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Morphology and molecular phylogeny of the anaerobic freshwater ciliate *Urostomides spinosus* nov. spec. (Ciliophora, Armophorea, Metopida) from China

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Abstract

The morphology and molecular phylogeny of a new metopid ciliate, *Urostomides spinosus* nov. spec., discovered in a freshwater ditch in Qingdao, China, were investigated using live observation, morphometry and protargol staining as well as molecular phylogenetic methods. Diagnostic features of the new species include a broadly obpyriform body carrying three posterior spines, eight somatic kineties, five preoral dome kineties with specialized row 3, adoral zone composed of about 28 membranelles, making a 270° turn around body axis. Phylogenetic analyses of the SSU rDNA sequence revealed that the genus *Urostomides* is monophyletic, but its interspecific relationships remained unresolved. Moreover, a closer relationship of the new species with the morphologically similar *Urostomides campanula* was not supported by the molecular data.

Keywords: Anaerobe; Apometopidae; Ciliary pattern; SSU rDNA; New species

Introduction

The ciliate fauna in anoxic environments gained increasing interests in recent years (Fernandes et al. 2018; Lynn 2008; Nitla et al. 2019). Anaerobes have unique life styles, hydrogenosomes, and symbiotic relationships with prokaryotes. These features make them appropriate objects for research on ecology and evolution of eukaryotic life in anoxia (Foissner 2016a; Hackstein 2018; Lewis et al. 2018, Lewis et al. 2020; Li et al. 2017; Rotterová et al. 2020; Wang et al. 2019). In spite of great research progress in this group of ciliates, their taxonomy and phylogeny remain largely unresolved because many nominal species have not been investigated with silver staining and molecular methods. The order Metopida Jankowski, 1980 within the class Armophorea Lynn, 2004 is a species-rich group (Bourland et al. 2014, Bourland et al. 2017a, Bourland et al. 2017b, 2018a, Bourland et al. 2018b; Bourland et al. 2020, Esteban et al. 1995, Jankowski 2007, Li et al. 2021, Omar et al. 2017: Rotterová et al. 2018, Silva-Neto et al. 2016, Vd'a ný and Foissner 2017a, 2017b, 2019, Vd'a ný et al. 2019). Within the Metopida, the family Apometopidae Foissner, 2016 originally included two genera, *Apometopus* Foissner, 2016 and *Cirranter* Jankowski,

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1964, that comprised two and one species, respectively (Foissner 2016b). Bourland et al. (2017b) redefined the genus *Urostomides* Jankowski, 1964 as obpyriform to clavate metopids with a four-rowed perizonal ciliary stripe, and synonymized *Apometopus* with *Urostomides*. *Urostomides* is known to be widely distributed, and contains nine species so far, namely *U. striatus* (McMurrich, 1884) Jankowski, 1964 (type species); *U. bacillatus* (Levander, 1894) Bourland et al., 2017; *U. caducus* (Kahl, 1927) Bourland et al., 2017; *U. campanula* (Kahl, 1932) Bourland et al., 2017; *U. darwini* (Kahl, 1927) Bourland et al., 2017; *U. denarius* (Kahl, 1927) Bourland et al., 2017; *U. pelobius* (Foissner, 2016) Bourland et al., 2017; *U. pullus* (Kahl, 1927) Bourland et al., 2017, and *U. pyriformis* (Levander, 1894) Bourland et al., 2017. The ciliature of these species has been studied, nevertheless, molecular data are unavailable for *U. pelobius* and *U. pyriformis*. Kovalchuk (1980) raised *Metopus pulcher* var. *tortus* Kahl, 1927 to species rank as *Metopus* (*Urostomides*) *tortus* Kahl 1927, where the subgenus was irrelevant. Esteban et al. (1995) also mentioned *Metopus tortus* without any description. However, none of them described the number of perizonal ciliary stripe rows of the species, which is the most important feature of the family Apometopidae. We do not see a reason to transfer the species to *Urostomides* at the present state of knowledge.

In 2018, we found a new species of *Urostomides*. The present work documents morphological, morphometric, and SSU rDNA sequence data of the new species.

Material and methods

Sample collection

Urostomides spinosus nov. spec. was collected from sediments of a freshwater ditch in Qingdao (36°03′51″N, 120°20′34″E), China in April 2018. The ditch was the connection between two ponds in Zhongshan Park with naturally accumulated sediments. The sediment was mainly composed of black mud and semi-decomposed leaves, rendering a strong sulfidic odor. After collection, the sample was taken to the laboratory with a sampling bottle and deposited in the anaerobic chamber under room temperature for 10 d to enrich ciliates. The cap of sampling bottle was on tight to prevent drying of the sample.

Morphological methods

Living cells were picked out using a micropipette and observed under light microscopes equipped with differential interference contrast illumination. Microphotographs were then taken at magnifications of 200–1000×. Protargol staining was performed following the method of Wilbert (1975) to reveal the ciliature and nuclear apparatus, using protargol reagent synthesized according to the protocol of Pan et al. (2013). All measurements were made from microphotographs with calibrated Zeiss ZEN2 software. Protargol-stained specimens were studied at 1000× magnification. Drawings of specimens were done with the aid of a drawing device.

DNA extraction and gene sequencing

Several cells were picked out using glass micropipettes and washed five times in filtered (0.22 μm pore size) water from the same habitat. Each of them was put into a 1.5 mL EP tube with 45 μl of ATL buffer and stored at room temperature for DNA extraction. Genomic DNA was extracted using a Blood & Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Q5® Hot Start High Fidelity DNA Polymerase (New England BioLabs, USA) was used to amplify the SSU rDNA with the primers 82F (5′-GAA ACT GCG AAT GGCTC-3′) (Jerome et al. 1996) and 18S-R (5′-TGA TCC TTC TGC AGG TTC ACC TAC-3′) (Medlin et al. 1988). PCR conditions followed Bai et al. (2020). The PCR products were detected using agarose gel and sequenced directly in both directions by four primers (82F, 18S-R, 900F and 900R) in TSINGKE (Qingdao, China). Contigs were assembled by Seqman (Swindell and Plasterer 1997).

Phylogenetic analyses

We created a dataset of 64 SSU rDNA sequences, including the newly obtained sequence. The sequences were aligned using the MAFFT algorithm (Katoh et al. 2002) on the GUIDANCE2 Server (http://guidance.tau.ac.il/) with default settings. Unreliable columns below confidence score 0.97 were removed. The alignment was manually edited using BioEdit 7.2.3 (Hall, 1999). The final dataset of unambiguously aligned characters consisted of 1696 positions. Phylogenetic trees were constructed by maximum likelihood (ML) and Bayesian methods. ML analysis was performed in RAxML 8.2.12 (Stamatakis, 2014) under the GTRGAMMA model. Node support was assessed by 1000 bootstrap pseudoreplicates. Bayesian analysis was performed using the GTR + I + G model with MrBayes 3.2.6. (Ronquist et al. 2012). Markov Chain Monte Carlo simulations were run for 10,000,000 generations, with a sampling frequency of 100 generations; first 10% of trees were removed as burn-in.

ZooBank registration: urn:lsid:zoobank.org:pub:43823340-7DD2-422D-ACEC-07B4F80F4B5C *Urostomides spinosus* nov. spec. urn:lsid:zoobank.org:act:0494E6DA-50B4-43C0-BA20-44425CD932FC

Results

Ciliophora Doflein, 1901
Armophorea Lynn, 2004

Metopida Jankowski, 1980
Apometopidae Foissner, 2016
Urostomides Jankowski, 1964

***Urostomides spinosus* nov. spec. (**Figs. 1**A-G,** 2**A-R;** Table 1**)**

Diagnosis: Body size about 25–45 × 20–35 μm in vivo. Body shape broadly obpyriform to campanulate, slightly dorsoventrally flattened, with three posterior spines. Cortical granules colorless and short rod-shaped, densely arranged in rows. Macronucleus ovoid, in preoral dome. Contractile vacuole terminal. Invariably eight somatic kineties including five widely spaced dome kineties; kinetal furrows prominent at kineties 2 and 3. Dome kinety 3 thigmotactically specialized. Adoral zone composed of 28 membranelles on average, approximatively horizontally arranged, making an about 270° turn around body axis. Freshwater habitat.

Type locality: Sediments of a freshwater ditch with black mud and semi-decomposed leaves in Qingdao (36° 03′51″N, 120°20′34″E), China.

Type material: The protargol slide containing the holotype (Fig. 2N, O) and some paratypes (registration number: ZWB201804170101) and eight further slides with paratypes (registration numbers: ZWB201804170102-09) were deposited in the Laboratory of Protozoology, Ocean University of China, Qingdao, China. The holotype and relevant paratypes were marked by black ink circles on back side of the slides.

Etymology: The species-group name *spinosus* (Latin adjective; thorny, spiny) refers to the fact that this species has several characteristic spines at its posterior end.

Morphology: Body size 25–45 × 20–35 μm in vivo and 20–35 × 17–22 μm after protargol staining (Table 1). Cells light grey at low magnification. Body broadly obpyriform to

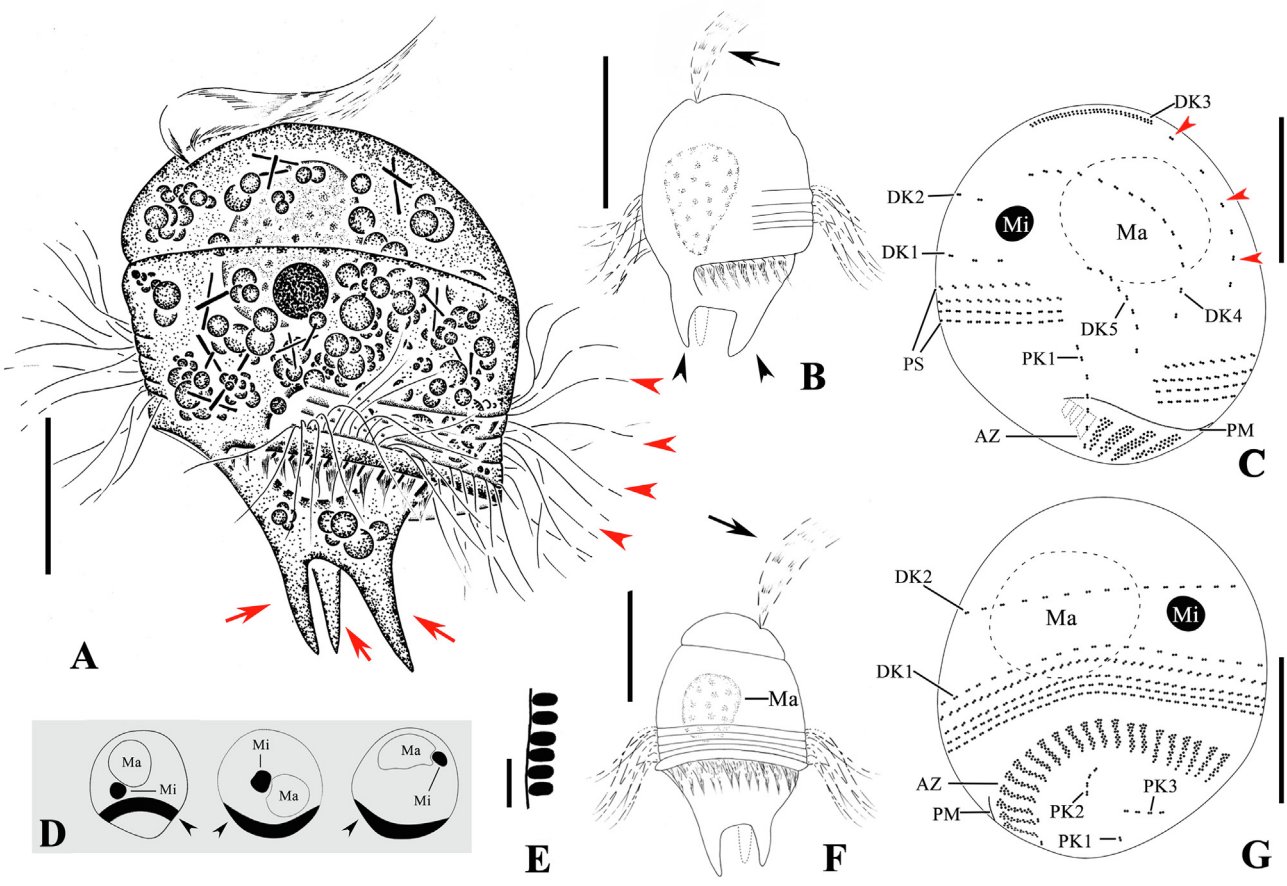

Fig. 1. A-G. *Urostomides spinosus* nov. spec. from life (A, B, E, F) and after protargol staining (C, D, G). **(A)** Ventral view of a representative specimen, arrowheads showing perizonal stripe cilia, arrows showing posterior spines. **(B, F)** Right (B) and left (F) views of specimens showing body shape, arrows showing cilia of preoral dome kinety 3, arrowheads showing posterior spines. **(C, G)** Ventral (C) and dorsal (G) views of the same, compressed specimen (holotype), arrowheads showing posterior dikinetids of preoral dome kinety 3. **(D)** Different perspectives showing nuclear apparatus, arrowheads showing adoral zone. **(E)** Cortical granules viewed from edge of cell. AZ, adoral zone; DK1-5, preoral dome kineties 1-5; Ma, macronucleus; Mi, micronucleus; PK1-3, postoral kineties 1–3; PM, paroral membrane; PS, perizonal ciliary stripe. Scale bars: 10 μm (A, C, G), 25 μm (B, F), 5 μm (E).

Table 1. Morphometric characteristics of *Urostomides spinosus* nov. spec. based on protargol stained specimens.

Characteristic[a]	Mean	M	SD	CV	Min	Max	n
Body, length	25.2	24.3	4.4	18	20.0	35.0	18
Body, width	23.2	22.0	4.2	18	17.0	22.0	18
Body, length–width, ratio	1.1	1.1	0.1	7	0.9	1.2	18
Anterior pole to posterior end of macronucleus, distance	14.6	14.0	3.6	24	10.0	22.0	10
Distance anterior pole to posterior end of macronucleus: body length, %	70	70	0.1	19	50	90	10
Macronucleus, length	10.7	10.6	1.8	17	8.0	13.5	28
Macronucleus, width	8.8	8.5	1.6	18	6.0	12.5	28
Adoral membranelles, number	28.2	28	1.8	6	25.0	32.0	30
Somatic kineties, number[b]	8.0	8.0	0	0	8.0	8.0	10
Preoral dome kineties, number	5.0	5.0	0	0	5.0	5.0	10
Postoral kineties, number	3.0	3.0	0	0	3.0	3.0	10
Perizonal ciliary stripes, number	4.0	4.0	0	0	4.0	4.0	28
Paroral membrane, length	10.8	10.0	2.4	22	8.0	17.0	15

[a] Measurements in μm. CV, coefficient of variation (%); M, median; Max, maximum; Min, minimum; n, number of specimens examined; SD, standard deviation.

[b] Posterior body kineties plus preoral dome kineties, excluding perizonal stripe rows.

campanulate, slightly dorsoventrally flattened (Figs. 1A, B, F, 2A–D, F). Preoral dome campanulate, wider than posterior body part, overhanging left margin, and occupying about 60% of body length (Figs. 1A, 2A–C). Two prominent cortical furrows along preoral dome kineties 2 and 3 (Figs. 1A, 2B, C). Posterior body part tapered into three prominent spines, approximately centrosymmetrically distributed, about 6 μm long in vivo, the left one slightly longer than the others (Figs. 1A, B, F, 2A–D); undiscernible in compressed or protargol-stained specimens. Cortical granules colorless, rod-like, about 1.0 × 0.5 μm in size in vivo, arranged perpendicularly to cell surface and in narrowly spaced rows, which form a refractive fringe underneath pellicle (Figs. 1E, 2E). Cytoplasm colorless, full of spherical particles (1 μm across) and rod-shaped structures (5.0 × 0.4 μm) (Figs. 1A, 2H). Macronucleus in preoral dome, globular to ovoid, size about 9 × 11 μm and outlines variable in different aspects after protargol staining (Figs. 1A, B, D, F, 2A, B, F). Micronucleus about 3 μm in diameter, globular, adjacent to concavity of macronucleus (Figs. 1D, 2G). Contractile vacuole terminally positioned, about 10 μm across, excretory pore and cytopyge not recognized (Figs. 1A, 2B, D). Locomotion by free-swimming quickly with distinctive side-to-side twitching movement while rotating about main body axis in the upper water column. Sluggish after water exposed to air.

Consistently eight somatic kineties including five widely spaced preoral dome kineties (Figs. 1C, G, 2I, J, N, P). Dikinetids of dome kineties 1 and 2 densely spaced, that is, about 1.7 μm apart. Anterior dikinetids of dome kineties 4 and 5 also densely spaced as in kineties 1 and 2, while those in posterior portion about 2.7 μm apart (Fig. 1C, G, 2I, J, N, P). Dikinetids in anterior part of dome kinety 3 densely spaced forming a zigzag structure (Figs. 1C, 2I, M, N, P), whereas posterior part of dome kinety 3 composed of about six loosely spaced dikinetids about 4 μm apart (Figs. 1C, 2J, P). Cilia in dome kinety 3 about 20 μm long in vivo, but cilia of other dome kineties lacking. Perizonal stripe composed of densely spaced dikinetids arranged in four spiraled rows, not aligned with adoral zone of membranelles, which starts nearby middle portion of body anteriorly and ends ahead of cytostome proximally (Figs. 1C, G, 2I–K, N, O). Rows 1–4 narrowly-spaced, dikinetids of rows 1 and 2 parallel to kinety axis, those of rows 3 and 4 obliquely inclined, never forming false kineties; cilia of perizonal stripe about 12 μm long. Invariably three postoral kineties, each composed of dikinetids; longest one just to right of proximal buccal portion composed of about eight dikinetids, optically intersecting with adoral membranelles (Figs. 1C, G, 2N, R). Caudal cilia not observed; nevertheless, in protargol preparations, four cilia from last dikinetid of postoral kineties 2 and 3 and last two dikinetids of postoral kinety 1 recognized, 1–2 μm in length; cilia from other dikinetids of postoral kineties lacking (Fig. 2K). Two extra dikinetids nearby the proximal membranelles in seven out of 10 investigated specimens (Fig. 2Q).

Adoral zone composed of 25–32 (on average 28) membranelles, begins on right margin and ends at slightly different levels on ventral surface, making an about 270° turn around main body axis (Figs. 1C, G, 2N, O). Proximalmost membranelle rhomboidal, next four or five membranelles getting longer; mid-adoral membranelles longest, about 5 μm long, composed of several inclined rows of basal bodies. Paroral membrane stichomonad, ca. 11 μm long on average (Figs. 1C, G, 2R).

Phylogenetic analysesy: The SSU rDNA sequence of *Urostomides spinosus* nov. spec. is 1651 bp long, with a GC content of 43.05%. The accession number in the NCBI is MW872361. *Urostomides bacillatus* is most similar to this new species with 96.99% sequence similarity.

The topologies of the phylogenetic trees from ML and BI analyses were identical, and thus only the ML tree topology is shown with support values derived from both algorithms

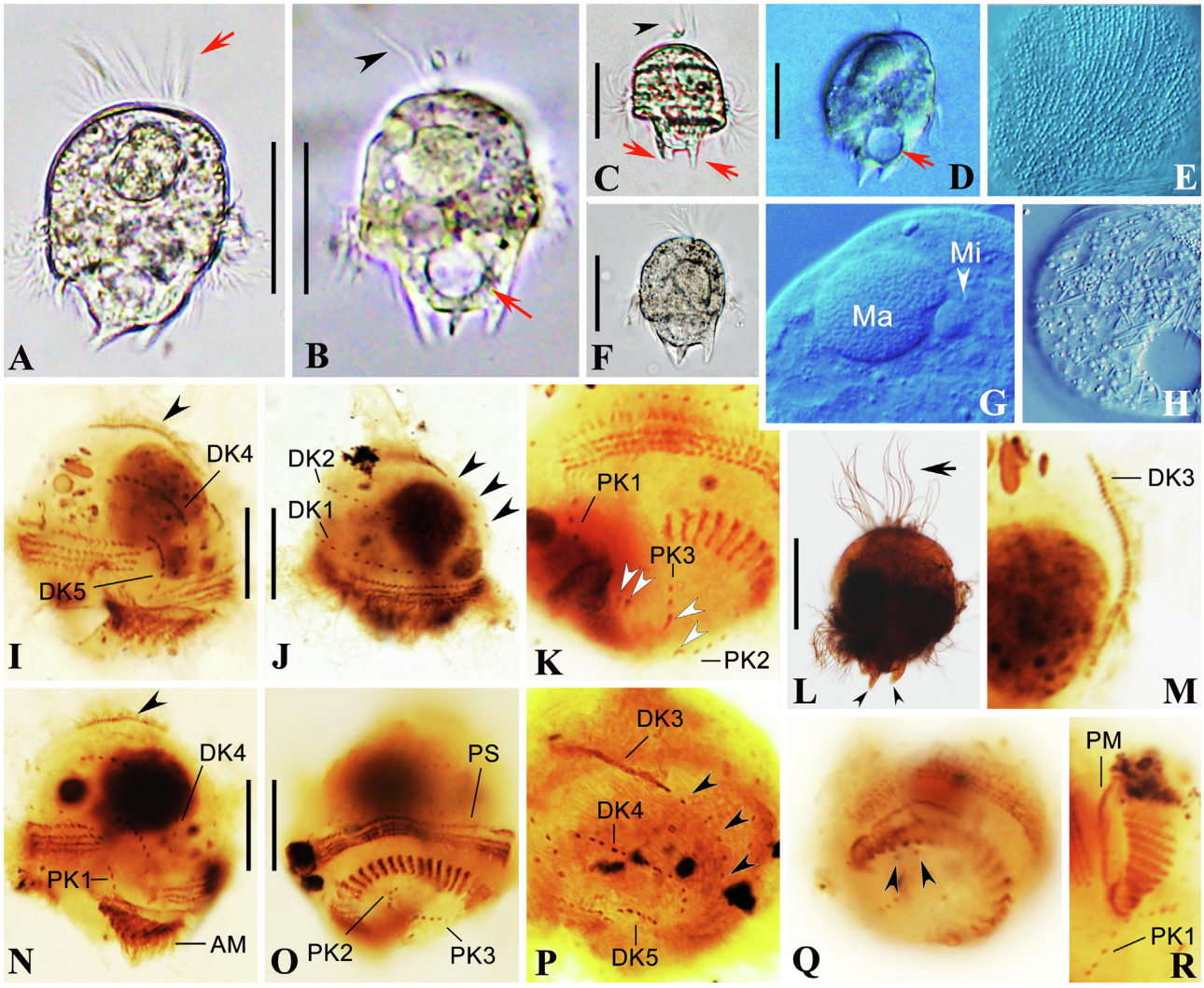

Fig. 2. A-R. Microphotographs of *Urostomides spinosus* nov. spec. from life with bright–field (A–C, F), and differential interference contrast (D, E, G, H) illuminations, and after protargol staining (I–R). **(A)** Right ventral view showing body shape, arrow showing cilia of preoral dome kinety 3. **(B)** Dorsal view showing body shape, arrowhead showing cilia of preoral dome kinety 3, arrow showing contractile vacuole. **(C)** Ventral view showing posterior spines (arrows) and cilia of preoral dome kinety 3 (arrowhead). **(D)** Right view showing body shape, arrow showing contractile vacuole. **(E)** Cortical granules at high magnification. **(F)** Left view showing dorsoventrally flattened body shape and posterior spines. **(G)** Macronucleus and micronucleus. **(H)** Spherical and rod-shaped inclusions. **(I)** Ventral view showing preoral dome kineties 3 (arrowhead), 4 and 5, perizonal ciliary stripe and adoral membranelles. **(J)** Dorsal view of preoral dome kinety 1–3, arrowheads showing posterior dikinetids of preoral dome kinety 3. **(K)** Posterior view of cell showing postoral kineties, arrowheads showing cilia from kinetids. **(L)** Strongly stained cell showing cilia of preoral dome kinety 3 (arrow) and posterior spines (arrowheads). **(M)** Showing preoral dome kinety 3. **(N)** Rightventral view of the holotype showing both ends of perizonal ciliary stripe and adoral membranelles, arrowhead showing preoral dome kinety 3. **(O)** Posterior dorsal view of the holotype showing perizonal ciliary stripe, adoral membranelles and postoral kineties. **(P)** Showing preoral dome kinety 3–5, arrowheads showing posterior dikinetids of preoral dome kinety 3. **(Q)** Posterior view showing 2 extra postoral dikinetids (arrowheads). **(R)** Proximal view of adoral zone, showing, inter alia, membranelles, paroral membrane and postoral kinety 1. AM, adoral membranelles; DK1–5, preoral dome kineties 1–5; Ma, macronucleus; Mi, micronucleus; PK1–3, postoral kineties 1–3; PM, paroral membrane; PS, perizonal ciliary stripe. Scale bars: 25 μm (A–D, F), 10 μm (I, J, L, N–Q).

(Fig. 3). The Armophorea were recovered paraphyletic, consisting of two unrelated clades of Metopida/Clevelandellida and Armophorida. The fully supported clade Metopida/Clevelandellida is comprised by the Clevelandellida forming an internal branch within the Metopida, and metopid families Metopidae and Apometopidae sister to Tropidoatractidae. The Apometopidae clade clusters with the Metopidae/Clevelandellidae cluster with full support. Members of the genus *Urostomides* group together with high support (98/1.00). Within the genus, *U. campanula* and *U. caducus*

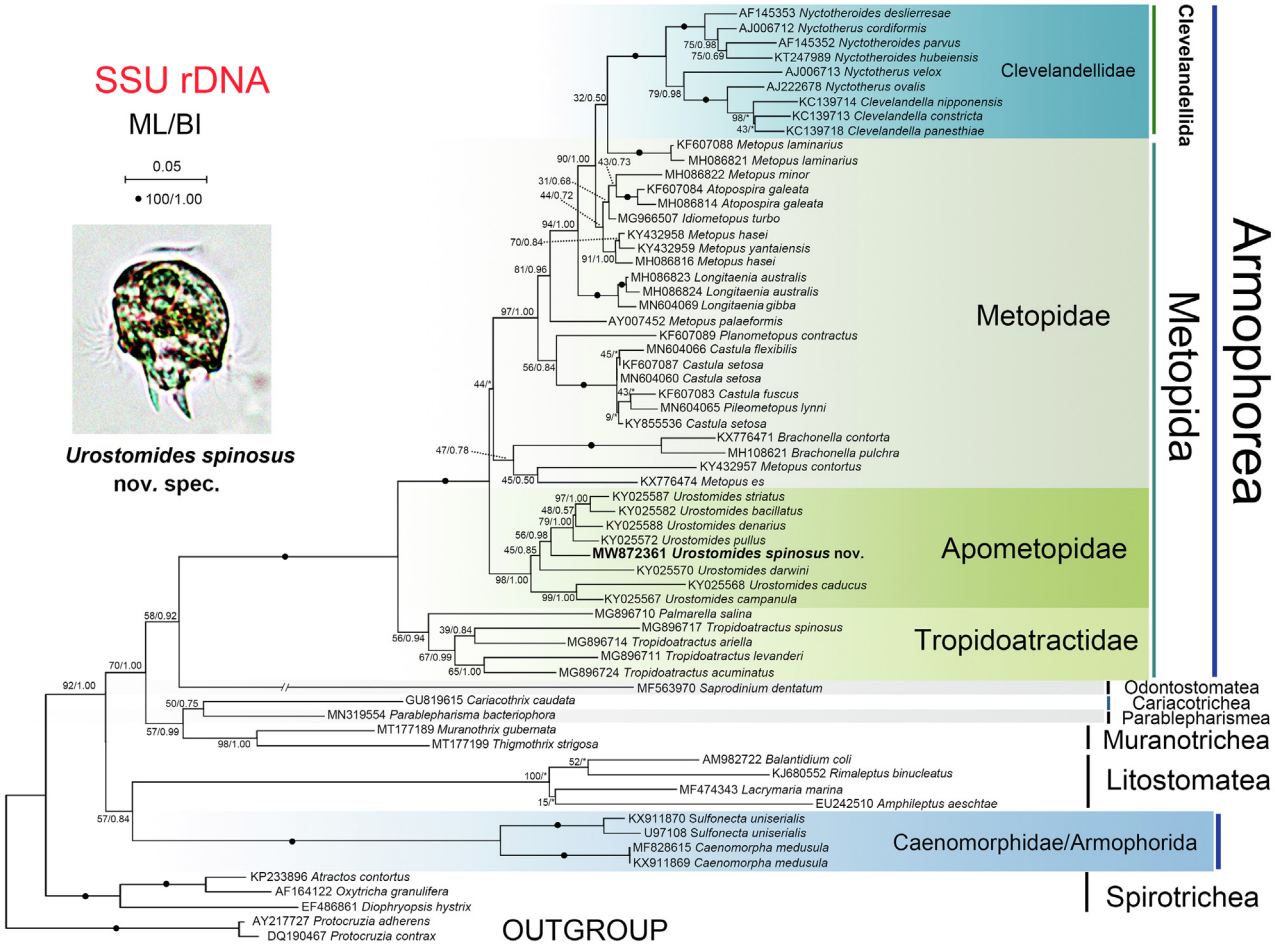

Fig. 3. Phylogenetic tree based on SSU rDNA sequences. The tree was constructed by the maximum likelihood method in RAxML (GTRGAMMAI model). The values at branches represent support in bootstrap values (maximum likelihood method)/posterior probabilities (Mrbayes method). New sequence in bold. Asterisks (*) reflect disagreements in topology between the BI and ML trees. The scale bar represents 5 changes per 100 positions.

branch off early. *Urostomides spinosus* nov. spec. clusters with the subclade formed by *U. pullus*, *U. denarius*, *U. bacillatus*, and *U. striatus* with low to high support (56/0.98).

Discussion

Identification of new species

Characterized by having an obpyriform, contorted body with anterior part twisted to the left and a perizonal stripe composed of four rows, our form should be assigned to the family Apometopidae (Bourland et al. 2017b; Foissner 2016b).

Based on its phylogenetic position, dominant preoral dome wider than the posterior body portion, five widely spaced dome kineties and a stichomonad paroral membrane, the current isolate undoubtedly belongs to the genus *Urostomides* (Bourland et al. 2017b; Foissner 2016b). We did not observe caudal cilia in live and protargol-stained specimens inferring its absence in the species. However, there are four cilia at the end of postoral kineties 1–3, being only about 1–2 μm long in protargol preparations. Elongated caudal cilia were not listed as a diagnostic feature when Foissner (2016b) erected *Apometopus* (junior synonym of *Urostomides* according to Bourland et al. 2017b). Bourland et al. (2017b) emended its diagnosis and mentioned elongated caudal cilia as a generic feature, although sometimes inconspicuous. All *Urostomides* species have caudal cilia, but they are inconspicuous in *U. darwini, U. striatus* and some populations of *U. denarius*, *U. pullus* (Bourland et al. 2017b). Considering its variation between species or even populations, caudal cilia may not be a good feature for a genus diagnosis among Metopida.

Our species can be easily distinguished from all known *Urostomides* species by having three posterior spines.

Though it most resembles *U. campanula, U. denarius*, and *U. pullus* in terms of the body size, shape of the preoral dome, and the number of dome kineties; *U. campanula* can be separated by having more somatic kineties (11–15 vs. 8) and a specialized dome kinety 3; *U. denarius* can be recognized by the number of somatic kineties (13–19 vs. 8) and the lack of a specialized preoral dome kinety 3; *U. pullus* has fewer adoral membranelles (15–25 vs. 25–32) and lacks the specialized preoral dome kinety 3 (Bourland et al. 2017b).

Another remarkable feature of the new species is the structure of dome kinety 3, whose anterior dikinetids are very narrowly spaced in a zigzag pattern and whose posterior dikinetids are loosely spaced. Till now, *Urostomides campunula* was the only species having a specialized preoral dome kinety 3 within the genus. Anterior dikinetids of dome kineties 4 and 5 are also more densely spaced than the posterior dikinetids but not in a zigzag pattern; such an arrangement is only seen in two species, viz. *U. pelobius* and *U. pyriformis*. Therefore, the above-mentioned traits might be synoapomorphies and thus have systematic significance.

Cirranter mobilis (Penard, 1922) Jankowski, 1964 is similar in body shape and size to *U. spinosus* nov. spec. (Jankowski 1964; Kahl 1932; Penard 1922; Sola et al. 1992). In the four descriptions, the cell size did not significantly differ (30–55 μm); only Kahl (1932) mentioned a small form of 20–22 μm. Sola et al. (1992) described two very short inconspicuous posterior prolongations, reminiscent of the spines in the new species. Those were, however, not mentioned in the other descriptions. According to Jankowski (1964) and Sola et al. (1992), *C. mobilis* has only one dome kinety, and thus cannot be confused with the new *Urostomides* species, which has five such rows.

Phylogenetic position of new species within the order Metopida Jankowski, 1980

As shown in the current analyses, five classes, Odontostomatea, Cariacotrichea, Parablepharismea, Muranotrichea, and Litostomatea, cluster in between the armophorean clades Metopida/Clevelandellida and Armophorida, once again confirming the non-monophyly of the Armophorea. The Metopida are a paraphyletic clade clustered with the endobiotic Clevelandellida. Both the family Apometopidae and the genus *Urostomides* are monophyletic (98/1.00), which is consistent with the recent study by Bourland et al. (2020). However, interspecific relationships among some *Urostomides* species remain unclear. The clustering of *U. spinosus* nov. spec. with four species of *Urostomides* was not well supported in this study, which shows a different position as compared with previous studies (Bourland et al. 2017b, 2018a, Bourland et al. 2018b, Bourland et al. 2020; Rotterová, et al. 2018). We anticipate adding more molecular markers and performing multi-gene or phylogenomic analyses would help to obtain a robust phylogeny of the genus in the future.

Author contributions

XH conceived and guided the study. WZ and SL conducted sampling and performed laboratory work. XH and SL identified the species. TZ did the phylogenetic analyses and the results interpretation. WZ drafted the manuscript, and SL, YB, KASA-R and XH made further revisions. KASA-R also polished the English. All authors read and approved the final version of manuscript.

CRediT authorship contribution statement

Wenbao Zhuang: Conceptualization, Investigation, Writing - original draft, Writing - review & editing, Visualization. **Song Li:** Investigation, Writing - review & editing. **Yang Bai:** Writing - review & editing, Visualization. **Tengteng Zhang:** Writing - review & editing. **Khaled A.S. Al-Rasheid:** Writing - review & editing, Funding acquisition. **Xiaozhong Hu:** Conceptualization, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition.

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