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European Journal of Protistology 63 (2018) 105–116

European Journal of PROTISTOLOGY

www.elsevier.com/locate/ejop

Morphology and systematics of two freshwater *Frontonia* species (Ciliophora, Peniculida) from northeastern China, with comparisons among the freshwater *Frontonia* spp.

Xinglong Cai[a,1], Chundi Wang[b,1], Xuming Pan[a,1], Hamed A. El-Serehy[c], Weijie Mu[a], Feng Gao[b,*], Zijian Qiu[a,*]

[a] *College of Life Science and Technology, Harbin Normal University, Harbin 150025, China*
[b] *Institute of Evolution & Marine Biodiversity, Ocean University of China, Qingdao 266003, China*
[c] *Zoology Department, College of Science, King Saud University, Riyadh 11451, Saudi Arabia*

Received 1 July 2017; received in revised form 6 January 2018; accepted 8 January 2018
Available online 12 January 2018

Abstract

The morphology and infraciliature of two *Frontonia* species, *F. shii* spec. nov. and *F. paramagna* Chen et al., 2014, isolated from a freshwater pond in northeastern China, were investigated using living observation and silver staining methods *Frontonia shii* spec. nov. is recognized by the combination of the following characters: freshwater *Frontonia*, size in vivo about 220–350 × 130–250 μm, elliptical in outline; 128–142 somatic kineties; three or four vestibular kineties, six or seven postoral kineties; peniculi 1–3 each with four kineties; single contractile vacuole with about 10 collecting canals. The improved diagnosis for *F. paramagna* is based on the current and previous reports. Comparisons among freshwater *Frontonia* are also provided. The small subunit ribosomal rRNA gene (SSU rDNA) sequences of the two species are characterized and phylogenetic analyses based on these sequences show that both species fall into the core clade of the genus *Frontonia*, and this genus is not monophyletic.

Keywords: Freshwater; *Frontonia*; *Frontonia shii* spec. nov; Phylogeny; SSU rDNA

Introduction

The ciliate genus *Frontonia* Ehrenberg, 1833 exhibits a much higher than expected level of morphospecies diversity, and it is undoubtedly the case that many new taxa are still awaiting discovery (Chen et al. 2014; Fan et al. 2011, 2013; Foissner 1987; Kahl 1931; Long et al. 2005, 2008; Pan et al. 2013a, 2013b; Song et al. 2009). Species assigned to this genus are commonly found in limnetic, brackish, and marine biotopes (Borror 1963, 1972; Burkovsky 1970; Kahl 1931; Long et al. 2005; Petz et al. 1995; Song and Wilbert 1989). In recent years, many species are commonly encountered in coastal areas (Fan et al. 2011, 2013; Liu et al. 2016; Liu M.J. et al. 2017; Liu W.W. et al. 2017; Long et al. 2005, 2008; Pan et al. 2013a,b; Xu et al. 2018). Compared with the brackish or marine *Frontonia* species, however, most freshwater forms are only insufficiently investigated: many of them are poorly defined, and their species characteristics need to be reviewed so that

*Corresponding authors.
E-mail addresses: gaof@ouc.edu.cn (F. Gao), qiuzijian48@163.com (Z. Qiu).
[1] Contributed equally.

https://doi.org/10.1016/j.ejop.2018.01.002
0932-4739/

species may be identified correctly (Bullington 1939; Kahl 1931).

Frontonia species can be distinguished by their body shapes, the structures of the oral apparatus, and general somatic ciliary patterns with the distinct preoral and postoral suture (Dragesco and Dragesco-Kernéis 1986; Fan et al. 2011, 2013; Foissner et al. 1994, 2002; Long et al. 2005, 2008; Pan et al. 2013a,b; Roque and Puytorac 1972). To date, more than ten freshwater species have been reported, for example: *Frontonia acuminata* (Ehrenberg, 1833) Bütschli, 1889; *F. vernalis* (Ehrenberg, 1833) Kahl, 1931; *F. atra* (Ehrenberg, 1833) Bütschli, 1889; *F. depressa* (Stokes, 1886) Kahl, 1931; *F. elliptica* Beardsley, 1902; *F. leucas* (Ehrenberg, 1833) Ehrenberg, 1838; *F. minuta* Dragesco, 1970; *F. pallida* Czapik, 1979; *F. paramagna* Chen et al., 2014 and *F. vesiculosa* Cunha, 1913 (e.g., Alekperov 2005; Bullington 1939; Dragesco 1960; Dragesco 1970; Dragesco and Dragesco-Kernéis 1986; Kahl 1931; Roque and Puytorac 1972). Detailed studies and comparisons among all the freshwater *Frontonia* species based on morphological data are needed.

As part of an on-going faunistic study of freshwater ciliates in northeastern China, the morphology and phylogeny of two *Frontonia* species, *F. shii* spec. nov. and *F. paramagna*, are published. In addition, morphological comparisons among the best-known freshwater *Frontonia* species are presented.

Material and Methods

Sample collection and identification

Frontonia shii spec. nov. and *F. paramagna* were collected on 2 June 2016 from a farmland pond 3.87 km northeast of the village Yutian at Hulan district in Harbin (45°93′87″N; 126°61′15″E), Heilongjiang province, northeastern China (water temperature 24 °C and pH 7.3). About 0.4 L water was collected from 0.1–0.5 m below the water surface using a sampling bottle. Ciliates were maintained in habitat water in Petri dishes as raw cultures at room temperature, and a clone was made for each species a week later (ca. 25 °C).

Isolated cells were observed and photographed in vivo using differential interference contrast microscopy. The silver carbonate (Ma et al. 2003) and Chatton-Lwoff (Wilbert and Song 2008) methods were used to reveal the infraciliature and argyrome. This method is also applicable for freshwater species, the key point is to fix cells with low-concentration fixative (10% formalin); additionally, the contractile vacuole pore can be stained sometimes. Counts and measurements of stained specimens were performed at magnifications of 100–1250×. Drawings were made with the help of a drawing device. Systematics and terminology are mainly according to Lynn (2008) and Gao et al. (2016).

DNA extraction, PCR amplification and sequencing

DNA was extracted using the DNeasy Blood and Tissue Kit (QIAGEN, Germany) according to the protocol of manufactures. DNA amplifications of the small subunit ribosomal RNA gene (SSU rDNA) were performed using Q5® Hot Start High-Fidelity DNA Polymerase (New England BioLabs, USA) with primers of 82F (5′-GAA ACT GCG AAT GGC TC-3′) and 18sR (5′-GAT CCT TCT GCA GGT TCA CCT AC-3′) (Elwood et al. 1985; Medlin et al. 1988). The touchdown PCR program was designed with the annealing temperature of 69–51 °C touch down (Wang P. et al. 2017). The PCR products were sequenced bidirectionally in TSINGKE Incorporated Company (Qingdao, China). The contigs were assembled by SeqMan (DNAStar).

Phylogenetic analyses

The newly characterized SSU rDNA sequences of *Frontonia shii* and *F. paramagna*, combined with 70 sequences downloaded from NCBI GenBank database (accession numbers as shown in Fig. 5) were used to construct the phylogenetic tree (Gao et al. 2017). Among them, three species of Prostomatea were selected as outgroups. All sequences were aligned using the GUIDANCE2 Server (http://guidance.tau.ac.il/ver2/) with default settings (Sela et al. 2015). The aligned sequences were further manually modified using the program BioEdit v7.0.1 (Hall 1999), generating a matrix of 72 taxa with 1766 characters. The maximum-likelihood (ML) analysis was performed in CIPRES Science Gateway using RAxML-HPC2 on XSEDE v8.1.24 (Stamatakis et al. 2008) with the model of GTR + I + G selected by Modeltest v.3.4 (Posada and Crandall 1998). The reliability of internal branches was assessed using a nonparametric bootstrap method with 1000 replicates (Huang et al. 2016). Bayesian inference (BI) tree was constructed using MrBayes on XSEDE v3.2.6 in CIPRES Science Gateway with the model of GTR + I + G selected by MrModeltest v2.0 (Nylander 2004). Markov chain Monte Carlo (MCMC) simulations were run with two sets of four chains for 6,000,000 generations with a frequency of 100 generations, and the first 6000 trees were discarded as burn-in (Chen et al. 2016). MEGA v6.06 (Tamura et al. 2013) was used to visualize tree topologies.

Results and Discussion

Class Oligohymenophorea de Puytorac et al., 1974

Order Peniculida Fauré-Fremiet in Corliss, 1956

Family Frontoniidae Kahl, 1926

Genus *Frontonia* Ehrenberg, 1833

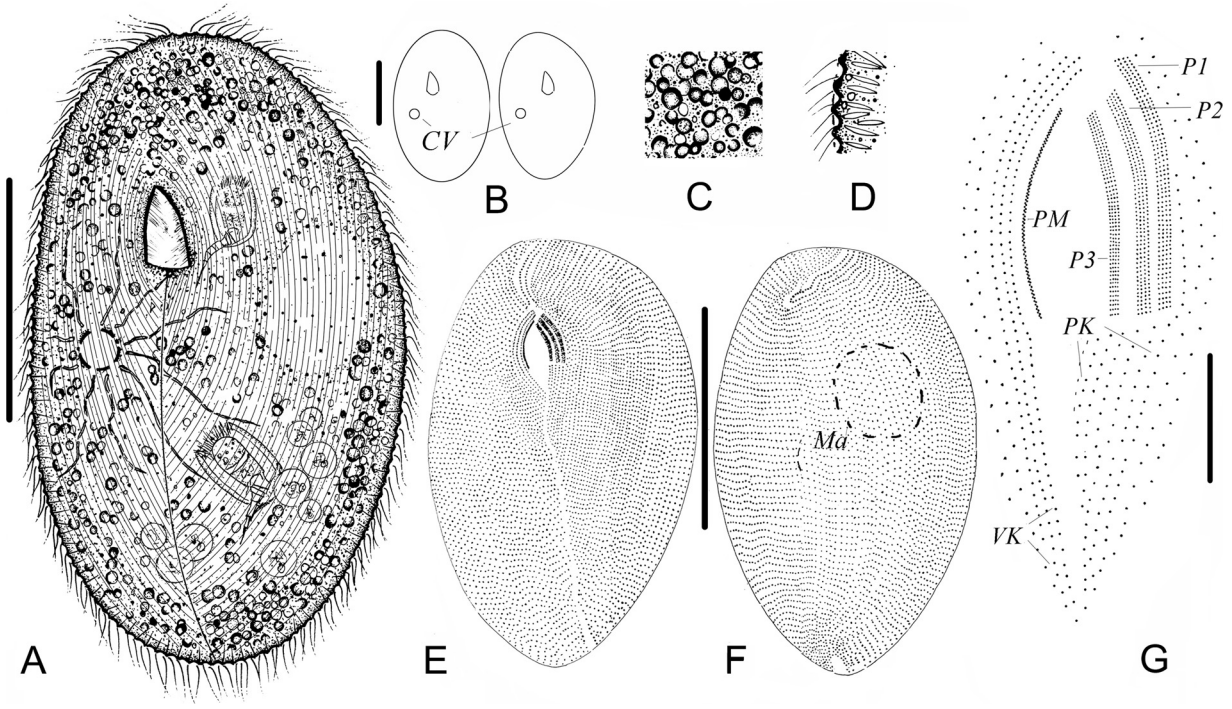

Fig. 1. A–G. Morphology and infraciliature of *Frontonia shii* spec. nov. from life (A–D) and after silver carbonate staining (E–G). (**A**) Ventral view of a representative individual. (**B**) Ventral view, to show contractile vacuole and different body shapes. (**C**) Part of cytoplasm, to show green algae. (**D**) Part of pellicle, to show extrusomes (about 8 μm long) forming a distinct seam. (**E, F**) Infraciliature of same specimen in ventral (E) and dorsal view (F). (**G**) Oral ciliature. CV, contractile vacuole; Ma, macronucleus; P1–3, peniculi 1, 2, 3; PK, postoral kineties; PM, paroral membrane; VK, vestibular kineties. Bars, 100 μm (A, B), 150 μm (E, F), 20 μm (G).

Table 1. Morphometric data of *Frontonia shii* spec. nov. (upper row), and *F. paramagna* (lower row).

Character	Min	Max	Mean	M	SD	CV	n
Body length	243	378	318.4	307	11.2	3.8	40
	340	473	395.5	382	24.8	6.3	40
Body width	146	271	203.3	211	16.2	7.5	40
	148	184	167.6	171	12.3	7.4	40
Number of somatic kineties	128	142	130.4	130	9.8	7.5	30
	150	157	152.7	154	13.6	8.9	30
Number of vestibular kineties	3	4	3.6	4	0.4	11.1	30
	3	3	3	3	0	0	30
Number of postoral kineties	7	8	7.1	7	0.8	11.3	30
	5	5	5	5	0	0	30
Number of ciliary rows in peniculus 1	4	4	4	4	0	0	11
	4	4	4	4	0	0	11
Number of ciliary rows in peniculus 2	4	4	4	4	0	0	11
	4	4	4	4	0	0	11
Number of ciliary rows in peniculus 3	4	4	4	4	0	0	11
	4	4	4	4	0	0	11

Data from silver nitrate-prepared specimens (body length, body width) and silver carbonate-prepared (remaining features) specimens. All measurements in micrometers. Abbreviations: CV = coefficient of variation (%); M = Median; Max = maximum; Mean = arithmetic mean; Min = minimum; n = number of specimens; SD = standard deviation.

Frontonia shii spec. nov. (**Figs. 1A–G**, **2A–M**; **Table 1**)

Diagnosis. Freshwater *Frontonia*, size in vivo about 220–350 × 130–250 μm (avg. 300 × 200 μm), elliptical in outline with both anterior and posterior ends slightly narrowed; buccal field approximately 13% of body length; extrusomes spindle-shaped, about 8 μm long; 128–142 somatic kineties; three or four vestibular kineties, six or seven postoral kineties; peniculi 1–3 each with four

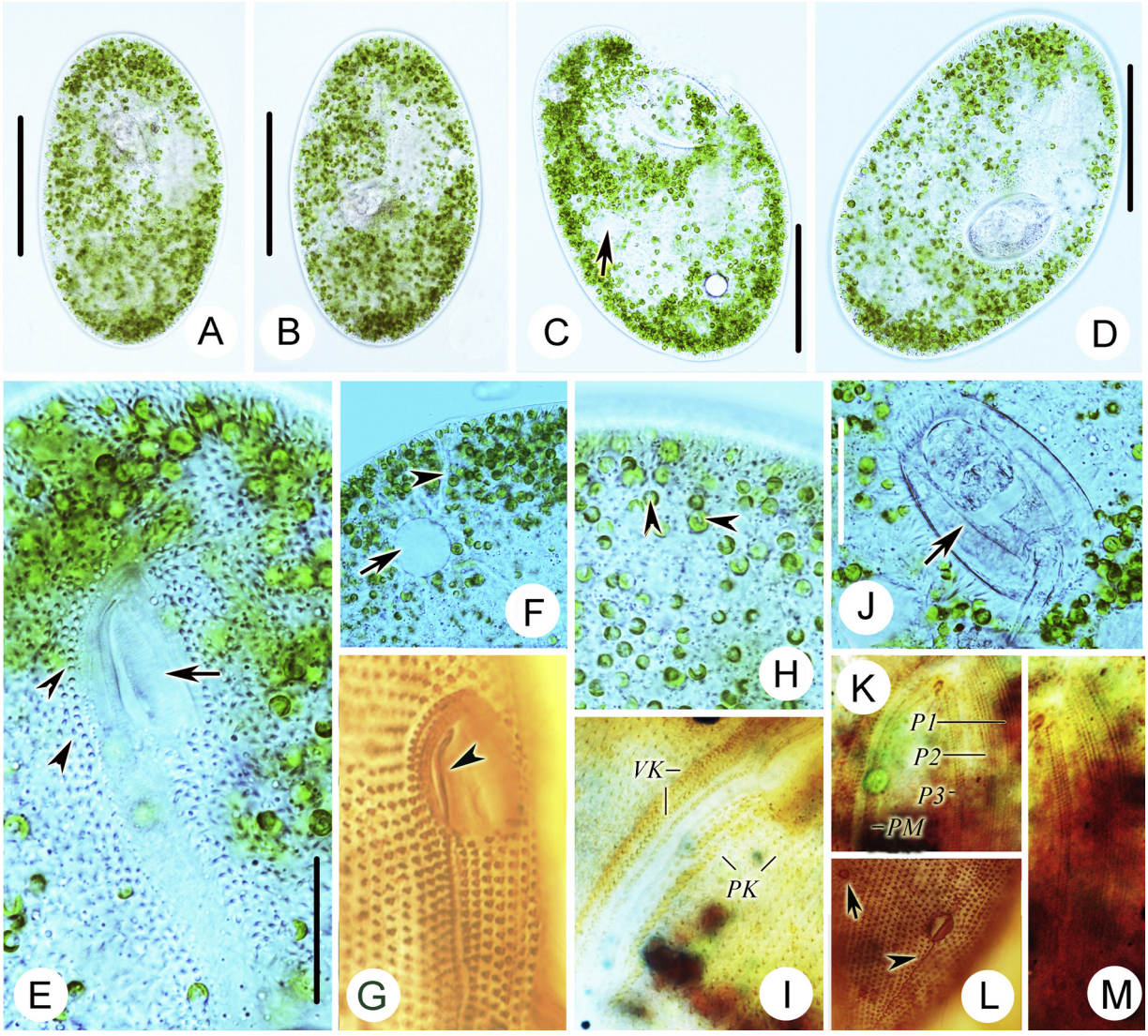

Fig. 2. A–M. *Frontonia shii* spec. nov. from life (A–D, bright field; F, H, J, DIC) and after silver nitrate- (G, L) and carbonate-staining (I, K, M). (**A**) Ventral view of a representative individual. (**B–D**) Different body shapes, arrow in (C) shows contractile vacuole. (**E**) Ventral view, to show buccal field (arrow) and vestibular kineties (arrowheads). (**F**) Ventral view, to show contractile vacuole (arrow), arrowhead shows collecting canal. (**G**) Oral ciliature of holotype specimen, to show argentophilic line (arrowhead). (**H, J**) Ventral views, showing green algae (arrowheads) and a rotifer (arrow). (**I**) Ventral view, to show postoral kineties and vestibular kineties. (**K, M**) Detailed ciliature of buccal area. (**L**) To show contractile vacuole pore (arrow) and postoral suture (arrowhead). P1–3, peniculi 1, 2, 3; PK, postoral kineties; PM, paroral membrane; VK, vestibular kineties. Bars, 150 μm (A, B), 100 μm (C, D), 40 μm (E), 20 μm (J).

kineties; one elongate-elliptical macronucleus centrally located; single contractile vacuole located right-ventrally in mid-region of cell, with about ten collecting canals.

Type location. A farmland pond 3.87 km northeast of the village Yutian at Hulan district in Harbin (45°93′87″N; 126°61′15″E), Heilongjiang province, northeastern China.

Type specimens. The slide containing the holotype specimen (Fig. 2G; silver nitrate-staining) is deposited in the Natural History Museum, London, UK with registration No. NHMUK 2018.2.26.1. A paratype slide is deposited in the Laboratory of Protozoology, Harbin Normal University with registration number CXL-2016092101.

Etymology. This species is named in honour of Professor Xinbai Shi, Harbin Normal University, Harbin, in recognition of his outstanding contributions to Protozoology.

Description. Body size in vivo usually about 220–350 × 130–250 μm (avg. 300 × 200 μm, n = 15). Body shape constant, wide-elliptical in outline with both anterior and posterior end broadly rounded (Figs. 1A, B, 2A–D). Ratio of length to width about 1.5:1 (Figs. 1A, 2A). Buccal cavity small and shallow, elliptic to triangular in outline, about 10–15% of body, located in anterior third of cell (Figs. 1A, 2E). Spherical, densely arranged single-celled green algae (likely *Chlorella* sp.), ca. 5 μm across and

without flagella, rendering cell green in colour (Figs. 1A, 2A–D, H). Extrusomes spindle-shaped, about 8 μm long (20–25 μm long after being ejected), densely arranged in cortex (Fig. 1A, D). Cytoplasm colourless, filled with irregular-shaped crystals (ca. 3 μm across), and randomly distributed food vacuoles filled with bacteria, rotifers and algae (Figs. 1A, 2A–D, J). Macronucleus ellipsoidal, about 70 × 50 μm, located in mid-region of body (Fig. 1A, F). Micronucleus not observed. Contractile vacuole located right-ventrally in mid-region of body, about 10–15 μm in diameter when fully expanded, contracting at about 1 min intervals (Figs. 1A, 2F); about 10 collecting canals, single contractile vacuole pore located right-ventrally (Figs. 1A, 2F, L). Body densely ciliated (Fig. 1A, D), somatic cilia about 10 μm long, caudal cilia approximately 15 μm long (Fig. 1A, D). Locomotion mostly by gliding back and forth on substrate or by swimming, moderately rapid while rotating anticlockwise along long body axis.

Somatic ciliature as shown in Figs. 1E–G, 2J, K. About 128–142 longitudinal somatic kineties, commencing at anterior end of cell, forming a conspicuous anterior suture that extends from anterior end of buccal cavity to dorsal side (Fig. 1E, F); posterior part of somatic kineties gradually shortened along posterior region of oral apparatus forming the postoral suture (Fig. 1E, F). Three or four vestibular kineties with close-set monokinetids (Figs. 1G, 2I). Seven or eight postoral kineties left of postoral suture, terminating anteriorly at buccal cavity and progressively shortened along postoral suture (Fig. 1E–G).

Buccal apparatus as shown in Figs. 1G, 2G, I, K. Three peniculi located on left wall of cavity: peniculi 1 and 2 about equally long, located close to each other, and each composed of four rows of kinetosomes. Paroral membrane double-rowed, located on right edge of buccal cavity (Figs. 1G, 2I, K). Several argentophilic lines left of paroral membrane (Fig. 2G).

Gene sequence data. The SSU rDNA sequence of *Frontonia shii* spec. nov. has been deposited in GenBank database with the accession number, length and G + C content as follows: MF279208, 1617 bp, 45.39%.

Remarks and comparison. It is widely accepted that the most important criteria for species identification and separation in *Frontonia* are the structure of peniculi 1–3, body size and shape, the position of the contractile vacuole (s) and the presence or absence of canals that drain into the vacuole and the habitat (Table 2; Foissner et al. 1994; Long et al. 2005, 2008; Petz et al. 1995; Roque 1961). The body size as well as the numbers of postoral and vestibular kineties can be various even among populations of the same species; the body colour of *Frontonia*, however, varies basically owing to the colour of their food (Dragesco and Dragesco-Kernéis 1986; Fan et al. 2011, 2013; Foissner et al. 1994, 2002). The spherical and densely arranged green algae, which render *F. shii* green in colour, are probably symbiotic: however, this assumption awaits further studies. Considering the body shape and size, the freshwater habitat, and the presence of four ciliary rows in each of peniculi 1–3, four species should be compared with the new species, namely *Frontonia paramagna*, *F. leucas*, *F. pallida* and *F. vernalis*.

Compared with *Frontonia paramagna*, *F. shii* spec. nov. has a different body shape (mainly elliptical body, right margin depressed in anterior third and posterior end narrowed vs. wide-elliptical in outline with both anterior and posterior ends broadly rounded in *F. shii*), and more somatic kineties (180–200 vs. 128–142 in *F. shii*) (Chen et al. 2014).

Frontonia leucas is more slender in shape (120–360 × 110–120 μm vs. 220–350 × 130–250 μm in *F. shii*), and has fewer somatic kineties (110–120 vs. 128–142) and postoral kineties (four vs. six or seven in *F. shii*) (Alekperov 2005; Carey 1992; Foissner 1987; Foissner et al. 1994; Gil and Pérez-Silva 1964a; Roque and Puytorac 1972; Tönniges 1914).

Frontonia pallida can be easily separated from *F. shii* by having a shorter body (150–160 μm vs. 220–350 μm in *F. shii*), fewer somatic kineties (60–66 vs. 128–142) and different numbers of kinety rows in peniculi 1–3 (four, three, two vs. four, four, four in *F. shii*) (Carey 1992; Czapik 1979).

Although both *Frontonia vernalis* and *F. shii* have symbiotic algae, the former is separated from the latter by having a different body shape (foot-shaped, that is somewhat broader at the anterior end vs. wide-elliptical in outline with both anterior and posterior end broadly rounded in *F. shii*), a smaller body size (197 × 108 μm on average vs. 300 × 200 μm on average in *F. shii*), more somatic kineties (175–190 vs. 128–142 in *F. shii*), and a different number of contractile vacuoles (two vs. one in *F. shii*) (Bullington 1939; Dujardin 1841; Ehrenberg 1838).

Frontonia paramagna Chen et al., 2014 (Fig. 3A–T; Table 1)

Some new characters were found in the Harbin population. Based on data from both the present study and the original description (Chen et al. 2014), an improved diagnosis is presented here.

Improved diagnosis. Freshwater *Frontonia* about 320–610 × 110–160 μm (avg. 400 × 150 μm) in vivo; dorsoventrally slightly flattened with conspicuously small buccal cavity; about 150–201 somatic kineties; three vestibular and 5–7 postoral kineties; peniculi 1–3 each with four kinetid rows; single macronucleus ellipsoidal; contractile vacuole located equatorially on right cell margin; about 15 collecting canals; extrusomes spindle-shaped.

Sampling site. A farmland pond 3.87 km northeast of the village Yutian at Hulan district in Harbin (45°93′87″N; 126°61′15″E), Heilongjiang province, northeastern China The site is close to the type locality (Chen et al. 2014).

Voucher slides. Two voucher slides (registration numbers CXL-20160921-01, 02) have been deposited in the Laboratory of Protozoology, Harbin Normal University, Harbin, China.

Table 2. Comparison of freshwater and soil *Frontonia* species.

Character	*F. vesiculosa*	*F. paramagna*	*F. leucas*	*F. pallida*	*F. acuminata*	*F. angusta*	*F. atra*	*F. depressa*	*F. elliptica*	*F. minuta*	*F. shii*	*F. terricola*
Body length in vivo	300–400	320–610	120–360	150–160	60–170	80–130,	100–250	60–93	80–220	70	300	70–110
Body shape	Fig. 4B	Fig. 4C	Fig. 4A	Fig. 4D	Fig. 4G	Fig. 4E	Fig. 4G	Fig. 4F	Fig. 4C	Fig. 4E	Fig. 4F	Fig. 4G
Number of SK	140–250	149–201	120–360	150–160	55–60	83–101	100–250	57–62	90–110	46–56	128–142	70–77
Ciliary rows in Peniculi 1–3	6, 6, 4	4, 4, 4	4–5, 4–5, 4–5	4, 3, 2	5–6, 5–6, 3–4	5, 5, 4	5, 4, 4	5, 5, 3	5–6, 5–6, 5–6	5, 5, 2	4, 4, 4	4, 4, 2
Number of VK	3	3	3	4	3	3 or 4	4	3	5	3	3 or 4	4
Number of PK	8	5–7	4 or 5	5	5	3–5	5 or 6	3	4	5	6 or 7	5–8
Number of CV	3–8	1	1	1	1	1	1	1	2	1	1	1
Position of CV	H	A	A	C	D	E	E	F	A	G	A	M
Number of CVP	5–10	1	1	2–5	1 or 2	2–6	4–6	1	2	2	1	2–5
Position of CVP	I	B	I	C	J	L	J	I	K	K	A	M
Collecting canals	Absent	Present	Present	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Present	Absent
Pigment	Absent	Absent	Absent	Absent	Present	Absent	Present	Absent	Absent	Absent	Absent	Absent
Symbiotic algae	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Present	Present	Absent
Data sources	3, 8	1, 2	3, 6, 7, 9–14	4,11	3, 6, 9, 12, 18	6, 9, 17	3, 6, 7, 9	3, 5, 6, 8,16	3, 6, 7	7, 15	1	5, 17

Abbreviations: CV = contractile vacuole; CVP = contractile vacuole pore; PK = postoral kineties; SK = somatic kineties; VK = vestibular kineties.

Position of CV and CVP: A, central on right margin of cell; B, central on left margin of cell; C, cell center to posterior half; D, behind (central) macronucleus; E, dorsolateral, posterior to buccal cavity; F, right-ventral in anterior third of cell; G, left in posterior third of cell; H, right portion of dorsal side; I, central portion of dorsal side; J, subequatorial portion of dorsal side; K, dorsal, anterior and posterior portion; L, mid-body; M, slightly in front of body center.

Data sources: 1, from the present work; 2, Chen et al. (2014); 3, Bullington (1939); 4, Czapik (1979); 5, Foissner (1987); 6, Kahl (1931); 7, Dragesco and Dragesco-Kernéis (1986); 8, Roque and Puytorac (1972); 9, Foissner et al. (1994); 10, Alekperov (2005); 11, Carey (1992); 12, Roque (1961); 13, Tönniges (1914); 14, Gil and Pérez-Silva (1964a); 15, Dragesco (1970); 16, Gil and Pérez-Silva (1964b); 17, Foissner et al. (2002); 18, Gil and Pérez-Silva (1964c).

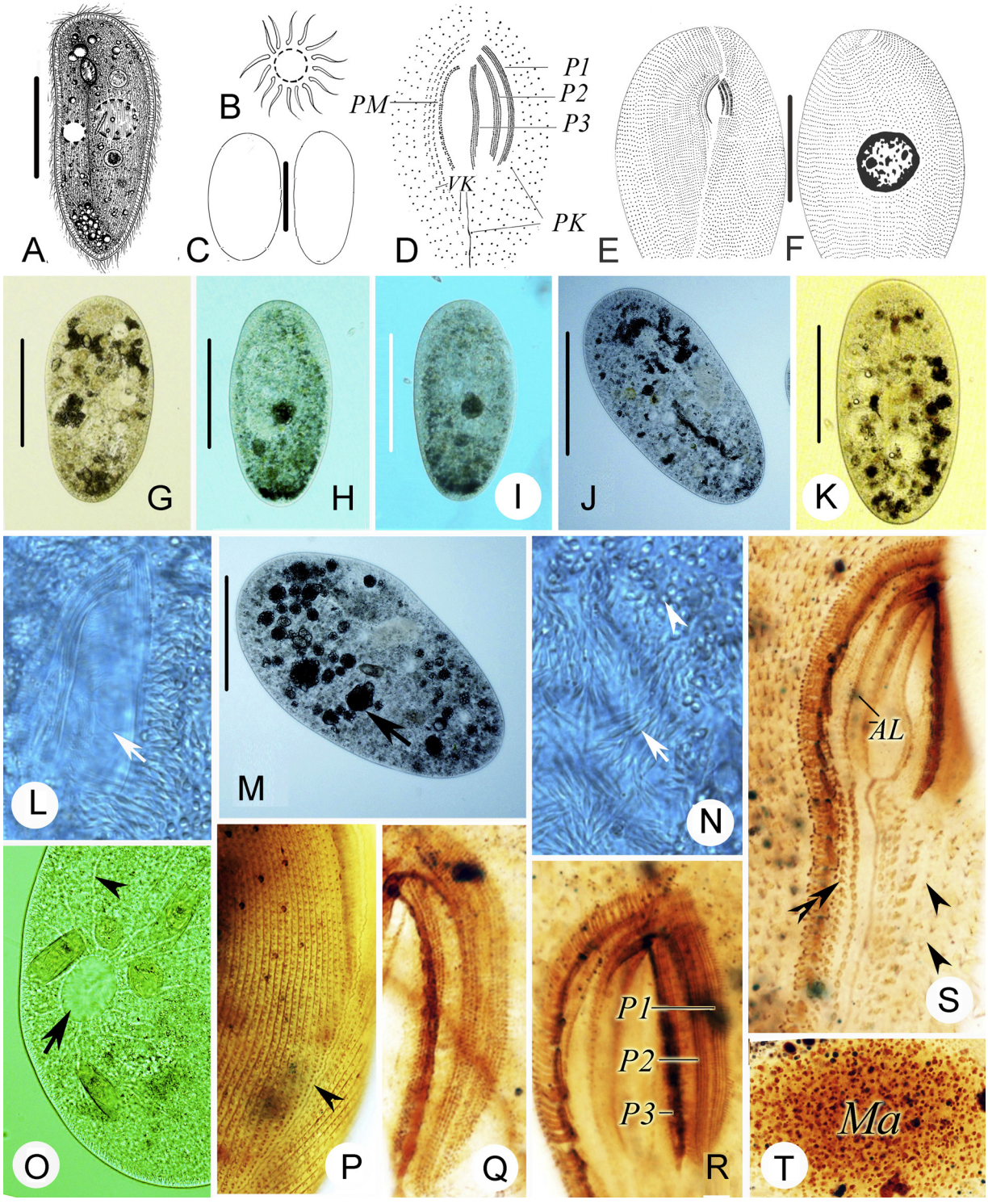

Fig. 3. A–T. *Frontonia paramagna* from life (A–C, G–N) and after silver carbonate staining (D–F, O–T). (**A**) Ventral view in vivo (from Chen et al. 2014). (**B**) Contractile vacuole with 15 collecting canals. (**C**) Different body shapes. (**D, Q–R**) Ciliature of buccal area. (**E, F**) Infraciliature of same specimen in ventral (E) and dorsal view (F). (**G**) Ventral view of a representative individual. (**H–K**) Different body shapes. (**L**) Ventral view, to show buccal field (arrow). (**M**) Ventral view, to show large black granules (arrow). (**N**) To show ejected (arrow) and not ejected (arrowhead) extrusomes. (**O**) Ventral view, to show contractile vacuole (arrow), arrowhead shows collecting canal. (**P**) To show postoral suture (arrowhead). (**S**) Ventral view, to show postoral kineties (arrowheads) and vestibular kineties (double arrowheads). (**T**) Macronucleus. AL, argentophilic lines; Ma, macronucleus; P1–3, peniculi 1, 2, 3; PK, postoral kineties; PM, paroral membrane; VK, vestibular kineties. Bars, 250 μm (A–E), 150 μm (G).

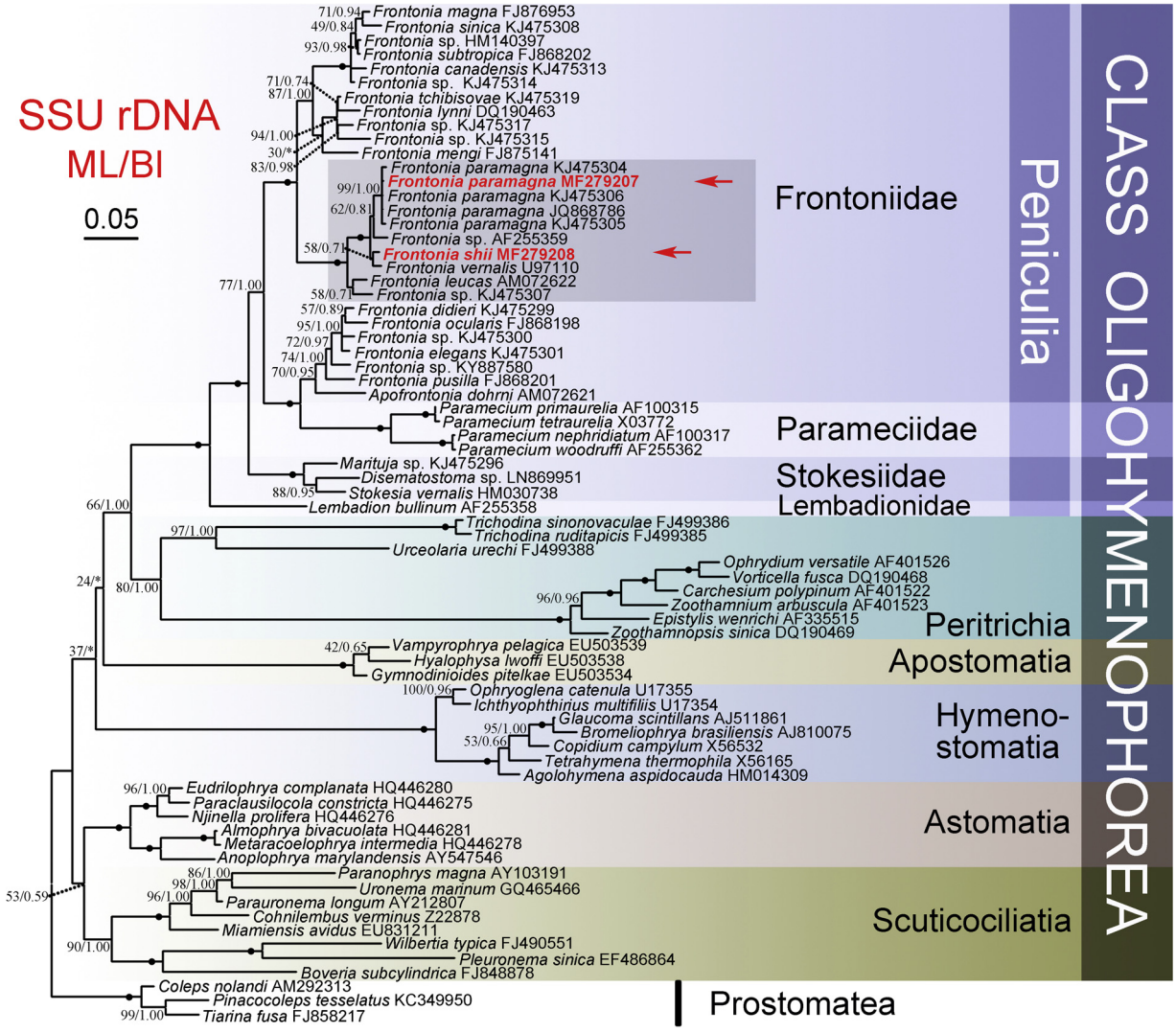

Fig. 4. Maximum likelihood tree based on SSU rDNA sequences. Numbers near the branches represent bootstrap values of ML and BI analyses. All branches are drawn to scale. The scale bar corresponds to 5 substitutions per 100 nucleotide positions. Asterisk (*) indicates the disagreement in topology between ML and BI trees. The black circles indicate the full support values (ML/BI: 100/1.00). The newly sequenced species in this work are in bold and marked with an arrow.

Description of our population. Body about 320–420 × 110–150 μm (avg. 370 × 130 μm, n = 11) in vivo, slender elliptical in outline with narrowed posterior end; dorsoventrally slightly flattened (Fig. 3C, G–K). Buccal cavity triangular in shape, about 12% of body length, located in anterior third of cell length (Fig. 3L). Cytoplasm colourless to slightly grey, often with many large (8–10 μm across) black granules at posterior end of body (Fig. 3G–K, M). Many dark-green food vacuoles and small, blue crystal granules (1–2 μm) distributed randomly in cytoplasm (Fig. 3G–K, M). Extrusomes spindle-shaped, about 5 μm long, but about 15 μm long when extruded (Fig. 3N). Macronucleus ellipsoidal, about 30 × 20 μm and located in mid-region of body (Fig. 3F, T). Micronucleus not observed. Contractile vacuole about 80 μm in diameter, located in mid-body right of cell median, with about 15 collecting canals (Fig. 3B, O). Contractile vacuole pore located on right dorsal surface. Somatic cilia about 10 μm long, and caudal cilia about 12 μm long. Locomotion by revolving moderately rapidly on substrate or by swimming while rotating clockwise along long body axis.

Somatic ciliature as shown in Fig. 3E, F, P–S. About 150–157 somatic kineties. Both anterior and postoral suture conspicuous, extend onto dorsal side. Buccal structure as shown in Fig. 3D, Q–S: three short vestibular kineties on right side of buccal cavity running from anterior vertex to postoral suture of cavity, with densely arranged kinetosomes. Three peniculi deeply located on left wall of cavity: peniculi 1 and 2 about equally long and close to each other, peniculus 3 slightly separated from peniculi 1 and 2 and curved to right. Each peniculus composed of four rows of kinetosomes (Fig. 3D, Q–S). Five or six postoral kineties (Fig. 3D,

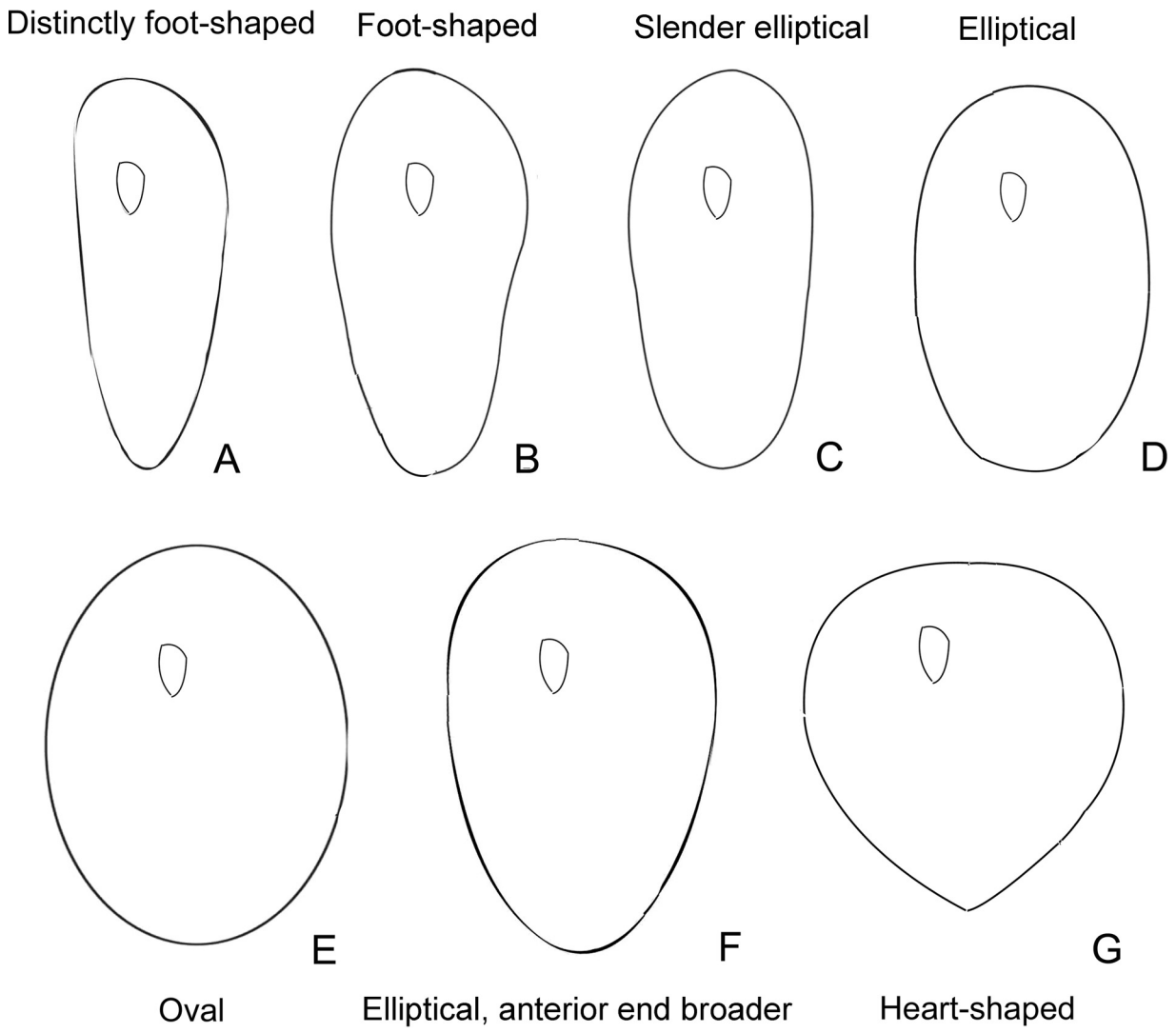

Fig. 5. A–G Different body shapes of freshwater and soil *Frontonia* species (see Table 2). (**A**) Distinctly foot-shaped; for example, in *F. leucas*. (**B**) Foot-shaped; for example, in *F. vesiculosa*. (**C**) Slender elliptical; for example, in *F. paramagna* and *F. elliptica*. (**D**) Elliptical; for example, in *F. pallida*. (**E**) Oval; for example, in *F. angusta* and *F. minuta*. (**F**) Elliptical, anterior end broader; for example, in *F. depressa*. (**G**) Heart-shaped; for example, in *F. acuminate*, *F. atra* and *F. terricola*.

S). Argentophilic lines located parallel to vestibular kineties (Fig. 3D, S).

Gene sequence data. The SSU rDNA sequence of our Harbin population of *F. paramagna* has been deposited in GenBank database with the accession number, length, and G + C content as follows: MF279207, 1623 bp, 45.16%.

Remarks and comparison. Our Harbin isolate appears similar to the type population (also from Harbin, Fig. 3A) described by Chen et al. (2014) in body size, shape, and colour, the pattern of ciliature, and the fact that both were found in freshwater habitats. There are some differences in the number of somatic kineties (150–157 vs. 180–200) and postoral kineties (five vs. six or seven in the original population). We consider these two differences as inter-population variability. Furthermore, 15 collecting canals were found in the present study whereas no collecting canal was observed or described in the original population. It cannot be excluded that Chen et al. (2014) did not check this feature or overlooked them.

Phylogenetic analyses. The phylogenetic trees of ML and BI share a similar topology; therefore, only the ML tree is shown in Fig. 5 with the support values from both ML and BI algorithms. The subclass Peniculia is monophyletic with four sub-clades corresponding to the families. The family Frontoniidae is paraphyletic with six *Frontonia* species and *Apofrontonia dohrni* grouping with the family Parameciidae in full support while the other *Frontonia* species falling into a fully supported clade.

The new species *F. shii* is sister to *F. vernalis*, with the sequence difference of 23 bp. It is worth mentioning that *F.*

vernalis was originally found in the Zoological Gardens of Berlin (not at the sea) among algae by Ehrenberg (1833). Later another form in the sea at Cadix and Copenhagen was found, which Ehrenberg (1838) doubted to be a different marine species. Bullington (1939) reported that he isolated the species from a large brackish pool at cold spring harbor and the Schoolhouse pond (brackish) at Woods Hole. Noticeably, the sequence under the name *F. vernalis*, deposited in GenBank (U97110) by Finlay et al. (1987), was isolated from a freshwater biotope. Although no morphological information was supplied, we believe that the identification made by Finlay et al. (1987) was correct.

SSU rDNA sequences of four other Chinese populations of *F. paramagna* in GenBank database with the accession numbers KJ475304, KJ475305, KJ475306 and JQ868786 were used to construct the phylogenetic tree (Fig. 5). The first three, collected in Guangzhou, Qingdao and Sichuan respectively, were reported in Zhao et al. (2016) without morphological data. The last one, collected in Harbin, was descripted by Chen et al. (2014). The five populations of *F. paramagna* cluster together with high support (99% ML, 1.00 BI), and the SSU rDNA sequence of the newly characterized population of *F. paramagna* differs from others in 2, 3, 6, and 7 bp, respectively (Wang C. et al. 2017).

As shown in Fig. 5, the genus *Frontonia* is divided into two clades, which is consistent with previous studies that *Frontonia* is not monophyletic (Fokin et al. 2006; Gao et al. 2008; Pan et al. 2013b). Noticeably, among all the *Frontonia* species for which molecular data are available, all the freshwater species form a fully supported clade, which indicates that all freshwater species having a common origin. Nevertheless, further investigations on *Frontonia* species with both detailed morphological and molecular data are needed to better understand their relationships.

Acknowledgements

This work was supported by the Natural Science Foundation of China (project number: 31501844, 31601866, 31430077), Heilongjiang Province Science Foundation for Youths (project number: QC2017017), University Nursing Program for Young Scholars with Creative Talents in Heilongjiang Province (UNPYSCT-2017180), the authors extend their sincere appreciation to the Deanship of scientific Research at King Saud University—Saudi Arabia for funding this work through Research Group number (RG-242). Many thanks are due to Prof. Weibo Song (OUC), who kindly read the draft and gave some helpful suggestions.

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