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Research article

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Systematic positions and taxonomy of two freshwater ciliates found in China, with establishments of one new family and two new species (Ciliophora, Oligohymenophorea)

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Abstract. The morphology and molecular phylogeny of two freshwater ciliates, *Pseudotetrahymena orientalis* gen. et sp. nov. and *Cyclidium paravorax* sp. nov., collected from Harbin, Northeast China, were investigated based on morphology of live specimens and ciliary pattern; the phylogenetic placements were inferred from the SSU-rRNA gene. Pseudotetrahymenidae fam. nov., assigned to the order Tetrahymenida Fauré-Fremiet in Corliss, 1956, can be distinguished from the other families by its membranelle 2 consisting of several clusters and the shape of the cell. The new species *C. paravorax* is similar to *C. vorax* Pan, 2020 in most morphological features except the membranelle 3, which is single-rowed. Phylogenetic analyses based on SSU-rRNA gene sequence indicate that Pseudotetrahymenidae lies in the periphery of the Tetrahymenida clade and forms an independent, fully supported clade. *Cyclidium paravorax* groups with *C. glaucoma* Müller, 1773, which supports the assignment of the new species to the genus *Cyclidium* Müller, 1773.

Keywords. *Pseudotetrahymena orientalis* gen. et sp. nov., *Cyclidium paravorax* sp. nov., morphology.

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Introduction

The ciliated protozoans are morphologically complex groups with a huge variety of species and a worldwide distribution in terrestrial, freshwater, brackish, marine, and hypersaline habitats (Chen *et al.* 2017; Wu *et al.* 2020, 2023; Li *et al.* 2021, 2024; Liu *et al.* 2022, 2024; Song *et al.* 2022, 2024; Jiang *et al.* 2023; Chi *et al.* 2024; Hao *et al.* 2024). Ciliates show high biodiversity, with over 4000 free-living species described to date (Gao *et al.* 2017).

The class Oligohymenophorea de Puytorac *et al.*, 1974 was established by Puytorac in 1974, and contains seven subclasses, namely Apostomatia Chatton & Lwoff, 1928, Astomatia Schewiakoff, 1896, Hymenostomatia Delage & Hérouard, 1896, Peniculia Fauré-Fremiet in Corliss, 1956, Peritrichia Stein, 1859, Scuticociliatia Small, 1967, and Urocentria Wang *et al.*, 2021. The cell size of Oligohymenophorea species is commonly small to medium, with the shape typically ovoid to elongate ovoid (Lynn 2008). In addition, species assigned to Oligohymenophorea are characterized by their oral apparatus consisting of four adoral membranelles: a distinct right paroral membrane composed of dikinetids and three left oral membranelles (polykinetids). In terms of taxonomy, ciliates in the class Oligohymenophorea have been studied for a long time. The first report of the class Oligohymenophorea was the description of *Cyclidium glaucoma* Müller, 1773 by Müller (Müller 1773; Lynn 2008). However, the taxonomy and systematics of Oligohymenophorea is still confused due to the limitations in techniques for species identification and the progress in molecular techniques (Gentekaki *et al.* 2014; Zhang *et al.* 2014; Wang *et al.* 2015). Therefore, it is necessary to carry out more work with an integrative method combining typical morphology and molecular approaches to resolve the taxonomic confusion in this complex group. The family Tetrahymenidae Corliss, 1952, belonging to Hymenostomatia Delage & Herouard, 1896, is mainly characterized by the following features: small, pyriform to elongate-ovoid cells that are free-swimming, and the oral structures (a paroral dikinetid that is ciliated along its entire length, and three oral polykinetids, each of equal number of rows of kinetosomes) (Lynn 2008).

The type genus *Tetrahymena* Furgason, 1940 is characterized by a four-part oral structure, and so far, over 80 species have been assigned to it (Quintela-Alonso *et al.* 2013; Liu *et al.* 2016; Lynn *et al.* 2018; Doerder 2019; Pan *et al.* 2019; Rataj & Vďačný 2020; Zhang & Vďačný 2021). Species of *Tetrahymena* can be divided into three infrageneric groups based on life cycle characteristics: the *rostrata* group, the *patula* group, and the *pyriformis* group (Corliss 1970; Lynn & Doerder 2012).

In recent years, many species and new taxa in the class Oligohymenophorea were discovered and reported from Northeast China, including ciliates in Hymenostomatia, Peniculia, and Scuticociliatia, indicating the potential diversity of undiscovered ciliates and highlighting the importance of conducting further research in the class Oligohymenophorea (Pan *et al.* 2017a, 2017b; Cai *et al.* 2018; Pan *et al.* 2019, 2020; Hao *et al.* 2022).

In the present study, two ciliates, *Pseudotetrahymena orientalis* gen. et sp. nov. and *Cyclidium paravorax* sp. nov., are described using observation in vivo and silver staining techniques (Foissner 1991). SSU-rRNA gene sequence data are also supplied for both species and their phylogenetic positions in the SSU-rRNA gene tree are estimated.

Material and methods

Sample collection, observation and identification

Pseudotetrahymena orientalis gen. et sp. nov. was collected on 11 October 2022 from a freshwater aquarium at Dafa international fish market in Harbin, Heilongjiang Province (45°44′9″ N, 126°35′41″ E), Northeast China (water temperature about 23°C). *Cyclidium paravorax* sp. nov. was collected on 23 November 2022 from a freshwater aquarium at Harbin Normal University, Harbin, Heilongjiang Province (45°52′3″ N, 126°33′2″ E), Northeast China (water temperature about 22°C). About 0.5 L of water was collected from 0.1 to 0.5 m below the surface using sterile sampling bottles (Fig. 1).

Samples of *Pseudotetrahymena orientalis* gen. et sp. nov. were kept in Petri dishes at room temperature with added unsterilized rice grains to enrich the growth of bacteria as a food source for the ciliates. Cells of *Cyclidium paravorax* sp. nov. were kept in monoclonal cultures in Petri dishes at room temperature (about 25°C) with filtered habitat water supplemented with sterilized rice grains to enrich the growth of

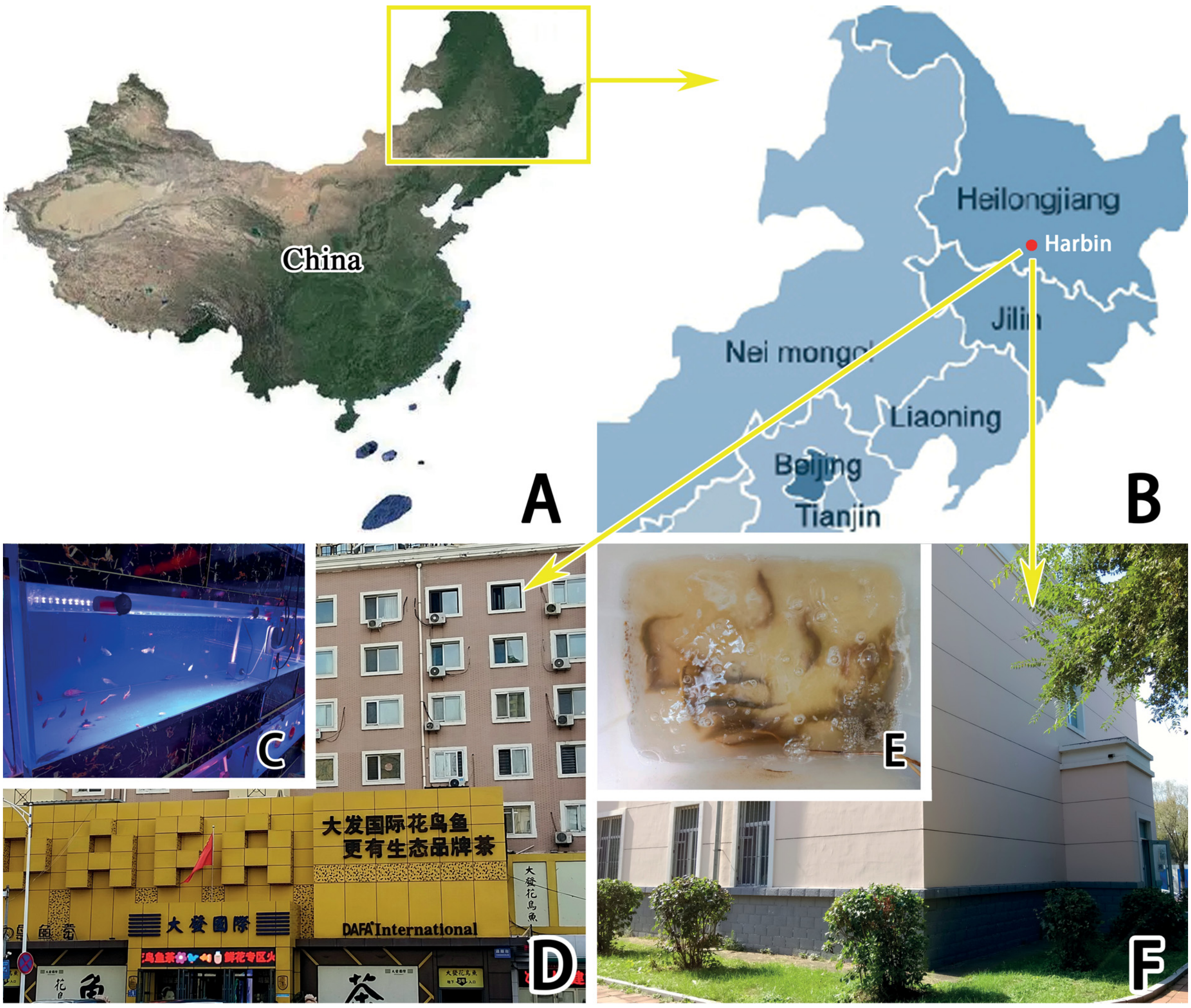

Fig. 1. Map and sampling sites. **A–B**. Map of China, showing the location of sampling sites in Harbin. **C–D**. Collecting site of Dafa international fish market (45°44′9″ N, 126°35′41″ E) from which *Pseudotetrahymena orientalis* gen. et sp. nov. was collected. **E–F**. Collecting site of Harbin Normal University (45°52′3″ N. 126°33′2″ E) where *Cyclidium paravorax* sp. nov. was collected.

bacteria as food for ciliates. Cells from Petri dishes were observed and photographed using differential interference contrast and bright-field microscopy (Zeiss Axio Imager A2, German) (Foissner 1991).

The silver carbonate method revealed the infraciliature and nuclear apparatus (Fernandez-Galiano 1976; Foissner 1992). Counts and measurements of sliver-stained specimens were performed at magnifications of 100 × to 1000 ×. Drawings of live and stained specimens are based on microscope observations, freehand sketches, and photomicrographs. Drawings of stained specimens were made with drawing devices (line drawing pens and tracing papers). Classification and terminology are according to Lynn (2008).

DNA extraction, PCR amplification and gene sequencing

About ten cells from the clonal culture of each species were washed with distilled water and genomic DNA was extracted using a DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. The SSU-rRNA gene was amplified with the primers Euk A: 5'-AAC CTG GTT GAT CCT GCC AGT-3' and EukB: 5'-TGA TCC TTC TGC AGG TTC ACC TAC-3' (Medlin *et al.* 1988). PCR condition for amplification of the SSU-rRNA gene was: denaturation for 5 min at 94°C, followed by 5 cycles of denaturation for 45 s at 94°C, annealing for 1 min 45 s at 56°C, extension for 2 min at 72°C, and other 25 cycles of denaturation for 45 s at 94°C, annealing for 1 min 45 s at 60°C, extension for 2 min at 72°C, and a final extension for 8 min at 72°C (Li *et al.* 2022; Liu *et al.* 2023). Bidirectional sequencing was performed by the Shanghai Sangon Biotechnology Company (Shanghai, China).

Analyses

Except for the two newly sequenced species, 61 SSU-rRNA gene sequences downloaded from the GenBank database were used in the analyses, as well as two species as the outgroup (for accession numbers, see Fig. 6). Sequences were aligned, and resulting alignments were refined by only trimming both ends using BioEdit ver. 7.0.5.2 (Hall 1999). Bayesian inference (BI) analysis was performed using MrBayes on XSEDE ver. 3.2.6 (Ronquist & Huelsenbeck 2003) on CIPRES Science Gateway (Miller *et al.* 2010) using the GTR+I+G evolutionary model as the best-fit model selected by MrModeltest ver. 2 (Nylander 2004) according to the Akaike Information Criterion (AIC). Markov Chain Monte Carlo (MCMC) simulations were run with two sets of four chains for 4 000 000 generations, with a sample frequency of every 100th generation. The first 10 000 trees were discarded as burn-in. A maximum likelihood (ML) tree was constructed using RAxML-HPC2 ver. 8.2.10 (Stamatakis *et al.* 2008) on the CIPRES Science Gateway (Miller *et al.* 2010) with the GTR+I+G evolutionary model as the best-fit according to the AIC criterion selected by the program Modeltest ver. 3.4 (Posada & Crandall 1998). Nodal support came from 1000 bootstrap replicates. TreeView ver. 1.6.6 and MEGA ver. 7 (Tamura *et al.* 2013) were used to visualize tree topologies. For the interpretation of bootstrap values and Bayesian posterior probabilities, we consider values ≥95 as high, from 71 to 94 as moderate, and from 50 to 70 as low (Hillis & Bull 1993; Alfaro *et al.* 2003).

Repository

HANU = Harbin Normal University

Abbreviations

M1 = membranelle 1
M2 = membranelle 2
M3 = membranelle 3
Ma = macronucleus
Mi = micronucleus
PK = postoral kineties
PM = paroral membrane
Sc = scutica
SK = somatic kineties

SK 1 = somatic kinety 1
SKn = somatic kinety n

Results

Taxonomy

Class Oligohymenophorea de Puytorac *et al.*, 1974
Subclass Hymenostomatia Delage & Hérouard, 1896
Order Tetrahymenida Fauré-Fremiet in Corliss, 1956

Family **Pseudotetrahymenidae** fam. nov.
urn:lsid:zoobank.org:act:A9FCE478-3C33-458D-AF4F-02505DF5BB74

Diagnosis

The outline of the cell is similar to the one in *Tetrahymena*. The adoral structure is of *Tetrahymena* type, composed of one paroral and three oral membranelles. M2 consists of several clusters, forming several parallel parts.

Type genus

Pseudotetrahymena gen. nov.

Genera included

Pseudotetrahymena gen. nov.

Genus ***Pseudotetrahymena*** gen. nov.
urn:lsid:zoobank.org:act:D5CB9EA2-346B-4228-99EF-ABC86B4B9BE8

Diagnosis

The size in vivo is about 165–230 × 125–210 µm; the anterior end pointed and the posterior end broadly rounded; the buccal field is big and deep, approximately 40% of body the length; 19 or 20 somatic kineties; two postoral kineties; M1 and M2 are approximately equal length; M2 consists of three parts (M2a, b, c); M3 is shorter than M1 and M2; one macronucleus; micronucleus is not observed; freshwater habitat.

Etymology

The genus-group name '*Pseudotetrahymena*' highlights the difference of the oral apparatus and outline from that of the genus *Tetrahymena*.

Type species

Pseudotetrahymena orientalis gen. et sp. nov. (monotypic genus).

Pseudotetrahymena orientalis gen. et sp. nov.
urn:lsid:zoobank.org:act:AD200533-1E68-4CF5-BE38-1CB4104F26A8
Figs 2–3; Table 1

Diagnosis

Size in vivo 165–230 × 125–210 µm; anterior end pointed and posterior end broadly rounded; buccal field big and deep, approximately 40% of body length; 19 or 20 somatic kineties; two postoral kineties;

M1 and M2 approximately equal length; M2 consists of three parts (M2a, b, c); M3 obviously shorter than M1 and M2; one macronucleus. Micronucleus not observed; freshwater habitat.

Etymology

The species-group name '*orientalis*' (Latin adjective; eastern, occurring in the Orient) refer to the fact that this species was discovered in East Asia.

Type material

Holotype

CHINA – **Heilongjiang Province** • Harbin City, Dafa international fish market [a freshwater aquarium]; 45°44′9″ N, 126°35′41″ E; 11 Oct. 2022; Xiang Wang leg.; HANU WX-20221011-01 (carbonate-stained slide with 6 specs, the holotype specimen marked with an ink circle).

Paratypes

CHINA – **Heilongjiang Province** • 5 specs; same collection data as holotype; HANU WX-20221011-02.

Type locality

A freshwater aquarium at Dafa international fish market in Harbin (45°44′9″ N, 126°35′41″ E), Northeast China.

Description

Size in vivo 165–230 × 125–210 μm; body shape flexible, anterior end narrowed and posterior end broadly rounded usually (Fig. 2A–B, E, H–I, K). When food vacuoles filled by food, 60% of posterior part of cell swells extremely (Fig. 2A, E). Ratio of length to width about 1.5:1. Buccal field usually big, deep, and oval, approximately 40% of body length (Fig. 2A, E, H–I; Table 1). Cortical granules spherical, arranged randomly on pellicle (Fig. 2H, J–K). Cytoplasm colorless to deep dark when food vacuoles filled with food (Fig. 2A, E, H–I, K), often containing several big food vacuoles filled with ingested *Tetrahymena* (food vacuoles about 40 μm in diameter) (Figs 2K, 3G). Single contractile vacuole (approximately 25 μm in diameter) positioned close to posterior end (Fig. 2K). Extrusomes not observed; single ellipsoid macronucleus, approximately 60 μm across after staining, usually squeezed by food vacuoles, no micronucleus observed (Fig. 3A–D, H). Length of somatic cilia approximately 10 μm, densely arranged. Locomotion mainly by swimming slowly, sometimes rotating forwards. Preying on *Tetrahymena*.

Altogether 19 or 20 SK arranged latitudinally, extending the entire length of body, consisting of monokinetids (Figs 2C–D, 3A–D). Two PK; PK1 and PK2 commence anteriorly at level posterior of PM and extend posteriorly nearly to end of cell (Figs 2F, 3C). Oral structure consisting of one PM and three oral membranelles (Figs 2C, F–G, 3E–F). PM generally C-shaped, composed of paired basal bodies, organized in zigzagging pattern, extending anteriorly to M2. (Figs 2F–G, 3E–F). M1 and M2 approximately equal in length, composed of four or three rows of kinetids (Figs 2F–G; 3E–F). M2 consisting of three parts, namely M2a, b, c. M2a not obvious anteriorly and conspicuous posteriorly, composed of two rows of kinetids (Figs 2F–G, 3E–F). M2b obvious, consisting of four rows of kinetids (Figs 2F–G, 3E–F). M2c divided into two parts, each composed of four rows of kinetids (Figs 2F–G, 3E). M3 shorter than M1 and M2, containing three rows of kinetids (Fig. 3E–F).

Gene sequences

The SSU-rRNA gene sequence of *Pseudotetrahymena orientalis* gen. et sp. nov. ahas been deposited in GenBank database with the accession number OR616813.

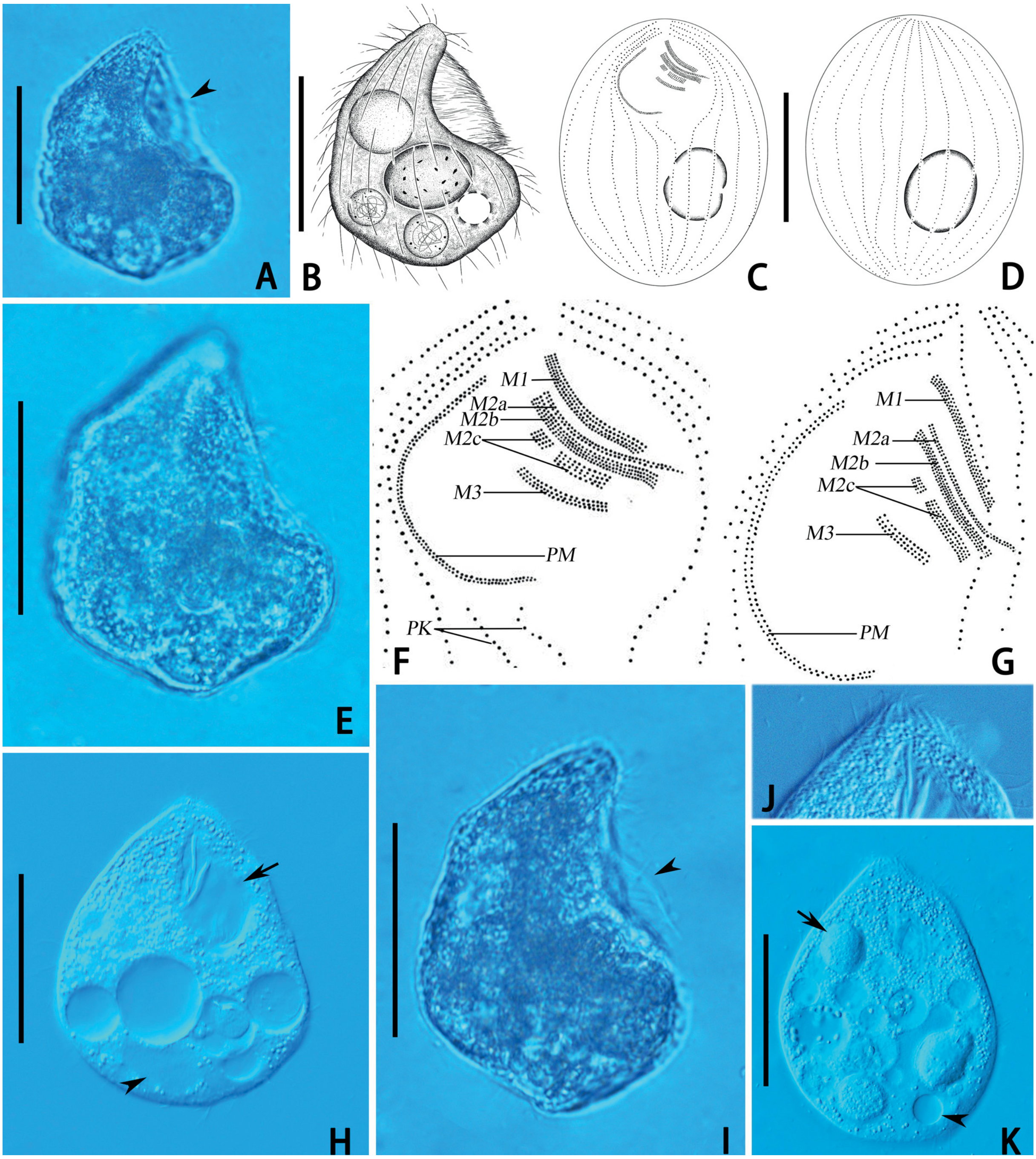

Fig. 2. Morphology and infraciliature of *Pesudotetrahymena orientalis* gen. et sp. nov. from life (A–B, E, H, I–K) and after carbonate-staining (C–D, F–G). **A–B**. Ventral views of the representative cell, arrowhead in A indicates oral field. **C–D**. Infraciliature of the holotype (HANU WX-20221011-01) showing the ciliary pattern and macronucleus. **C**. Ventral view. **D**. Dorsal view. **E**. Dorsal view of representative cell. **F–G**. Details of oral structure. **H**. Arrowhead shows macronucleus, arrow indicates oral field. **I**. Arrowhead shows oral cilia. **J**. Details of cortical granules. **K**. Arrow shows big food vacuole filled with ingested *Tetrahymena*; arrowhead marks contractile vacuole. M1 = membranelle 1; M2a = membranelle 2a; M2b = membranelle 2b; M2c = membranelle 2c; M3 = membranelle 3; PM = paroral membrane. Scale bars = 95 µm.

Subclass Scuticociliatia Small, 1967
Order Pleuronematida Fauré-Fremiet in Corliss, 1956
Family Cyclidiidae Ehrenberg, 1838
Genus *Cyclidium* Müller, 1773

Cyclidium paravorax sp. nov.
urn:lsid:zoobank.org:act:EFC1DA92-72AD-4EA2-936F-12805A06A0EA
Figs 4–5; Tables 1–2

Diagnosis

The cell about 20–30 × 15–25 μm in vivo; the buccal field occupies about 40–45% of the body length; nine somatic kineties; SKn extends to posterior sub-terminally; one or two macronuclei and one micronucleus; M1 consists of two longitudinal rows and is obviously shorter than M2; M2 has a long-triangle shape; M3 consists of four basal bodies in one row; the scutica is mainly composed of two pairs of kinetosomes; single caudal cilium; freshwater habitat.

Etymology

The species-group name '*paravorax*' is a composite of the Greek adjective 'para-' (beside) and the species-group name '*vorax*', indicating its similarity to *Cyclidium vorax* Pan, 2020.

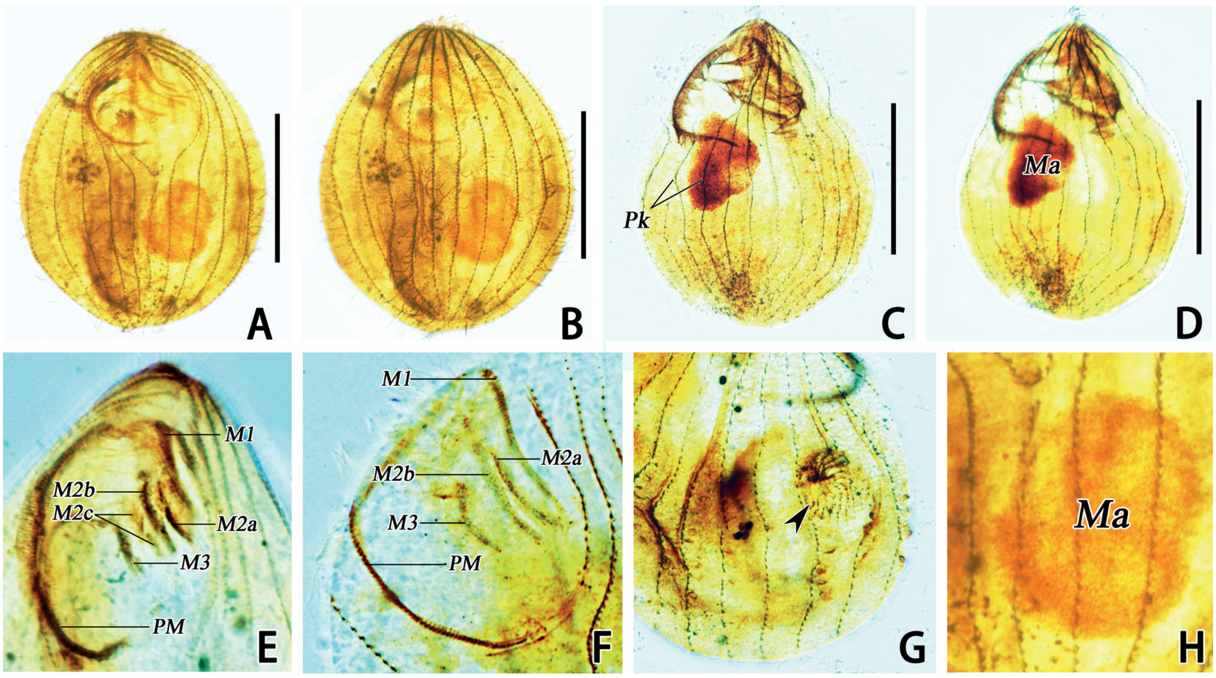

Fig. 3. Photomicrographs of *Pesudotetrahymena orientalis* gen. et sp. nov. after silver carbonate staining, paratypes (HANU WX-20221011-02), showing the whole cell (A–D) and details of the buccal region (E–H). **A**. Ventral view. **B**. Dorsal view. **C–D**. Different form of specimen exhibiting infraciliatures and macronucleus. **E–F**. Details of the adoral zone. **G**. Arrowhead shows stained *Tetrahymena* in food vacuole. **H**. Macronucleus. M1 = membranelle 1; M2a = membranelle 2a; M2b = membranelle 2b; M2c = membranelle 2b; M3 = membranelle 3; PM = paroral membrane; Ma = macronucleus; PK = postoral kineties. Scale bars = 95 μm; E–H not to scale.

Table 1. Morphometric characterization of *Pesudotetrahymena orientalis* gen. et sp. nov. (Po), *Cyclidium paravorax* sp. nov. (Cp), *Cyclidium vorax* Pan, 2020 (Cv; Pan *et al.* 2020), *Cyclidum glaucoma* Müller, 1773 (Cg; Pan *et al.* 2020), *Cyclidium sinicum* Pan, 2017 (Cs; Pan *et al.* 2017), and *Cyclidium varibonneti* Song, 2000 (Cvb; Song 2000). Data from silver carbonate-stained and protargol-prepared specimens. Measurements in µm. CV = coefficient of variation (%); M = Median; Max = maximum; Mean = arithmetic mean; Min = minimum; N = number of specimens; SD = standard deviation.

Character	Species	Max	Min	Mean	M	CV	SD	N
Body length, µm	Po	234	166	193.2	192	8.6	16.7	17
	Cp	30	20	24.6	24.0	13.2	3.3	24
	Cv	34	45	39.3	40	7.8	3.1	15
	Cg	18	12	13.2	13	14.4	1.9	15
	Cs	32	21	28.3	27	21.4	5.7	15
	Cvb	19	12	13.5	–	10.9	1.46	17
Body width, µm	Po	208	124	164.5	168	12.1	19.8	19
	Cp	25	15	19.7	19.5	15.2	3.0	24
	Cv	34	25	28.5	29	8.5	2.4	13
	Cg	12	8	10.3	1	7.1	0.7	15
	Cs	16	12	13.2	13	9.1	1.2	15
	Cvb	13	8	9.8	–	9.2	0.22	17
Buccal field length, µm	Po	94	64	76.8	75.0	10.2	7.9	20
	Cp	15	9	11.2	11.0	11.2	1.2	24
	Cv	24	17	20.4	20	10.1	2.1	18
	Cg	8	6	7.4	7	6.1	0.43	12
	Cs	15	10	12.2	12	9.1	1.1	13
	Cvb	13	9	9.7	–	6.8	0.66	17
Somatic kineties, number	Po	22	18	19.7	20	5.4	1.1	9
	Cp	10	9	9.2	9.0	4.4	0.4	24
	Cv	10	9	9.6	10	13.6	0.5	13
	Cg	9	8	8.3	8	3.7	0.3	13
	Cs	11	11	11	11	0	0	13
	Cvb	13	11	11.7	–	5.1	0.6	16
Postoral kineties, number	Po	2	2	2.0	2.0	0.0	0.0	10
Macronucleus length, µm	Po	88	48	60.0	55.0	16.1	11.4	14
	Cp	11	5	6.9	7.0	19.2	1.3	16
	Cv	14	8	11	11	16.5	2	13
	Cg	5	3	4.1	4	2.5	0.1	15
	Cvb	5	3	3.9	–	18.5	0.72	16
Macronucleus, number	Po	1	1	1	1	0	0	22
	Cp	2	1	1.3	1	35.3	0.5	23
	Cv	2	1	1.5	1	33.7	0.5	27
	Cg	1	1	1	1	0	0	15
	Cs	2	2	2	2	0	0	13
	Cvb	2	2	2	–	0	0	>100
Basal bodies in SKn, number	Cp	13	10	11.4	11.0	9.3	1.1	18
	Cv	13	11	11.7	12	8.2	0.9	11
	Cg	12	11	11.1	11	1.8	0.2	11
	Cs	13	12	12.6	13	3.8	0.4	14
	Cvb	11	10	10.4	–	4.9	0.51	11
Basal bodies in SK 1, number	Cp	14	13	13.1	13	2.6	0.3	15
	Cs	13	12	12.6	13	4.6	0.5	14
	Cvb	16	14	15.2	–	3.9	0.58	13

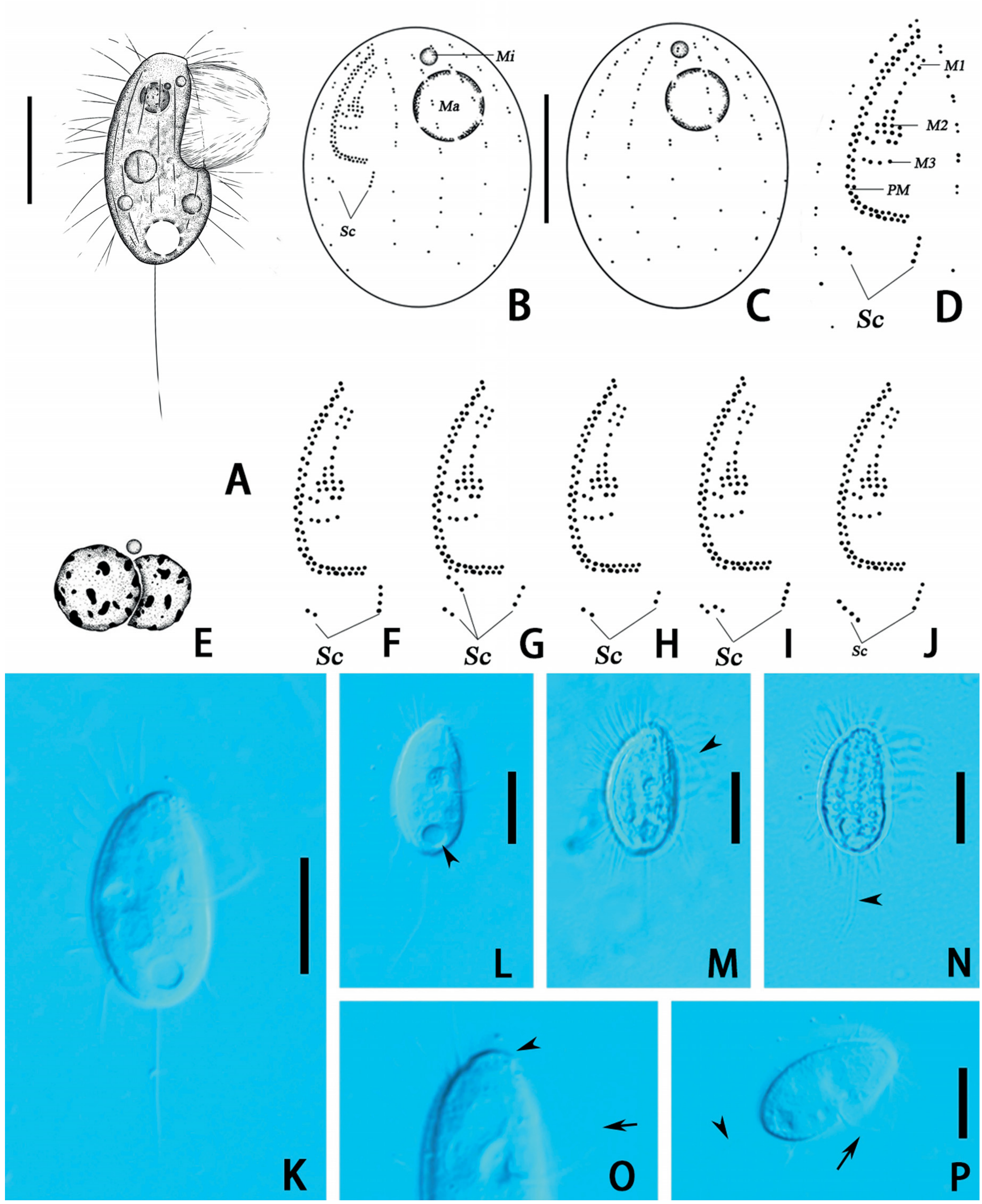

Fig. 4. Morphology and infraciliature of *Cyclidium paravorax* sp. nov. **A, K–N**. Ventral views of representative specimens (paratytes; HANU WX-20221123-02); arrowhead in L marks contractile vacuole, in M depicts paroral membrane and in N indicates caudal cilium. **B–C**. Infraciliature of the holotype (HANU WX-20221123-01) to show the ciliary pattern. **B**. Ventral view. **C**. Dorsal view. **D**. Details of oral structure. **E**. Macronuclei and micronucleus. **F–J**. Patterns of scutica. **O**. Arrowhead shows flat section, arrow shows paroal membrane. **P**. Arrowhead marks caudal cilium, arrow show paroal membrane. M1 = membranelle 1; M2 = membranelle 2; M3 = membranelle 3; Ma = macronucleus; Mi = micronucleus; PM = paroral membrane; Sc = scutica. Scale bars = 15 µm.

Type material

Holotype

CHINA – **Heilongjiang Province** • Harbin City, Harbin Normal University; 45°52′3″ N, 126°33′2″ E; 23 Nov. 2022; Xiang Wang leg.; HANU WX-20221123-01 (carbonate-stained slide with 22 specs, the holotype specimen marked with an ink circle).

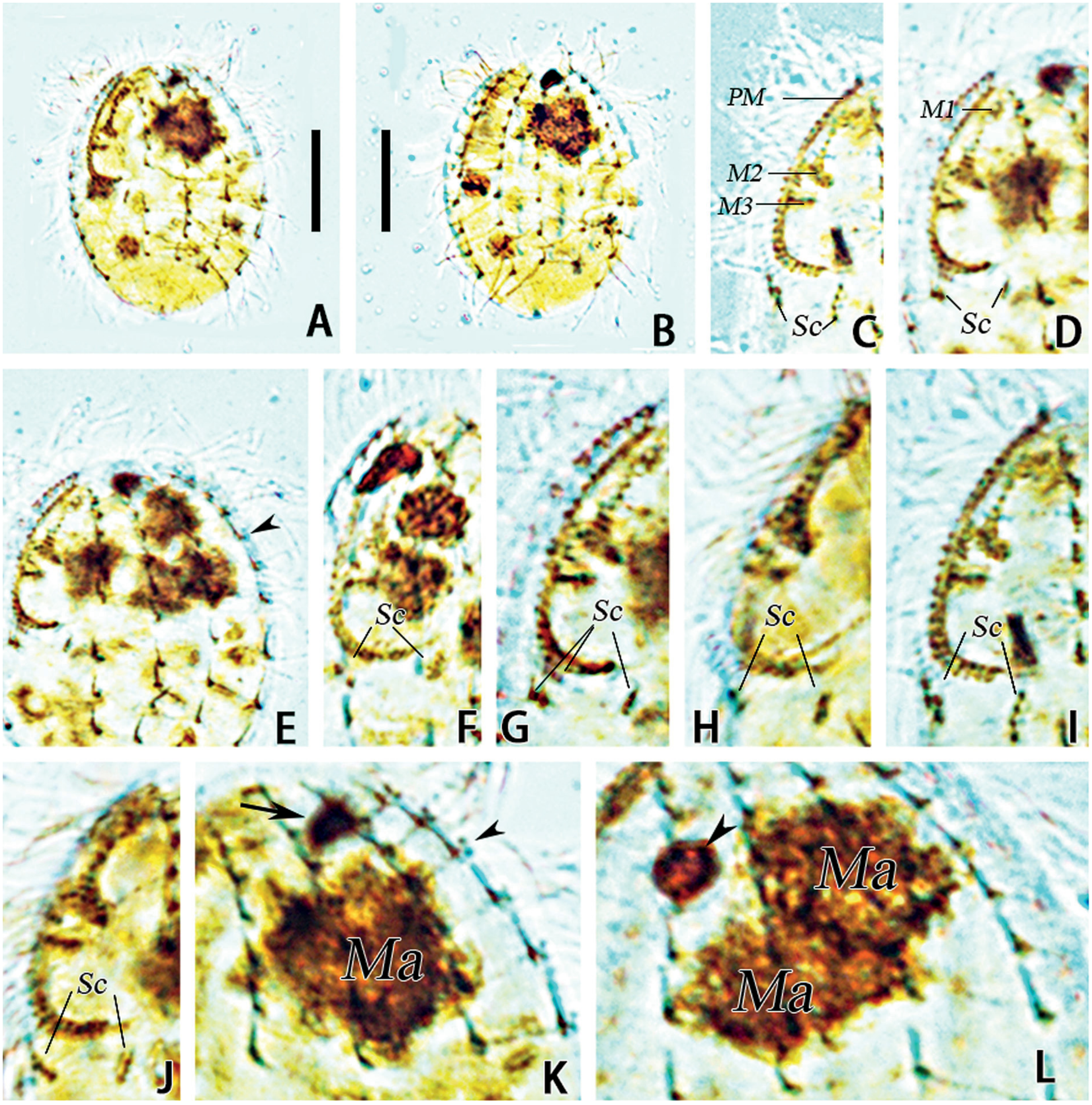

Fig. 5. Photomicrographs of *Cyclidium paravorax* sp. nov. after silver carbonate staining; paratytes (HANU WX-20221123-02). **A–B**. Whole cell. **A**. Ventral View. **B**. Dorsal view. **C–I**. Details of the buccal region. **C–D**. Details of buccal area. **E**. Arrowhead indicate dikinetid. **F–J**. Patterns of scutica after staining. **K–L**. Macronuclei and micronucleus. **K**. Arrowhead indicates dikinetid, arrow shows micronucleus. **L**. Arrowhead marks micronucleus. M1 = membranelle 1; M2 = membranelle 2; M3 = membranelle 3; Ma = macronucleus; PM = paroral membrane; Sc = scutica. Scale bars = 15 µm.

Table 2. Comparison between *Cyclidium paravorax* sp. nov. and *Cyclidium bonneti* Grolière, 1980 (Grolière 1980). Measurements in µm.

Character	*Cyclidium paravorax* sp. nov.	*Cyclidum bonneti* Grolière, 1980
Body size, µm (base on silver staining)	20–30 × 15–25	18–28 × 11–18
Length of buccal field/cell length	4.5/10	3/4
Somatic kineties, number	9–10	14–16
Macronucleus, number	1–2	2
Basal bodies in SKn, number	10–13	16

Paratypes

CHINA – **Heilongjiang Province** • 39 specs; same collection data as holotype; HANU WX-20221123-02.

Type locality

A freshwater aquarium at Harbin Normal University, Harbin (45°52′3″ N, 126°33′2″ E), Northeast China.

Description

Body about 20–30 × 15–25 µm in vivo, ellipsoidal with flat section in anterior end, posterior end slightly wider (Fig. 4A, K–N). Ratio of length to width about 1.25:1. Buccal field about 40%–45% of body length, with prominent paroral membrane on ventral side (Fig. 4A, K–P; Table 1). Cortex smooth. Somatic cilia and cilia of paroral membrane about 6 µm long (Fig. 4K–N), single caudal cilium about 13 µm long (Fig. 4A, K, N, P). Cytoplasm colorless, containing several (approximately 1 µm in diameter) bacteria-filled food vacuoles and variable-sized (0.5–1 µm) granules (Fig. 4A, K, N). Contractile vacuole located near posterior end of cell, approximately 4 µm in diameter (Fig. 4A, K–L, N). Extrusomes not observed; one or two globular macronuclei approximately 7 µm in diameter (eight out of 23 cells examined with two macronuclei); one micronucleus approximately 2 µm across after staining (Figs 4E, 5K–L). Locomotion by swimming fast, sometimes motionless for long periods.

Altogether 9 or 10 SK extending from near anterior end to near posterior end (Figs 4B–C, 5A–B), all consisting of dikinetids in its anterior half and widely positioned monokinetids posteriorly (Fig. 5E, K). SKn consisting of 10 to 13 kinetids; SK 1 composed of 14 kinetids. PM generally L-shaped, occupies about 40%–45% of body length, composed of zigzagging structure (Figs 4D, 5C). M1 consists of two longitudinal rows of monokinetids, shorter than M2 (Figs 4D, 5D); M2 long-triangle shaped, composed of three longitudinal rows and 8 horizontal rows (Figs 4D, 5C); M3 obviously smaller and shorter than M1 and M2, bearing one row consisting of four basal bodies (Figs 4D, 5C). Scutica patterns diversified (Figs 4F–J, 5F–J), arranged in two groups mainly (Figs 4D, F, 5F), located near posterior end of PM; each part composed of two and four basal bodies.

Gene sequences

The SSU-rRNA gene sequence of *Cyclidium paravorax* sp. nov. has been deposited in GenBank database with the accession number OR466404.

Phylogenetic positions of Pseudotetrahymena orientalis gen. et sp. nov. and Cyclidium paravorax sp. nov.

The maximum likelihood and Bayesian inference trees have almost identical topologies, therefore only the maximum likelihood tree is shown here (Fig. 6). *Pseudotetrahymena orientalis* gen. et sp. nov. lies in the periphery of the Tetrahymenida clade. *Cyclidium paravorax* sp. nov. clusters with the clades formed by *Cyclidium* sp. KX853100, *C. glaucoma* Z22879, *C. glaucoma* EU032356 and *C. glaucoma* KY476313.

Discussion

Establishment of the new family

The order Tetrahymenida Fauré-Fremiet in Corliss, 1956 is mainly characterized by small to medium-sized cell with typically ovoid outline and holotrichous somatic ciliation. The oral structures consists of a right paroral (undulating membrane) and three left oral polykinetids (membranelles) situated in the oral

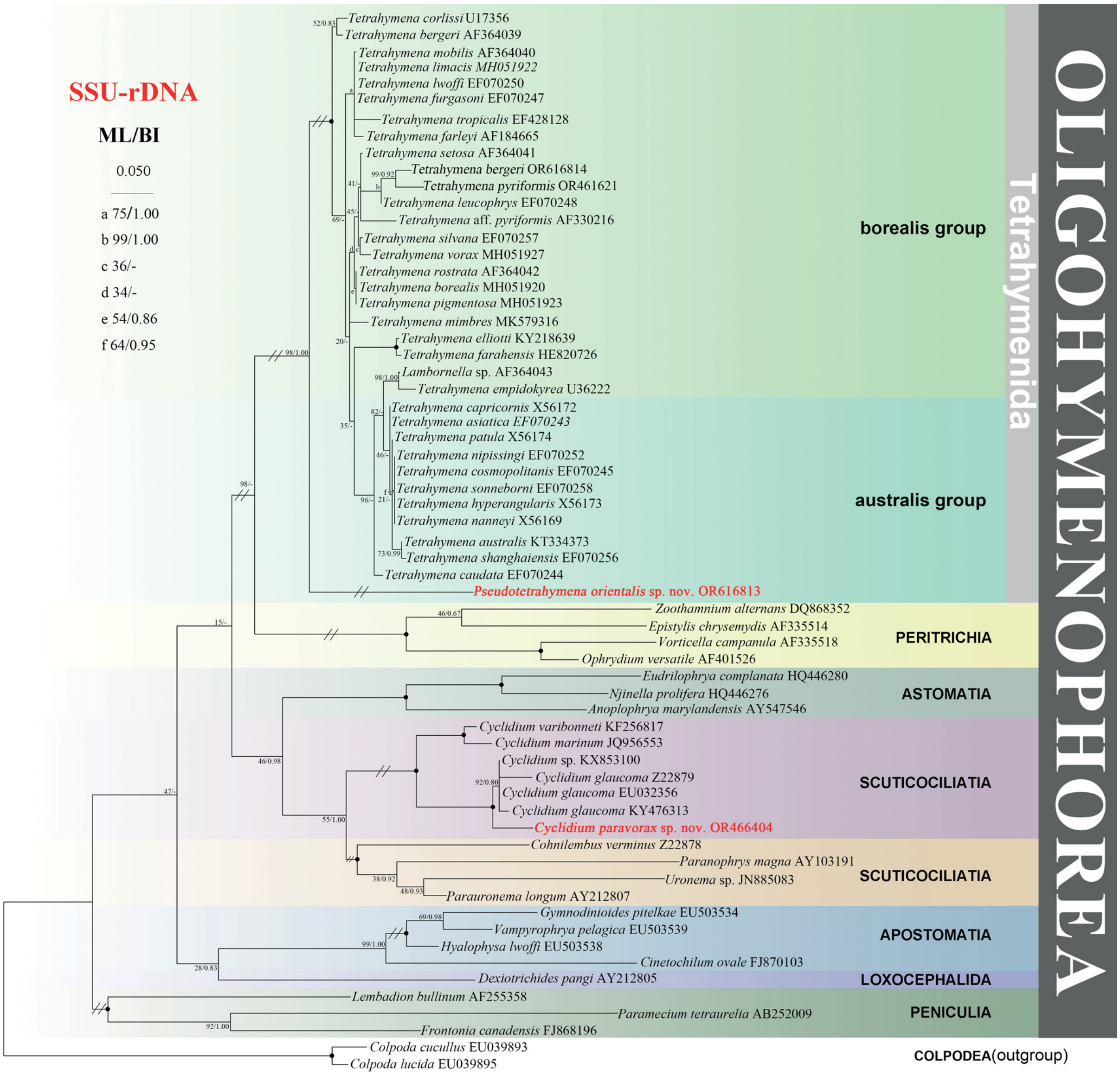

Fig. 6. The maximum likelihood tree inferred from the SSU-475 rRNA gene sequences, showing the positions of *Pseudotetrahymena orientalis* gen. et sp. nov. and *Cyclidium paravorax* sp. nov. (in red). Numbers at nodes represent the bootstrap values of maximum likelihood out of 1000 replicates and the inference. Fully supported (100%/1.00) branches are marked with solid circles. The scale bar corresponds to five substitutions per 100 nucleotide positions.

cavity. Members of this order exhibit a complex life cycle in histophagous and parasitic species, and are commonly found in freshwater habitats, sometimes terrestrial (Lynn 2008).

Pseudotetrahymenidae fam. nov. should be assigned to the Tetrahymenida based on its morphological characteristics and habitat. In terms of the structure of the oral apparatus and morphology of the cell, the following families should be compared with Pseudotetrahymenidae: Glaucomidae Corliss, 1971, Spirozonidae Kahl, 1926, Tetrahymenidae Corliss, 1952, Turaniellidae Didier, 1971, Trichospiridae Kahl, 1926 (Roux 1901; Corliss 1952, 1971; Foissner *et al.* 1981; Ganner & Foissner 1989).

Pseudotetrahymenidae fam. nov. differs from Glaucomidae by the following characters: the absence of a small group of kinetosomes (X group) (vs present in Glaucomidae) , M1 and M2 composed of three or four rows of kinetids (vs more than three rows in Glaucomidae), and the cell size (165–230 µm vs usually 40–70 µm in Glaucomidae) (Corliss 1971; Lynn 2008; Pan *et al.* 2017b).

Pseudotetrahymenidae fam. nov. can be separated from Spirozonidae by: the somatic kineties arranged latitudinally (vs somatic kineties on left side and dorsal left mild torsional in Spirozonidae), the caudal cilia absent (vs caudal cilia present, forming a ring at the posterior end of cell in Spirozonidae), and the paroral membrane having continuous dikinetids (vs paroral membrane having isolated dikinetids at anterior end in Spirozonidae) (Foissner *et al.* 1981).

Though Pseudotetrahymenidae fam. nov. resembles Tetrahymenidae in the body shape and the position of the paroral membrane, which begins at the level of M2, it can be distinguished from Tetrahymenidae in having M2 composed of several parts (vs only one part in Tetrahymenidae) (Corliss 1952; Lynn 2008).

Pesudotetrahymenidae fam. nov. can be clearly distinguished from Turaniellidae by the following features: latitudinally arranged right ventral kineties (vs curving left, twisting anterior of the oral region, sometimes abruptly, to run parallel to the anterior suture in Turaniellidae), longitudinal somatic kineties (vs one or more somatic kineties interrupted by left edge of buccal cavity in Turaniellidae), and a paroral membrane entirely consisting of ciliated kinetosomes (vs anterior part consisting of ciliated and posterior part of non-ciliated kinetosomes in Turaniellidae) (Ganner & Foissner 1989; Lynn 2008).

When compared with Trichospiridae, Pesudotetrahymenidae fam. nov. can be separated by the following features: longitudinal somatic kineties (vs a special band of cilia associated with a pellicular ridge that spirals dextrally posteriorly, ending in a transverse ring of cilia in Trichospiridae), the absence of caudal cilia (vs caudal cilia forming a tuft in Trichospiridae), and the deep buccal cavity containing one paroral membrane and three oral membranelles (vs oral structures as anterior extensions of several somatic kineties invaginating into a shallow cavity in Trichospiridae) (Roux 1901; Lynn 2008).

Comparison of *Cyclidium paravorax* sp. nov. with congeners (Tables 1–2)

The most important diagnostic criteria for species identification and separation in the genus *Cyclidium* are the pattern of oral membranelles 1–3, the ratio of oral field to the body length, the number of somatic kineties, the type of kineties constituting somatic ciliary rows, the number of macronuclei and micronuclei, and the termination position of the posterior end of somatic kineties (Borror 1972; Grolière 1980; Didier & Wilbert 1981; Agamaliev 1983; Alekperov 2005).

Cyclidium paravorax sp. nov. can be separated from *C. vorax* by its smaller body size (20–30 × 15–25 µm vs 35–40 × 18–20 µm in *C. vorax*), M2 composed of three longitudinal lines and eight horizontal rows (vs four longitudinal lines and ten horizontal rows), and M3 having one row (vs two rows in *C. vorax*) (Pan *et al.* 2020).

Cyclidium paravorax sp. nov. can be distinguished from *C. sinicum* Pan *et al.*, 2017 by having nine or ten somatic kineties (vs stably 11 in *C. sinicum*), somatic kineties consisting of dikinetids in their anterior half and widely positioned monokinetids posteriorly (vs comprising loosely spaced monokinetids in *C. sinicum*), and M3 single-rowed (vs two-rowed in *C. sinicum*) (Pan *et al.* 2017).

Cyclidium paravorax sp. nov. differs from *C. varibonneti* Song, 2000 by having shorter buccal field (about 45% of body length vs 75% in *C. varibonneti*), nine or ten somatic kineties (vs mostly 11 or 13 in *C. varibonneti*), and occurring in a freshwater habitat (vs marine habitat for *C. varibonneti*) (Song 2000).

Cyclidium paravorax sp. nov. can be distinguished from *C. bonneti* Grolière, 1980 by fewer somatic kineties (nine or ten vs 14–16 in *C. bonneti*), and a smaller ratio of buccal field relative to the body length (45% vs > 60% in *C. bonneti*) (Grolière 1980).

Compared with *C. glaucoma*, *C. paravorax* sp. nov. differs in the following features: larger body size (20–30 × 15–25 µm vs 12–18 × 8–12 µm in *C. glaucoma*), and smaller buccal field to body length ratio (45% vs 56% in *C. glaucoma*) (Pan *et al.* 2020).

Comments on scutica of *Cyclidium*

Scutica is a structure composed of non-ciliated kinetosomes in diverse patterns which exists widely in scuticociliates (Borror 1963; Thompson 1967; Lynn 2008; Fan *et al.* 2011; Foissner *et al.* 2014; Pan *et al.* 2020; Hao *et al.* 2022; Poláková *et al.* 2023). Scutica of ciliates assigned to the genus *Cyclidium* mainly consist of two pairs of basal bodies (Song 2000; Pan *et al.* 2017a; Pan *et al.* 2020; Poláková *et al.* 2023). In present study, five distinct patterns of scutica were observed in *C. paravorax* hereafter named types I–V (Figs 4F–J, 5F–J).

Scutica type I contains two parts composed of two and four kinetosomes, respectively (Figs 4F, 5F). Kinetosomes of type II are divided into three parts consisting of two, three and three kinetosomes each (Figs 4G, 5G). Type III is composed of four basal bodies divided into two pairs (Figs 4H, 5H). Type IV consists of eight basal bodies divided into two parts, two pairs of kinetosomes forming one part, another four kinetosomes forming one row (Figs 4I, 5I). In type V, two rows of kinetosomes forms shape V, each row consisting of four basal bodies (Figs 4J, 5J). Such a phenomenon indicates the possibility of intraspecific diversity of scutica among scuticociliates. Therefore, it is necessary to get more morphological data to confirm the intraspecific diversity of scutica for scuticociliates, not just for the genus *Cyclidium*.

Phylogenetic analyses

The newly obtained SSU-rDNA sequence of *Pseudotetrahymena orientalis* gen. et sp. nov. is most similar to *Tetrahymena setosa* AF364041, with which it shares a molecular similarity of about 99.28% but still exhibits a difference of 15 nucleotides, which support the validity of *P. orientalis* as a distinct species. Besides, Pseudotetrahymenidae fam. nov. emerges as the earliest-branching clade within Tetrahymenida and forms a sister clade to all the other Tetrahymenida species with high support (ML/BI, 99/1.00). This phylogenetic position, combined with the morphological characteristics of *P. orientalis*, further supports the establishment of Pseudotetrahymenidae.

Cyclidium paravorax sp. nov. groups with *Cyclidium* sp. KX853100, *C. glaucoma* Z22879, *C. glaucoma* EU032356, and *C. glaucoma* KY476313 with full support, indicating a close relationship between these species.

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Conflicts of interest

The authors declare that they have no conflict of interest.

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