluded. Ventral locomotor cilia are distributed in two separate longitudinal bands extending from U09 to approximately U88 (Fig. 3*c*, *d*, 6*a*, 7*b*); each band is approximately club-shaped anteriorly, but narrowing considerably from the posterior pharyngeal region; bands approach each other immediately behind the hypostomion, but remain separate throughout their entire length; individual cilia are ~8–10 μm long; two denser tufts of longer cilia (10–14 μm) are present in the posterior region (U88) (Fig. 3*d*, 5*d*).

Digestive tract

Mouth of small size (~4–5 μm in diameter), projecting slightly ventrally (Fig. 3*a*, *c*, 7*b*) and leading into a 31 μm long pharynx (Fig. 3*a*, 4*b*, 7*b*); pharynx very muscular, showing a solid bulb anteriorly (11 μm in diameter) and posteriorly (14.5 μm); two cuticular teeth are visible within the anterior bulb (Fig. 3*a*); pharynx connected to a sack-like intestine; pharyngeo-intestinal junction at U22; intestine straight, wider anteriorly, narrowing posteriorly, anus ventral at U88.

Nephridial system

A pair of conspicuous, tubular protonephridia are present in trunk region, extending from past the pharyngo-intestinal

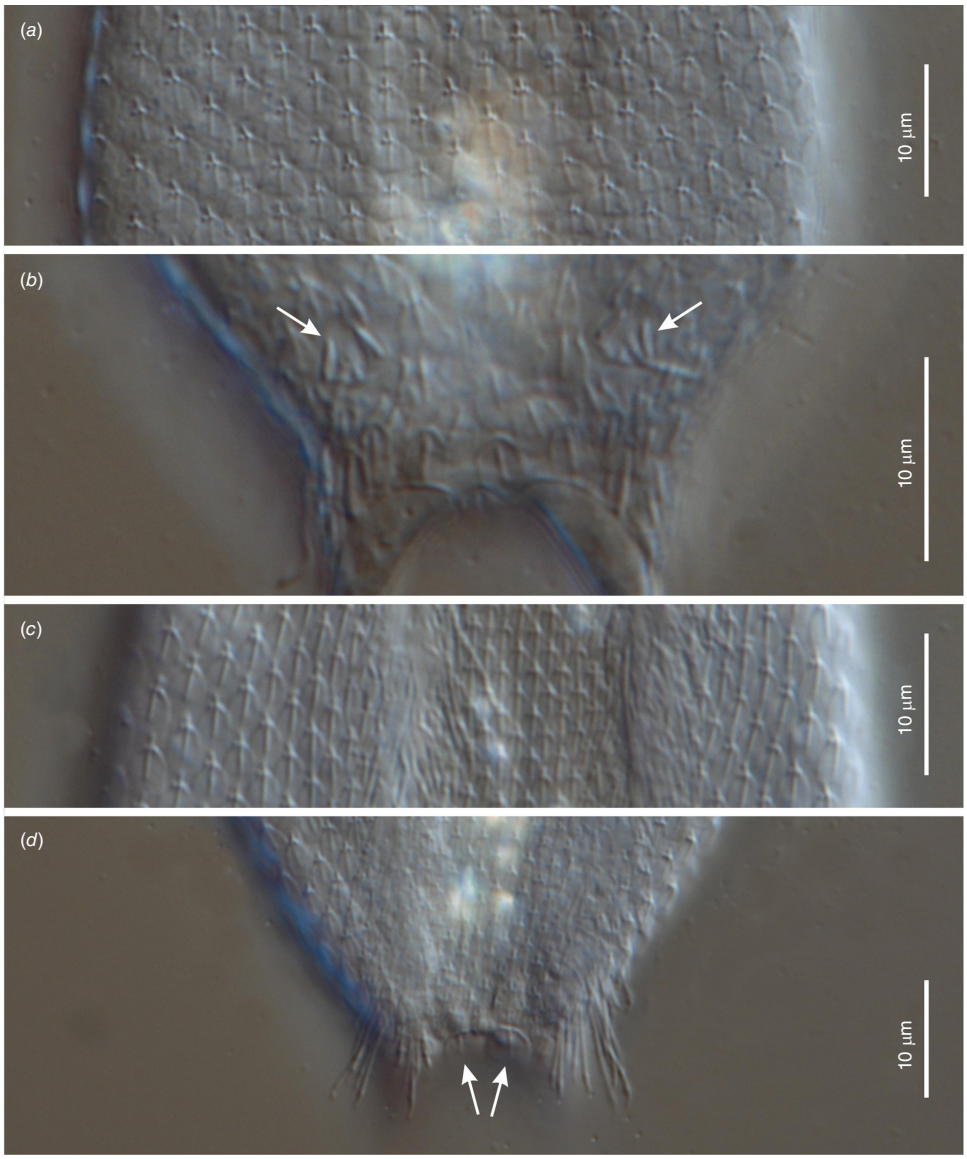

Fig. 5. Differential interference contrast photomicrographs showing morphological details of of the holotypic specimen of *Litigonotus ghinii* gen. nov., sp. nov. (*a*) Close up of the mid-trunk, dorsal view, showing the cuticular covering made of pedunculated scales. (*b*) Close up of the posterior trunk region, dorsal view, showing the cuticular covering made of keeled scales; arrows indicate the double keeled scales carrying the posterior bristles. (*c*) Close up of the mid-trunk region, ventral view, showing the pedunculated scales covering also the interciliary field. (*d*) Close up of the posterior trunk region, ventral view, showing the two perianal keeled scales (arrows).

junction (U38) to approximately mid-body (U49); each protonephridium includes a proximal, straight portion containing (1–2) vibrating flagella, and somewhat convoluted posterior region that apparently empties outwards (Fig. 3*a*, 8*a*).

Reproductive tract

Adult specimens were in the parthenogenetic phase (i.e. no developing sperm were seen) showing a large egg filling much of the trunk region (Fig. 3*a*, 4*a*, 6*a*). During the observation, one of the specimens released its egg (60 × 38 µm). The shell of the laid egg appears smooth, without ornamentation (Fig. 6*e*).

Variability and remarks

The general appearance and the cuticular characteristics of the other studied adult specimens reflect those of the holotype (e.g. head plates, pedunculated scales covering most of the body, non-pedunculated, keeled scales covering the posterior end, a rather short pharynx bearing two robust bulbs) (Fig. 6*a*, 7*c*). Some variability concerned (i) the total body length, which ranged from 135 to 148 µm (mean = 139.5 µm, s.d. = 3.4, n = 3); (ii) the furca length, spanning from 16.0 to 18.0 µm (mean = 16.8 µm, s.d. = 1.0, n = 3); notably, the size of the adhesive tubes did not vary among the measured specimens (constantly 10.2 µm long); (iii) the number of the alternating columns of scales, which numbered as low as 34 and as high as 45 (mean = 38.2 µm, s.d. = 4.6, n = 4). These data indicate the new taxon is characterised by rather limited morphological intraspecific variability. One trait seemed not to fit this statement: the maximum width at the mid trunk, which varied from 42 to 58 µm; however, this significant variation depends on the development stage of the egg inside the specimen; therefore,

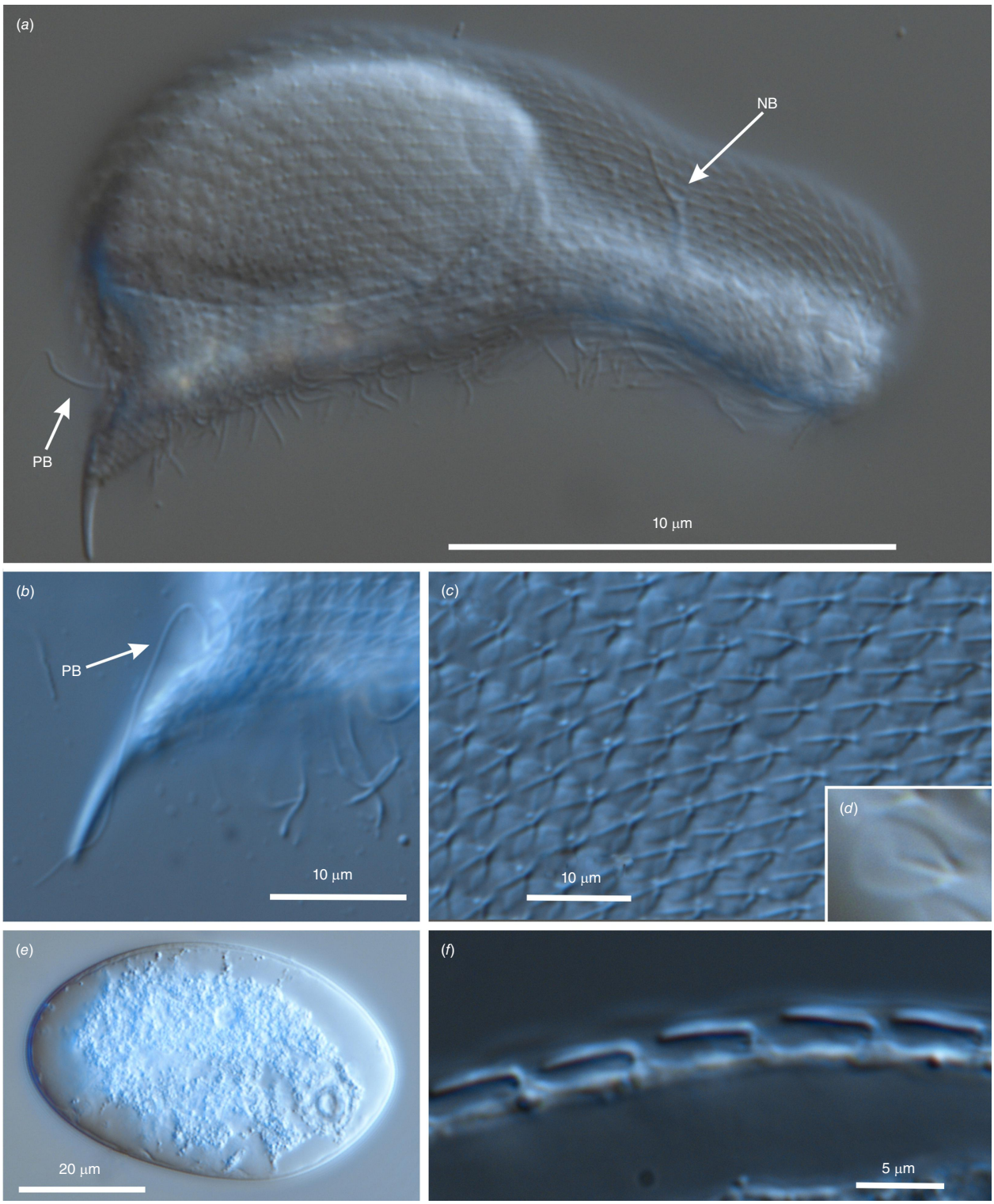

Fig. 6. Differential interference contrast photomicrographs showing the morphology of *Litigonotus ghinii* gen. nov., sp. nov. (*a*) An adult specimen with a large egg inside, lateral view, arrows indicate neck and posterior bristles. (*b*) Detail of the posterior region, lateral view, showing the furca and the posterior bristle (BP). (*c*) Close up of the mid-trunk region, lateral view, showing the cuticular covering made of pedunculated scales. (*d*) Close up of a scale. (*e*) The egg immediately after being laid. (*f*) Detail of the dorsal trunk region, lateral view, showing the scales originating from a peduncle.

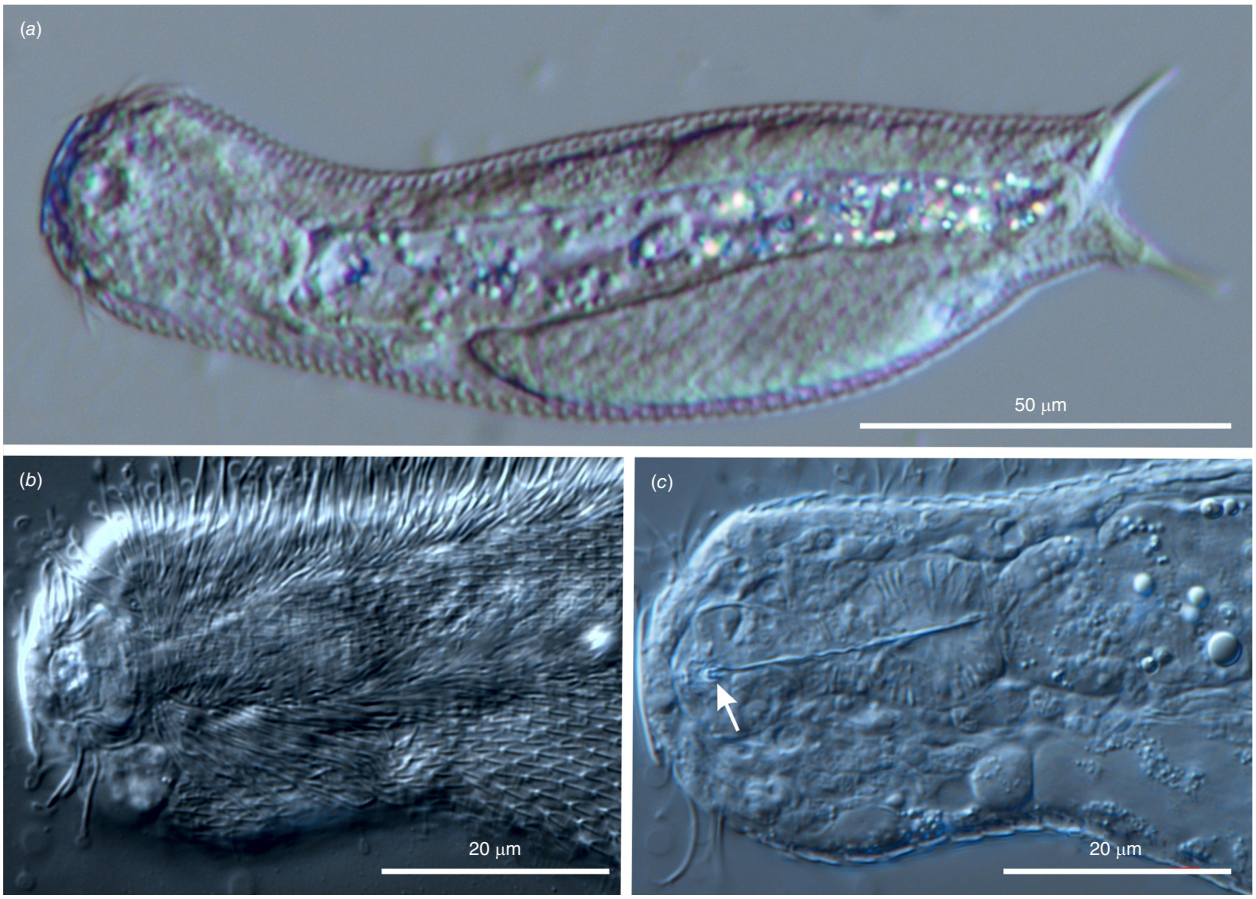

Fig. 7. Differential interference contrast photomicrographs showing the morphology of *Litigonotus ghinii* gen. nov., sp. nov. (*a*) Habitus of an adult specimen, ventrolateral view. (*b*) Close up of the anterior region, ventral view, showing locomotor cilia, interciliary field, mouth and hypostomion. (*c*) Same region at different focal plane, showing the pharynx with two bulbs, and the pharyngeal teeth (arrows).

the trunk width is an unreliable trait for comparisons among specimens.

The general look of the five individuals without an appreciable egg inside (subadults) is very similar to that of the adults (Fig. 8); the main difference regards the body length, which varied from 114 to 128.8 µm (mean = 122.7 µm, s.d. = 6.4, n = 5); notably, the pharynx seems to reach quite soon the full length during the growth of these gastrotrichs since the smallest specimen had a pharynx 28 µm long whereas that of the longest was 30 µm, the same length as the pharynx of the holotype.

Taxonomic and phylogenetic affinities

Based on morphology, and especially on the form of the cuticular scales, the studied specimen could be affiliated by current taxonomic criteria either to the genus *Aspidiophorus*, which includes species bearing pedunculated scales (Voigt 1902; Todaro *et al.* 2019), or to *Heterolepidoderma*, which includes species possessing keeled scales (Remane 1927; Garraffoni and Melchior 2015; Todaro *et al.* 2019). Since pedunculated scales cover most of the body of the studied specimens, their affiliation to *Aspidiophorus* would seem most appropriate. Such a taxonomic decision has been made recently also for the Swedish marine species *Aspidiophorus gullmarsfjordensis* Kånneby & Todaro, 2017, whose members are covered mainly by pedunculated scales but also bear a few keeled scales on the posterior trunk region (Kånneby and Todaro 2017). Although the Scandinavian and Tuscan species share the unusual combination of pedunculated scales and keeled scales, the specimens of the two taxa are distinguished by several morphometric characteristics. For example, specimens of the Swedish species are larger (186–190 µm *v*. 135–148 µm in total length), have a three-lobed head (*v*. five-lobed in the new species), and its interciliary field is bare, whereas in the Tuscan specimens are entirely covered by scales.

Among the freshwater members of the genus *Aspidiophorus*, there are also species showing combinations of pedunculated scales covering most of the body, and keeled scales in the posterior region, e.g. *A. ophiodermus* Balsamo, 1983 and *A. schlitzensis* Schwank, 1990 (Balsamo 1983; Schwank 1990).

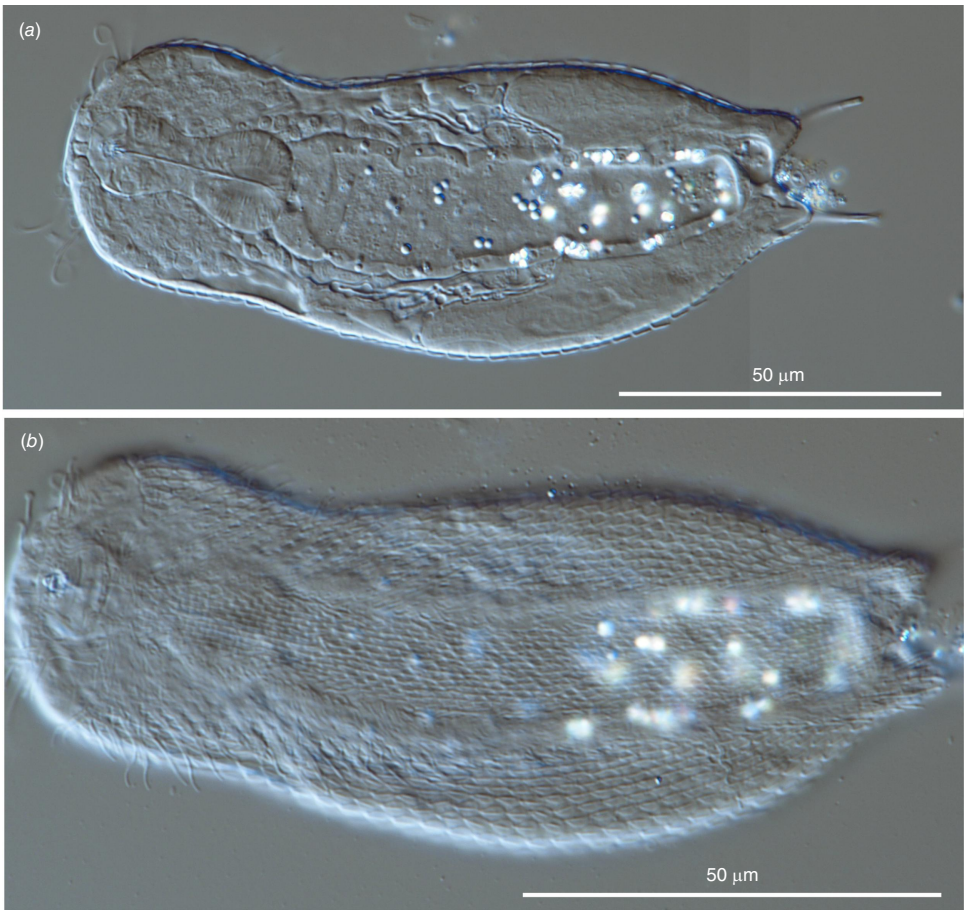

Fig. 8. Differential interference contrast photomicrographs showing the morphology of *Litigonotus ghinii* gen. nov., sp. nov. (*a*) Habitus of a subadult specimen, showing the internal anatomy. (*b*) Habitus of the same specimen, ventral view.

The specimens of the current study are easily distinguished from both of them due to a number of morphological traits, which include but are not limited to the presence of two robust bulbs in the pharynx (absent in the other two species) and the shape of the pedunculated scales. In fact, *A. schlitzensis* scales appear smooth (Schwank 1990), lacking the weak slight keel present in the scales of the new species, whereas in *A. ophiodermus*, the keel extends as a short spiny process beyond the scale itself (Balsamo 1983) in contrast with the new species, where the keel of the scale stops approximately halfway down the scale. In a comparative framework it is worth mentioning that currently none of the species affiliated to *Heterolepidoderma* bear pedunculated scales. Consequently, it is reasonable to consider the specimens under study belonging to an undescribed species and hence new to science. Genetic information, particularly the knowledge of the entire sequence of the mitochondrial genome, represents formidable additional traits helpful in characterising the new species.

As mentioned earlier, traditional taxonomy based on the current understanding of the anatomical traits could lead to the Tuscan species being affiliated to either *Aspidiophorus* or *Heterolepidoderma*; however, our phylogenetic analyses based on the sequences of small and large subunit ribosomal RNA genes consistently indicate that the new species is unrelated to either genus.

In all the analyses, the studied species appears sister to *Chaetonotus* aff. *subtilis* in a robustly supported subset of a larger clade, which includes mostly planktonic species belonging to the families Dasydytidae and Neogosseidae (Fig. 1, Supplementary Fig. S1, S2). Furthermore, our analyses found the genus *Aspidiophorus* polyphyletic, with the marine species, *A. parameditterraneus* Hummon, 1974, *A. polystictos* Balsamo & Todaro 1987, *A. tentaculatus* Wilke, 1954 and *Aspidiophorus* sp., clustering together along the non-monophyletic Chaetonotidae branch, and the freshwater taxa, *A. ophiodermus* Balsamo, 1983, *A. tetrachaetus* Kisielewski, 1986, and two *Aspidiophorus* spp., spread over the tree (Fig. 1, Supplementary Fig. S1, S2). Similarly, our phylogenetic analyses found *Heterolepidoderma* non-monophyletic (Fig. 1; Supplementary Fig. S1, S2). Previous studies based on molecular traits have also found Chaetonotidae, *Aspidiophorus*, and *Heterolepidoderma* to be non-monophyletic, highlighting the need for a revision of these taxa (e.g. Kånneby *et al.* 2013; Kolicka *et al.* 2020; Rataj Križanová and Vďačný 2024). Although such a task is beyond the scope of the present work, not including the new species in *Aspidiophorus* or *Heterolepidoderma* and creating a new genus may facilitate it.

In our analysis, the sister taxon of the new genus is *Chaetonotus* aff. *subtilis*. The morphology of the organisms corresponding to the sequences of *Chaetonotus* aff. *subtilis* (i.e. MN496212/MN496279, MN496228/MN496295) is unknown; however, the name suggests that these specimens are morphologically similar to *C. subtilis* Kolicka, Kotwicki & Dabert, 2018. Specimens of the latter species have the body covered with three-lobed scales, each bearing a simple spine; pedunculated scales are absent. The striking cuticular dissimilarities and the relatively high number of molecular differences suggest that *L. ghinii* and *C.* aff. *subtilis* belong to two distinct evolutionary lines. Consequently, we propose the name *Litigonotus ghinii* gen. nov., sp. nov. for the specimens found in Tuscany.

Concluding remarks

It is fascinating to note that the ancestral homeland of the new species found in the botanical garden of the University of Pisa remains a mystery, as the geographic origin of the founding population is unknown. Interestingly, this phenomenon is not unique to this particular species; other species found in ponds at different botanical gardens or Palm houses share similar stories (e.g. Kolicka *et al.* 2013). By contrast, the significant difference in the number of specimens collected on the two sampling dates left no doubt that the new species reaches a higher abundance in summer than in winter. These contrasting observations further indicate the need for ongoing research and monitoring to understand better our environment's biodiversity and ecological changes (Saponi *et al.* 2024, in press).

The state-of-the-art analysis of gastrotrichs' mitochondria is certainly very preliminary given the lack of sequences from other representatives of the phylum; therefore, we would like to underline how important it is to obtain mitochondrial sequences from other gastrotrich species to resolve the intricate systematic history of these organisms. Indeed, it has already been demonstrated that in other groups of metazoans a phylogenomic approach based on the entire mitochondrial marker allowed the resolution of similar debated taxonomic questions (Timmermans *et al.* 2014; López-López and Vogler 2017; Poliseno *et al.* 2017). Furthermore, the possibility of verifying any similarities or differences in the energy metabolism of gastrotrichs that have colonised ecologically different environments and have different reproductive modalities would be fascinating (Monnens *et al.* 2020).

Finally, the parallel between the highly conserved mitochondrial genome of the two freshwater Chaetonotidae studied so far, and the generally short length of the phylogenetic branches leading to the different lineages of freshwater gastrotrichs (also symptomatic of little variability) has not escaped our notice. The acquisition and analysis of mitogenomic data of additional taxa showing a different trend could shed light on the origin of Chaetonotidae and help clarify the deep phylogenetic relationships within the family (Kolicka *et al.* 2020). To simplify communication during this challenging task, we propose the name Oiorpata nom. nov. to define the clade that includes members of the current families Chaetonotidae, Dasydytydae, and Neogosseidae as it emerges consistently from the cladistic analyses performed so far (e.g. Hochberg and Litvaitis 2000; Kieneke *et al.* 2008; Kånneby *et al.* 2013; Kånneby and Todaro 2015; Garraffoni *et al.* 2017; Kolicka *et al.* 2020; Rataj Križanová and Vďačný 2024). Oiorpata is an alternative term (in Scythian language) for the Amazons, the mythical female warriors. Here, the name alludes to the primary female status (parthenogenetic) of the members of this clade. Oiorpata could also be extended to include members of the families Dichaeturidae and Proichthydidae, although none of them have been included in the molecular phylogenetic analysis conducted so far.

Supplementary material

Supplementary material is available online.

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Data availability. The data that support this study are available in the article and accompanying online supplementary material. Sequence of the ribosomal operon of the new species has been deposited in the NCBI GenBank database under Accession number OR915722. The complete mitochondrial genome of the new species has been deposited in NCBI under Accession number PP105008.

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