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Morphology and systematics of two freshwater urostylid ciliates, with description of a new species (Protista, Ciliophora, Hypotrichia)

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Abstract

The morphology of two freshwater urostylid species, *Neourostylopsis flava* spec. nov. and *Pseudourostyla subtropica* Chen et al., 2014, isolated from freshwater ponds in northern and southern China, respectively, was investigated following examination of specimens in vivo and following protargol staining. *Neourostylopsis flava* spec. nov. is distinguished from its congeners by the following characteristics: body size 150–220 × 50–75 μm in vivo; yellow in colour; bright yellow to yellow-brownish spherical cortical granules densely arranged along marginal cirral rows and in irregular short rows on dorsal side; adoral zone with 40–55 membranelles; six to eight frontal, three or four buccal, two pretransverse ventral and seven to nine transverse cirri; 27–40 midventral pairs extending to about anterior 55% of cell; four or five left and four right marginal rows; freshwater habitat. A redescription of a freshwater population of *P. subtropica* is also provided. Phylogenetic analyses based on small subunit ribosomal DNA sequences shows that *P. subtropica* and *N. flava* spec. nov. group with their congeners and both *Neourostylopsis* and *Pseudourostyla* are monophyletic.

Keywords: Freshwater ciliate; Morphology; *Neourostylopsis flava* spec. nov.; Phylogeny; *Pseudourostyla subtropica*; SSU rDNA

Introduction

In recent years there have been numerous reports on the morphology and phylogeny of hypotrich ciliates, mainly due to the application of silver staining methods to reveal characters of taxonomic and morphogenetic importance, and of molecular technologies to reveal their evolutionary

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relationships (Chen et al. 2013, 2015; Fan et al. 2014a,b; Foissner et al. 2014; Heber et al. 2014; Hu and Kusuoka 2015; Jung et al. 2014, 2015; Kumar et al. 2014, 2015; Kumar and Foissner 2015; Küppers 2014; Lu et al. 2014; Luo et al. 2014; Lv et al. 2015; Park et al. 2013; Shao et al. 2014a,b, 2015; Singh and Kamra 2015; Spakova et al. 2014). Urostylids are one of the most diverse and complex groups of hypotrichs and consequently have been the focus of much of this research (Berger 2006; Chen et al. 2010, 2014; Dai and Xu 2011; Huang et al. 2012, 2014; Jankowski 1979; Jerka-Dziadosz 1970; Kumar et al. 2010; Paiva et al. 2009; Suganuma 1973; Yi et al. 2009).

The urostylid genus *Metaurostylopsis* was established by Song et al. (2001) with *M. marina* the type species by original designation. The generic placement of two species of *Metaurostylopsis*, however, has been questioned, i.e., *M. songi* Lei et al., 2005, which Berger (2006) suggested should be excluded from *Metaurostylopsis*, and *M. flavicana* Wang et al., 2011, the systematics of which remains unclear due to discordance between the morphogenetic and molecular data (Song et al. 2011). Nevertheless, both species were retained in the genus *Metaurostylopsis* pending further information (Berger 2006; Song et al. 2001, 2011).

Based on a combination of morphological and molecular data, Chen et al. (2013) established the genus *Neourostylopsis* which they defined as follows: marine or brackish Urostylidae with frontal and transverse cirri clearly differentiated; buccal cirri present; two frontoterminal cirri; midventral complex composed of midventral pairs only and not exceeding the halfway point of the cell; more than one row of marginal cirri on each side which derive from individual anlagen within each parental row; caudal cirri lacking. Hitherto, three species were assigned to *Neourostylopsis*: *N. flavicana* (Wang et al., 2011) Chen et al., 2013 (type species), *N. songi* (Lei et al., 2005) Chen et al., 2013 and *N. orientalis* Chen et al., 2013.

The genus *Pseudourostyla* was established by Borror (1972) with *P. cristata* (Jerka-Dziadosz, 1964) Borror, 1972 as the type species by original designation. Six further species were subsequently described, namely *P. levis* Takahashi, 1973, *P. muscorum* (Kahl, 1932) Borror, 1972, *P. nova* Wiackowski, 1988, *P. pelotensis* Paiva and Silva-Neto, 2006, *P. cristatoides* Jung et al., 2012 and *P. subtropica* Chen et al., 2014. Species separation and identification remains problematic in this genus because some are insufficiently studied (Berger 2006; Chen et al. 2014; Kahl 1932; Oberschmidleitner and Aescht 1996; Paiva and Silva-Neto 2006; Wiackowski 1988).

In this paper, the morphology of two freshwater urostylid ciliates, *Neourostylopsis flava* spec. nov. and *Pseudourostyla subtropica* Chen et al., 2014, is investigated and their evolutionary relationships are analyzed based on small subunit ribosomal DNA (SSU rDNA) sequence data. *Pseudourostyla subtropica* is reinvestigated because it was isolated from freshwater pond whereas it was previously reported from a brackish water habitat (Chen et al. 2014).

Material and Methods

Neourostylopsis flava spec. nov. was collected on 27 March 2011 from a freshwater pond (36°03′ 48.4″N; 120°20′41.5″E) in Qingdao, China (water temperature 14 °C, pH 7.3). *Pseudourostyla subtropica* was isolated on 26 April 2012 from a freshwater pond (salinity 0‰) near Daya Bay (22°42′31.0″N; 114°45′08.6″E) in Guangdong province, China (water temperature 21 °C, pH 7.4). In each case, about 0.5 L water was collected from 0.1 to 0.5 m below the water surface using a sampling bottle. Ciliates were maintained in habitat water in Petri dishes as raw cultures at room temperature (ca. 25 °C) with rice grains to enrich the growth of bacteria as food resources. Three to five cells of each species were collected with a micropipette and rinsed four times with autoclaved seawater to remove other protists.

Isolated cells were observed and photographed in vivo using differential interference contrast microscopy (Olympus BX53, Tokyo, Japan). The protargol method used to reveal the infraciliature (Wilbert 1975); the protargol reagent was made according to Pan et al. (2013). Counts and measurements of stained specimens were performed at magnifications of 100–1250×. Drawings were made with the help of a camera lucida (Song 2001). Terminology follows Berger (2006).

Genomic DNA of *Neourostylopsis flava* spec. nov. and *Pseudourostyla subtropica* was extracted using the DNeasy Tissue kit (Qiagen, CA). The PCR amplifications of SSU rDNA sequences were performed with universal primers (Medlin et al. 1988). Purified PCR product of the appropriate size was inserted into the pMD^{TM}18-T vector (Takara Biotechnology, Dalian Co., Ltd.) and sequenced on an ABI-PRISM 3730 automatic sequencer (Applied Biosystems). The SSU rDNA sequences of *N. flava* spec. nov. and *P. subtropica* are deposited in the GenBank database with accession numbers KR013238 and KR013239, respectively. Other sequences used in the study were obtained from GenBank database (accession numbers shown in Fig. 6). The accession numbers of seven oxytrichids used in the analyses are as follows: *Cyrtohymena citrina* AY498653, *Gastrostyla steinii* AF164133, *Laurentiella strenua* AJ310487, *Oxytricha longa* AF164125, *Paraurostyla weissei* AJ310485, *Pleurotricha lanceolata* FJ748886 and *Stylonychia mytilus* AF164123. Sequences were aligned using Clustal W implemented in BioEdit 7.0 (Hall 1999) enabling pairwise analysis. Two oligotrichids (*Parastrombidinopsis minima* DQ393786, *Stenosemella nivalis* FJ196074) and two choreotrichids (*Strombidium stylifer* DQ631805, *Novistrombidium orientale* FJ422988) were used as the outgroup. Phylogenetic relationships were determined by maximum likelihood and bayesian inference analyses. The maximum likelihood analysis was conducted using RAxML-HPC2 on XSEDE (8.1.11) (Stamatakis 2006; Stamatakis et al. 2008) via the CIPRES Science Gateway website (http://www.phylo.org/sub_sections/portal) using the GTR + I (=0.6986) + G (=0.4938) model as selected by

Modeltest v.3.4 (Posada and Crandall 1998). The reliability of internal branches was estimated by bootstrapping with 1000 replicates. The Bayesian inference analysis was performed with MrBayes v3.2.3 (Ronquist et al. 2012) via the CIPRES Science Gateway using the GTR + I + G model which was selected as the best model by MrModeltest v.2.0 (Nylander 2004). The chain length of Markov chain Monte Carlo simulations was 1,000,000 generations with a sampling frequency of 100 generations. The first 25% of sampled trees was discarded as burn-in. Phylogenetic trees were visualized with TreeView v.1.6.6 (Page 1996) and MEGA v.4 (Tamura et al. 2007).

Results and Discussion

Neourostylopsis flava spec. nov. (Figs 1A–F, 2A–M; Table 1)

Diagnosis

Freshwater *Neourostylopsis*; body in vivo 150–220 × 50–75 μm, elliptical to spindle-shaped; bright yellow to yellow-brownish spherical cortical granules, elliptical in lateral view, ca. 1 μm across, densely arranged along marginal cirral rows and in irregular short rows on dorsal side, rendering cell yellow in colour; contractile vacuole at about 40% of body length near left cell margin; adoral zone with 40–55 membranelles; six to eight frontal cirri; three or four buccal cirri; two pretransverse ventral cirri; seven to nine transverse cirri; 27–40 mid-ventral cirral pairs; four or five left and four right marginal cirral rows; 49–98 macronuclear nodules.

Type locality

A freshwater pond at Zhongshan Park in Qingdao (36°03′48.4″N; 120°20′41.5″E), northern China.

Type deposition

A protargol slide (registration number FYB-2011032701-01) with the holotype specimen (Fig. 1E, F) marked with a circle, and two paratype slides (registration numbers: FYB-2011032701-02, 03) with protargol-stained specimens, are deposited in the Laboratory of Protozoology, Ocean University of China. An additional paratype slide is deposited in the Natural History Museum, London (registration number NHMUK 2015.7.24.1).

Etymology

The species-group name '*flava*' refers to its bright yellow to yellow-brownish body colour. Female gender.

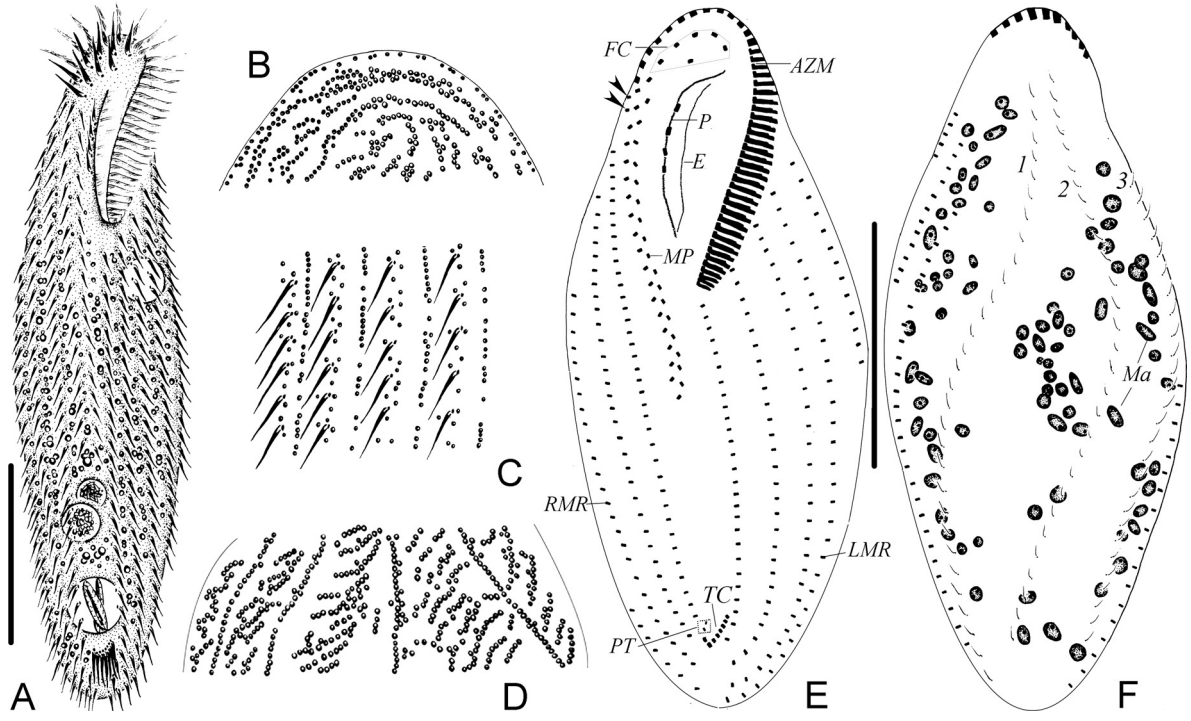

Fig. 1. (**A**–**F**) Morphology and infraciliature of *Neourostylopsis flava* spec. nov. from life (A–D) and after protargol staining (E, F). (**A**) Ventral view of a representative individual. (**B**) Anterior part in dorsal view showing arrangement of cortical granules. (**C**) Ventral view, to show marginal cirri and cortical granules. (**D**) Dorsal view, to show cortical granules. (**E**, **F**) Ventral (E) and dorsal (F) view of same specimen (holotype), showing infraciliature and nuclear apparatus, arrowheads in (E) mark frontoterminal cirri. We designate the inner marginal row as row 1 on both sides. AZM, adoral zone of membranelles; E, endoral; FC, frontal cirri; LMR, left marginal cirral rows; Ma, macronuclear nodules; MP, midventral pairs; P, paroral; PT, pretransverse ventral cirri; RMR, right marginal cirral rows; TC, transverse cirri; 1–3, dorsal kineties. Bars, 50 μm (A, E, F).

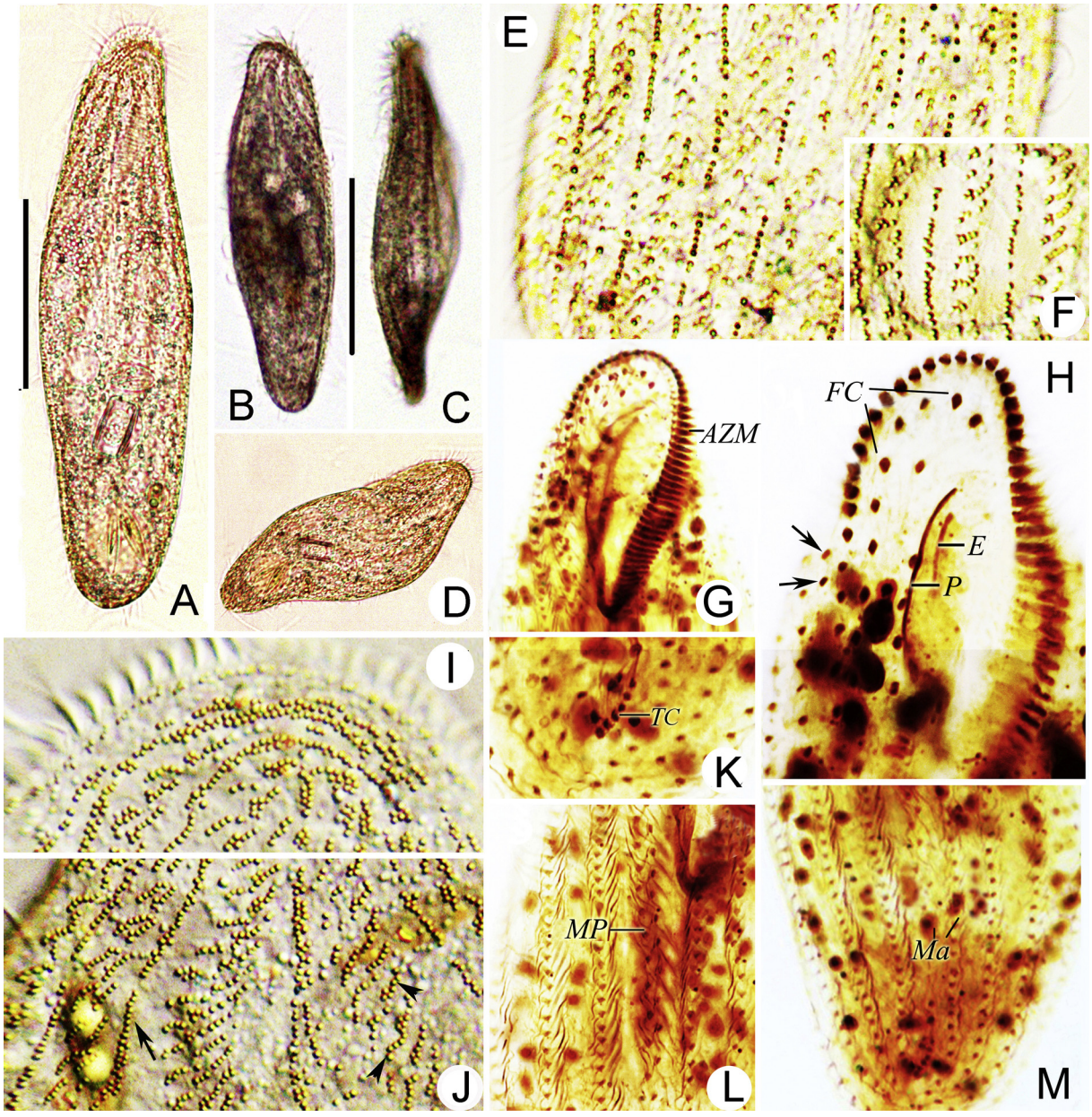

Fig. 2. (**A**–**M**) Photomicrographs of *Neourostylopsis flava* spec. nov. from life (A–F, I, J) and after protargol staining (G, H, K–M). (**A**) Ventral view of a representative individual. (**B**) Another individual, to show a different body shape. (**C**) Left lateral view of a representative individual. (**D**) Ventral view of a contracted cell. (**E**, **F**) Ventral views, to show cortical granules. (**G**, **H**) Ventral views of buccal field, arrows show frontoterminal cirri, endoral is not completely stained in (H). (**I**, **J**) Dorsal views, to show cortical granules, arrow and arrowheads in (J) show cortical granules in lines along dorsal kineties and cortical granules regularly grouped between dorsal kineties, respectively. (**K**, **L**) Ventral views (holotype), to show transverse cirri (K), marginal rows (L) and midventral pairs (L). (**M**) Posterior region, to show macronuclear nodules. AZM, adoral zone of membranelles; E, endoral; FC, frontal cirri; Ma, macronuclear nodules; MP, midventral pairs; P, paroral; TC, transverse cirri. Bars, 60 μm (A), 80 μm (B, C).

Description

Body size approximately 150–220 × 50–75 μm in vivo, usually ellipsoidal or spindle-shaped with both ends rounded and slightly narrowed (Figs 1A, 2A, B). Dorsoventrally flattened about 3:1, flexible and slightly contractile (Fig. 2C, D). Spherical, densely packed cortical granules, ca. 1 μm across, bright yellow to yellow-brownish giving cell yellow appearance even at low magnification (Figs 1B–D, 2I, J), arranged either in lines along dorsal kineties or regularly grouped between dorsal kineties (Fig. 2E, F). Cytoplasm colourless to yellowish, filled with many lipid droplets (ca. 2–3 μm across), irregular-shaped crystals (ca. 3–4 μm across), and food vacuoles (ca. 4–10 μm across) containing small ciliates, flagellates, diatoms and bacteria (Figs 1A, 2A).

Table 1. Morphometric characterization of *Neourostylopsis flava* spec. nov. (Nf) and the Daya Bay population of *Pseudourostyla subtropica* Chen et al., 2014 (Ps).[a]

Character	Species	Min	Max	Mean	SD	*M*	CV	*N*
Body, length	Nf	132	192	157.8	15.5	158	9.9	20
	Ps	348	604	459.3	58.4	478	12.7	25
Body, width	Nf	49	85	67.1	9.5	64	14.2	20
	Ps	112	385	215.1	56.4	201	26.2	25
Adoral zone, length	Nf	40	74	56.7	8.0	58	14.2	20
	Ps	134	215	175.0	22.9	182	13.1	25
Adoral membranelles, number	Nf	40	55	44.7	3.7	45	8.4	20
	Ps	144	212	165.2	23.0	170	13.9	25
Frontal cirri, number	Nf	6	8	7.3	0.5	7	5.5	20
	Ps	38	48	41.9	2.8	42	6.8	20
Buccal cirri, number	Nf	3	4	3.8	0.4	4	11.1	20
	Ps	1	1	1.0	0	1	0.0	20
Frontoterminal cirri, number	Nf	2	2	2.0	0	2	0	20
	Ps	2	2	2.0	0	2	0.0	20
Midventral pairs, number	Nf	27	40	32.2	3.3	32	10.5	20
	Ps	20	32	23.5	2.6	22	11.1	20
Transverse cirri, number	Nf	7	9	7.7	0.5	8	7.4	20
	Ps	9	13	11.1	0.9	11	8.2	20
Pretransverse cirri, number	Nf	2	2	2.0	0	2	0	20
Left marginal cirral rows, number	Nf	4	5	4.9	0.2	5	5.9	20
	Ps	10	12	11.0	0.7	11	6.6	20
Cirri in left marginal row 1[b], number	Nf	20	37	27.2	5.0	26	18.5	15
Cirri in left marginal row 2[b], number	Nf	20	38	28.3	5.5	29	19.5	15
Cirri in left marginal row 3[b], number	Nf	18	39	29.7	5.6	30	19.1	15
Cirri in left marginal row 4[b], number	Nf	21	41	30.7	5.6	31	18.3	15
Cirri in left marginal row 5[b], number	Nf	22	37	28.8	5.1	28	17.8	15
Cirri in innermost left marginal cirral row, number	Ps	34	48	43.1	3.4	43	7.9	20
Cirri in outmost left marginal cirral row, number	Ps	5	12	7.8	2.1	8	28.1	20
Right marginal cirral rows, number	Nf	4	4	4.0	0	4	0	20
	Ps	6	9	7.6	0.6	8	9.0	20
Cirri in right marginal row 1[b], number	Nf	30	45	37.3	4.7	4	12.7	15
Cirri in right marginal row 2[b], number	Nf	28	46	38.4	5.2	37	13.6	15
Cirri in right marginal row 3[b], number	Nf	25	48	37.6	6.1	39	16.3	15
Cirri in right marginal row 4[b], number	Nf	24	43	34.5	6.1	38	17.9	15
Cirri in right marginal innermost cirral, number	Ps	16	29	23.0	3.5	25	15.4	20
Cirri in right marginal outmost cirral row, number	Ps	42	58	48.4	3.6	46	7.5	20
Dorsal kineties, number	Nf	3	3	3.0	0	3	0	20
	Ps	13	15	13.7	0.5	14	4.3	15
Macronuclear nodules, number	Nf	49	98	75.2	14.6	78	19.5	20
	Ps	105	210	146.4	14.6	160	10.1	25

[a] All measurements in μm. Abbreviations: CV, coefficient of variation (%); M, median; Max, maximum; Mean, arithmetic mean; Min, minimum; *N*, number of specimens; SD, standard deviation.

[b] Numbered from inside to outside.

Contractile vacuole (ca. 15 μm across) at about 40% of body length near left cell margin, contracting at intervals of about 3 min (Fig. 1A). Locomotion by slow to moderately fast swimming while rotating clockwise around long body axis, sometimes slowly crawling on substrate.

Infraciliature as shown in Figs 1E, F, 2G, H, K, L. Adoral zone prominent, approximately 35% of body length, comprising 40–55 membranelles, cilia ca. 15–20 μm long in vivo (Figs 1A, 2G). Paroral slightly longer than endoral (Figs 1E, 2H). Three or four buccal cirri arranged alongside paroral, cilia about 10–12 μm long in vivo. Six to eight frontal cirri (about 15–20 μm long) arranged approximately in two rows, their posterior ends continuous with midventral complex (Figs 1E, 2H). Two frontoterminal cirri inconspicuously separated from frontal cirri (Figs 1E, 2H). Midventral complex consists of 27–40 pairs, closely adjacent to frontoterminal cirri and extending to about 55% of cell length (Fig. 1E). Seven to nine transverse cirri about 20 μm long, arranged in J-shaped pseudo-row, not projecting beyond posterior body margin (Figs 1E, 2K). Two pretransverse ventral

Table 2. Morphometric comparison of *Neourostylopsis flava* spec. nov. with three congeners.

Character	*N. flava*	*N. flavicana*	*N. orientalis*	*N. songi*
Body size in vivo	150–220 × 50–75 μm	130–200 × 30–60 μm	120–200 × 40–60 μm	90–150 × 20–35 μm
Body colour	Yellow	Yellow-brownish	Grey or colourless	–
Cortical granule colour	Bright yellow to yellow brownish	Bright yellow	Yellow	Colourless
Adoral membranelles, number	40–55	33–45	25–31	28–47
Frontal cirri, number	6–8	4–8	5	5
Frontoterminal cirri, number	2	2	2	2–3
Midventral pairs, number	27–40	13–17	8–10	9–12
Left marginal rows, number	4–5	4	3	3
Right marginal rows, number	4	3	3	3
Transverse cirri, number	7–9	7–10	5–7	6–7
Dorsal kineties, number	3	3	3	3
Contractile vacuole, position	At 40% of cell length	Anterior of mid-body	At 40% of cell length	Anterior of mid-body
Habitat	Freshwater	Brackish	Brackish	Marine
Source	Present work	Wang et al. (2011)	Chen et al. (2013)	Lei et al. (2005)

cirri located anterior of right transverse cirri. Four or five left, and constantly four right marginal cirral rows with cilia 10–12 μm long in vivo (Figs 1E, 2H). Three dorsal kineties, kinety 3 shortened anteriorly (Fig. 1F). Numerous (49–98) macronuclear nodules scattered throughout body, individual nodules spherical to ellipsoidal, about 10 × 10–25 μm after protargol staining (Figs 1F, 2H). Micronuclei not observed.

Comparison with similar taxa

Hitherto, three species were assigned to the genus *Neourostylopsis*: *N. songi*, *N. flavicana* and *N. orientalis* (Fig. 4A–S; Table 2). The new species is the first record of *Neourostylopsis* from freshwater, all records of other congeners being from saline waters (Chen et al. 2013; Lei et al. 2005; Wang et al. 2011). The reason is that *Neourostylopsis*

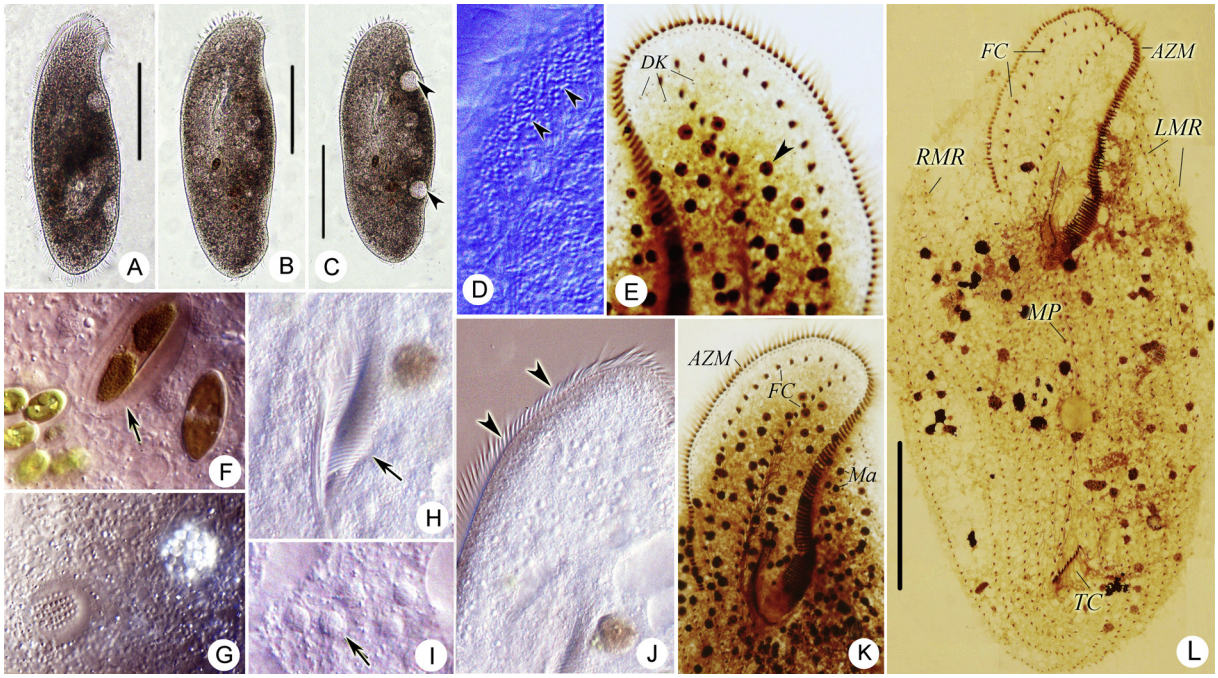

Fig. 3. (**A**–**L**) Photomicrographs of *Pseudourostyla subtropica* Chen et al., 2014 from life (A–D, F–J) and after protargol staining (E, K, L). (**A**) Ventral view of a representative individual. (**B**, **C**) Two other individuals with different body shapes, arrowheads in (C) mark contractile vacuoles. (**D**) Ventral view, arrowheads mark larger cortical granules. (**E**) Dorsal view showing dorsal kineties and macronuclear nodules (arrowhead). (**F**, **G**) Ventral views to indicate food vacuoles, arrow in (F) shows food vacuole containing flagellates. (**H**, **J**) Ventral views, arrow in (H) and arrowheads in (J) show adoral zone of membranelles. (**I**) Ventral view, arrow shows macronuclear nodules. (**K**, **L**) Ventral views, showing infraciliature and nuclear apparatus. We designate the inner marginal row as row 1 on both sides. AZM, adoral zone of membranelles; DK, dorsal kineties; FC, frontal cirri; LMR, left marginal row; Ma, macronuclear nodules; MP, midventral pairs; RMR, right marginal row; TC, transverse cirri. Bars, 80 μm (L), 100 μm (E), 150 μm (A–C).

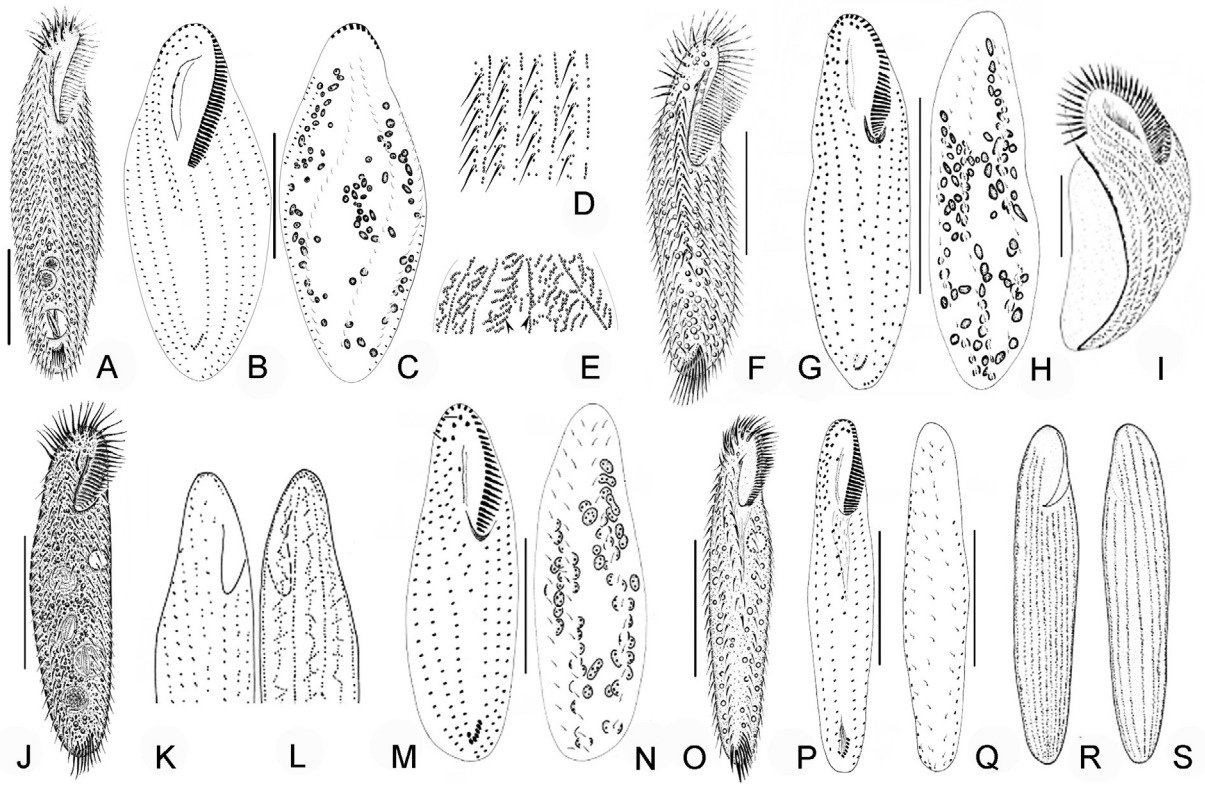

Fig. 4. (**A**–**S**) Comparative illustrations of four *Neourostylopsis* species. (**A**–**E**) *N. flava* spec. nov. (from present work). (**F**–**I**) *N. flavicana* (from Wang et al. 2011). (**J**–**N**) *N. orientalis* (from Chen et al. 2013). (**O**–**S**) *N. songi* (from Lei et al. 2005). Bars, 30 μm (A), 50 μm (B, C, F, I, J, M, N, O–Q), 80 μm (G, H).

was originally marine and *N. flava* has undergone a transition to freshwater. The evidence could come from the SSU rDNA tree: all other species in this part of the tree (*N. songi*, *N. flavicana*, *N. orientalis, Anteholosticha gracilis, Bergeriella ovata* and *Monocoronella carnea*) are marine or brackish, which indicates that marine forms are ancestral. Although *N. flava* spec. nov. and *N. flavicana* share a number of features such as a body size of about 200 × 50 μm, a yellow body colour, bright-yellow cortical granules and the number and arrangement of transverse cirri, the former differs from the latter in having greater numbers of buccal cirri (three or four vs. one), midventral pairs (27–40 vs. 13–17), right marginal rows (four vs. three) and adoral membranelles (40–55 vs. 33–45) and the freshwater (vs. brackish water) habitat (Fig. 4F–I; Wang et al. 2011). In addition, the SSU rDNA sequences of *N. flava* spec. nov. and *N. flavicana* differ at 70 bp sites, which also supports the validity of *N. flava* spec. nov. as a separate species.

Compared to *Neourostylopsis songi*, *N. flava* spec. nov. has a larger body size (150–220 × 50–75 μm vs. 90–150 × 20–35 μm in vivo), higher numbers of frontal cirri (six to eight vs. five), midventral pairs (27–40 vs. 9–12) and marginal rows (four or five left, four right vs. three left, three right), bright yellow to yellow-brownish (vs. colourless) cortical granules and pretransverse ventral cirri present (vs. absent). Furthermore, the habitat limnetic (vs. marine) (Fig. 4O–S; Lei et al. 2005).

Neourostylopsis flava spec. nov. can be easily separated from *N. orientalis* by its body colour (yellow vs. grey or colourless), higher numbers of adoral membranelles (40–55 vs. 25–31), frontal cirri (six to eight vs. five), midventral pairs (27–40 vs. eight to ten) and marginal rows (four or five left, four right vs. three left, three right), pretransverse ventral cirri (present vs. absent), and habitat (freshwater vs. brackish water) (Fig. 4J–N; Chen et al. 2013).

Genus *Pseudourostyla* Borror, 1972

Pseudourostyla subtropica Chen et al., 2014 (Fig. 3A–L; Table 1)

A redescription is supplied based on the present population from Daya Bay, southern China.

Deposition of specimens. Three protargol slides containing voucher specimens are deposited in the Laboratory of Protozoology, Ocean University of China with registration numbers FYB-2012042601-01, 02, 03.

Description of Daya Bay population. Body 250–550 × 100–180 μm in vivo, usually elliptical in outline with both ends rounded and slightly narrowed, ratio of length to width about 3:1–4:1, dorsoventrally flattened about 2.5:1, flexible but not contractile (Fig. 3A–C). Two types of

cortical granules, both of which colourless, spherical, and distributed throughout cortex: type 1 large (ca. 1 μm across) and densely packed, type 2 small (ca. 0.5 μm across) and relatively sparsely distributed (Fig. 3D, I). Cytoplasm colourless, filled with numerous granular inclusions and lipid droplets (ca. 5–7 μm long) giving cell a dark appearance (Fig. 3G). Food vacuoles contain mainly bacteria, flagellates (ca. 20–50 μm long) and small ciliates (ca. 20 μm long) (Fig. 3F). Two contractile vacuoles (ca. 60 μm in diameter when fully expanded) near left body margin, one about 1/3 down body length, the other about 2/3 down body length, pulsating at intervals of 5–10 s (Fig. 3C). Locomotion by crawling slowly on substrate, although occasionally stationary, or by swimming whilst rotating about long body axis.

Infraciliature as shown in Fig. 3E, K. Adoral zone occupies about 35% of cell length (Fig. 3E, K), composed of 144–212 membranelles; undulating membranes optically intersect in their posterior quarter, endoral slightly longer than paroral; one buccal cirrus right of undulating membranes. Frontal cirri strong, numbering 38–48, arranged in two arcs forming a bicorona (Fig. 3K, L). Midventral complex composed of 20–32 pairs, extending to transverse cirri. Nine to 13 transverse cirri arranged in J-shaped pseudo-row, with cilia approximately 35 μm long in vivo. Ten to 12 left and six to nine right marginal cirral rows, with cilia 15–20 μm long

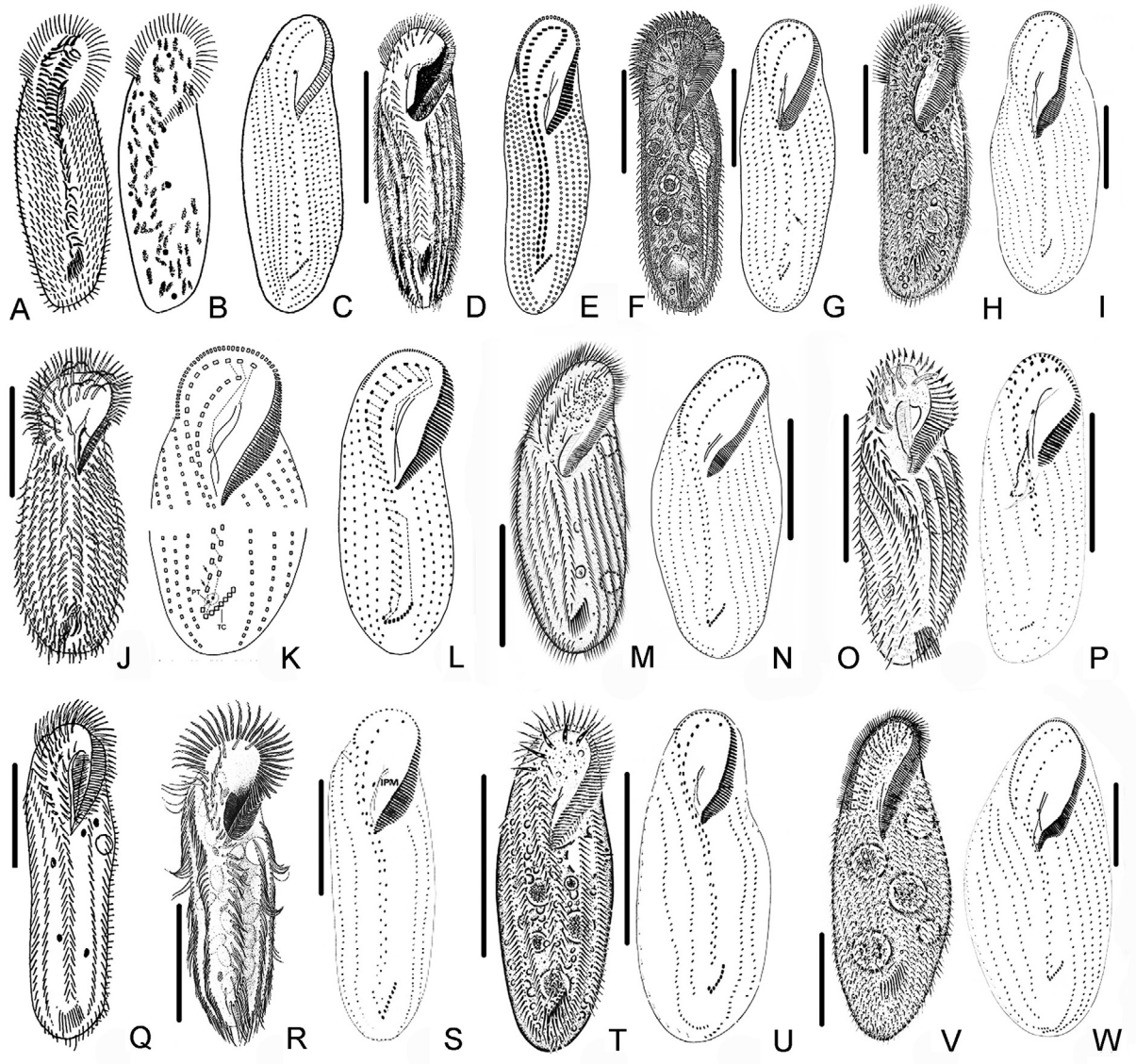

Fig. 5. (**A**–**W**) Comparative illustrations of some *Pseudourostyla* species. (**A**–**I**) *P. cristata* (A, B, from Jerka-Dziadosz 1964; C, from Borror 1972; D, from Borror and Wicklow 1983; E, from Jin and Ng 1989; F, G, from Oberschmidleitner and Aescht 1996; H, I, from Chen et al. 2010). (**J**–**L**) *P. levis* (J, from Takahashi 1973; K, from Takahashi 1988; L, from Takahashi and Suhama 1991). (**M**, **N**) *P. cristatoides* (from Jung et al. 2012). (**O**, **P**) *P. pelotensis* (from Paiva and Silva-Neto 2006). (**Q**) *P. muscorum* (from Kahl 1932). (**R**–**U**) *P. nova* (R, S, from Wiackowski 1988; T, U from Chen et al. 2014). (**V**, **W**) *P. subtropica* (from Chen et al. 2014). Bars, 100 μm.

in vivo. Thirteen to 15 dorsal kineties (Fig. 3E). Numerous (105–210) macronuclear nodules and two to six micronuclei.

Comparison and remarks. Seven morphospecies have been assigned to the genus *Pseudourostyla*: *P. cristata* (Jerka-Dziadosz, 1964) Borror, 1972 (Fig. 5A–I); *P. levis* Takahashi, 1973 (Fig. 5J–L); *P. muscorum* (Kahl, 1932) Borror, 1972 (Fig. 5Q); *P. nova* Wiackowski, 1988 (Fig. 5R–U); *P. peloten-sis* Paiva and Silva-Neto, 2006 (Fig. 5O, P); *P. cristatoides* Jung et al., 2012 (Fig. 5M, N); and *P. subtropica* Chen et al., 2014 (Fig. 5V, W) (Berger 2006; Chen et al. 2014; Jung et al. 2012; Kahl 1932; Oberschmidleitner and Aescht 1996; Paiva and Silva-Neto 2006; Wiackowski 1988). It is widely accepted that the most important criteria for species identification and separation in *Pseudourostyla* are: (1) the body size and shape; (2) the colour and arrangement of cortical granules; (3) the numbers and arrangement of the adoral membranelles, buccal cirri, frontal cirri, frontoterminal cirri, transverse cirri, left marginal cirral rows, right marginal cirral rows, and dorsal kineties, and; (4) the appearance and number of macronuclear nodules (Berger 2006; Kahl 1932; Oberschmidleitner and Aescht 1996; Paiva and Silva-Neto 2006; Wiackowski 1988).

The present population of *P. subtropica* corresponds well with the type population of Chen et al. (2014) in terms of body shape, colour and arrangement of cortical granules, general features of the infraciliature, the position of the contractile vacuoles, and the nuclear apparatus. The only significant difference is the habitat, the Daya Bay population was isolated from freshwater whereas the original population was discovered in brackish water with salinity 4.5‰ (Chen et al. 2014).

Molecular phylogeny based on SSU rDNA sequences. The length (bp), GC content and GenBank accession numbers of the SSU rDNA sequences of

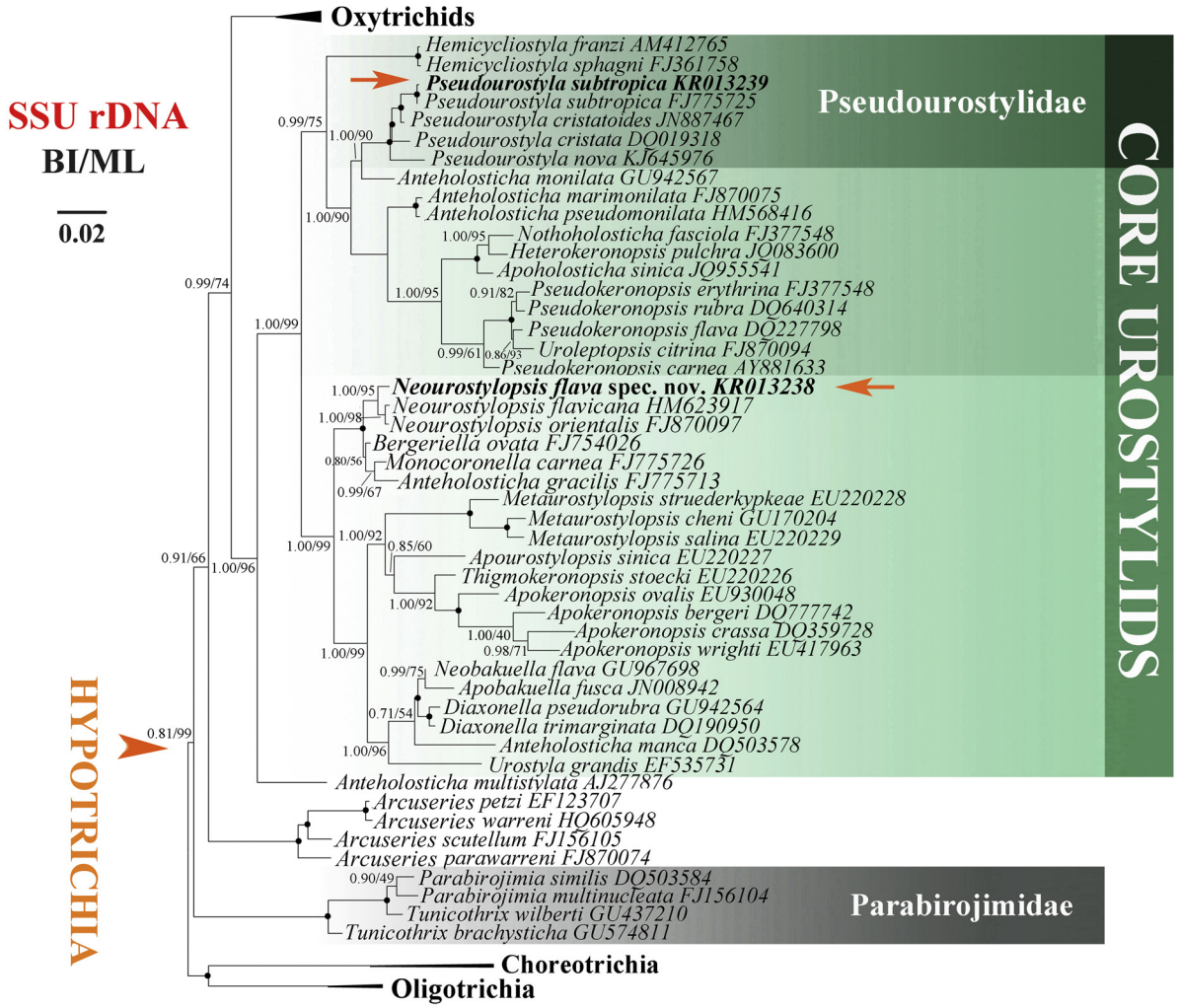

Fig. 6. Maximum likelihood tree inferred from SSU rDNA sequences, showing the positions of *Neourostylopsis flava* spec. nov. and the Daya Bay population of *Pseudourostyla subtropica* (in bold and arrowed). Numbers at nodes represent the posterior probability of Bayesian inference and the bootstrap values of maximum likelihood out of 1000 replicates. The scale bar corresponds to two substitutions per 100 nucleotide positions. Nodes which were fully supported are represented by filled circles.

Neourostylopsis flava spec. nov. and the Daya Bay population of *Pseudourostyla subtropica* are: 1724 bp, 44.5%, KR013238 and 1727 bp, 45.4%, KR013239, respectively.

The topologies of the SSU rDNA trees constructed using Bayesian inference and maximum likelihood analyses were almost identical, therefore only the maximum likelihood tree is shown in Fig. 6. Both *Pseudourostyla subtropica* and *Neourostylopsis flava* spec. nov. are placed in the "core" of the urostyloids clade. Four *Pseudourostyla* species, namely *P. subtropica*, *P. cristata*, *P. cristatoides* and *P. nova*, form a monophyletic assemblage with full support, which then clusters with *Anteholosticha monilata* (1.00 BI, 90% ML). For *P. subtropica*, there is only 1 bp difference in the SSU rDNA sequences between the Daya Bay population and the original population (Chen et al. 2014).

The three *Neourostylopsis* species for which SSU rDNA gene sequence data are available, i.e., *N. flava* spec. nov., *N. orientalis* and *N. flavicana*, form a highly supported clade (1.00 BI, 95% ML) that is sister to the clade comprising *Monocoronella carnea*, *Bergeriella ovata* and *Anteholosticha gracilis*. This is consistent with the morphological similarities of all taxa within these two clades, i.e., the number and arrangement of the adoral membranelles, frontal cirri and dorsal kineties and the presence of buccal and frontal cirri (Hu and Suzuki 2004; Liu et al. 2010).

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