ACTA
PROTOZOOLOGICA

Acta Protozool. (2013) 52: 35–49
http://www.eko.uj.edu.pl/ap
doi:10.4467/16890027AP.13.004.0832

Studies on Three Diverse *Frontonia* Species (Ciliophora, Peniculida), with Brief Notes on 14 Marine or Brackish Congeners

Xuming PAN[1,2]*, Weiwei LIU[2]*, Zhenzhen YI[1], Xinpeng FAN[3], Khaled A. S. AL-RASHEID[4] and Xiaofeng LIN[1]

[1]Key Laboratory of Ecology and Environmental Science in Guangdong Higher Education, South China Normal University, Guangzhou, China; [2]Laboratory of Protozoology, Institute of Evolution and Marine Biodiversity, Ocean University of China, Qingdao, China; [3]School of Life Science, East China Normal University, Shanghai, China; [4]Zoology Department, King Saud University, Riyadh, Saudi Arabia

Abstract. Living observation and silver impregnation methods were used to investigate the morphology and infraciliature of three *Frontonia* ciliates (*F. guangdongensis* spec. nov., *F. ocularis* Bullington, 1939 and *F. schaefferi* Bullington, 1939) that were isolated from coastal waters of the South China Sea. *Frontonia guangdongensis* spec. nov. may be recognized by the combination of the following characteristics: cells about 160 × 35 μm *in vivo*; elongated body with right margin depressed in anterior third; length to width ratio 4:1 to 5:1; three or four vestibular and four or five postoral kineties; peniculi 1 and 2 each with four rows of kineties, peniculus 3 with two rows; one contractile vacuole in mid-body region right of cell median; brackish water habitat. A key based on morphological data for fourteen marine or brackish water *Frontonia* species found in China is also provided. In addition, the small subunit (SSU) rDNA gene was sequenced for *F. ocularis* Bullington, 1939. Our phylogenetic analyses support the contention that the genus *Frontonia* is not monophyletic.

Key words: *Frontonia guangdongensis*, *Frontonia ocularis*, *Frontonia schaefferi*, new species, South China Sea.

INTRODUCTION

The class Oligohymenophora exhibits a much higher than expected level of morphospecies diversity, and it is undoubtedly the case that many new forms are still awaiting discovery (Fan *et al.* 2010, 2011b, c; Pan *et al.* 2010, 2011; Zhang *et al.* 2011; Gao *et al.* 2012a, b). The genus *Frontonia* was established by Ehrenberg (1838) and is commonly found in freshwater, brackish, and marine biotopes (Kahl 1931, Borror 1963, Burkovsky 1970, Song and Wilbert 1989, Petz *et al.* 1995, Long *et al.* 2005). Members of the genus can be distinguished by their body shape, the structure of their oral apparatus, and their general somatic ciliary pattern with its distinct preoral and postoral suture (Roque and de Puytorac 1972; Dragesco and Dragesco-Kernéis 1986; Foissner *et al.* 1994; Fan *et al.* 2011a, 2013).

*Co-first author.
Address for correspondence: Zhenzhen Yi, Key Laboratory of Ecology and Environmental Science in Guangdong Higher Education, South China Normal University, Guangzhou 510631, China; Tel.: +86-20-85210644; E-mail: zyi@scnu.edu.cn

Recent investigations have revealed a high diversity of *Frontonia* species in Chinese coastal waters, and the presence of new or poorly known taxa have highlighted the need to conduct further studies on this genus (Long *et al.* 2005, 2008; Fan *et al.* 2011a, 2013; Pan *et al.* 2013). During the past seven years fourteen *Frontonia* species have been isolated from coastal waters of China seas, namely: *F. didieri* Long *et al.*, 2008, *F. elegans* Fan *et al.*, 2013, *F. lynni* Long *et al.*, 2005, *F. magna* Fan *et al.*, 2011, *F. mengi* Fan *et al.*, 2011, *F. guangdongensis* spec. nov., *F. multinucleata* Long *et al.*, 2008, *F. cularis* Bullington, 1939, *F. pusilla* Fan *et al.*, 2013, *F. canadensis* Roque and Puytorac, 1972, *F. schaefferi* Bullington, 1939, *F. sinica* Fan *et al.*, 2013, *F. subtropica* Pan *et al.*, 2013, and *F. tchibisovae* Burkovsky, 1970. The great diversity of *Frontonia* species highlights the need for a key to the identification of these species that draws together the morphological data in an ordered form (Figs 5, 6; Table 1).

As part of an on-going faunistic study of marine ciliates in southern China, the morphology and infraciliature of three *Frontonia* species, *F. guangdongensis* spec. nov., *F. ocularis* Bullington, 1939, and *F. schaefferi* Bullington, 1939 were studied, along with the molecular phylogenetic assignment of *F. ocularis*. In addition, a key to the marine and brackish water *Frontonia* species found in China is provided.

MATERIALS AND METHODS

Frontonia guangdongensis spec. nov. was collected from a coastal shrimp culturing area in Nansha, Guangdong Province (23°43′N, 113°33′E) on 9 November 2008, when the water temperature was 17°C, salinity 28‰, and pH 7.5. *Frontonia ocularis* Bullington, 1939 was collected from a mangrove wetland in Zhuhai, Guangdong Province (21°48′N, 113°13′E) on 28 November 2008, when the water temperature was 17°C, salinity 26‰, and pH 8.1. *Frontonia schaefferi* Bullington, 1939 was isolated from a mangrove wetland near Shenzhen, Guangdong Province (22°32′N, 113°40′E) on 22 April 2009, when the water temperature was 25°C, salinity 21‰, and pH 7.8. In each case, sand (the top 5 cm layer) or sediment plus seawater were taken from the original sites.

Cells were observed using bright field and differential interference contrast microscopy at 100 × to 1,000 × magnification. Protargol (Wilbert 1975), Chatton-Lwoff (Wilbert and Song 2008), and silver carbonate (Ma *et al.* 2003) methods were used to reveal the infraciliature and argyrome. Counts and measurements of stained specimens were performed at a magnification of 100–1,000 ×. Drawings were made with the help of a camera lucida. Terminology is mainly according to Corliss (1979).

Total genomic DNA was extracted from five cells for each species using the REDExtract-N-Amp Tissue PCR Kit (Sigma, St. Louis, USA) as described in Miao *et al.* (2011). The universal primers Euk A (5' – AAC CTG GTT GAT CCT GCC AGT – 3') or 82 F (5' – GAA ACT GCG AAT GGC TC – 3') and Euk B (5'– TGA TCC TTC TGC AGG TTC ACC TAC – 3') were used for PCR amplifications of the SSU rRNA gene (Medlin *et al.* 1988). Cloning and sequencing were performed according to Gao *et al.* (2012). Although three *Frontonia* species are described in this paper, we failed to extract DNA from either *F. guangdongensis* or *F. schaefferi* due to the low number of specimens of these species. The DNA analysis, therefore, is limited to *F. ocularis.*

A total of 49 taxa were used for phylogenetic analysis, including the one newly sequenced and twelve previously sequenced *Frontonia* spp. (for Genbank accession numbers, see Fig. 7). *Coleps nolandi* Nitzsch, 1827 and *C. hirtus* Nitzsch, 1827 were used as the outgroup taxa. Sequences were aligned using Clustal W implemented in BioEdit 7.0 (Hall 1999). The final alignment of 1622 characters was used to construct phylogenetic trees according to the method described by Liu *et al.* (2012). Briefly, the hierarchical nested likelihood ratio test implemented in MrModeltest (Nylander 2004) was used to select the best-fit model of nucleotide substitution, and this was then used for Bayesian inference (BI) analysis using MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003). The program was run for 1,000,000 generations with a sample frequency of 100 and a burn-in of 2,500 generations. PhyML version 2.4.4 (Guindon and Gascuel 2003) was used for the maximum likelihood (ML) analysis with the best model GTR + I (= 0.3301) + G (= 0.4754) selected by the program Modeltest version 3.4 (Posada and Crandall 1998). The reliability of internal branches was assessed using a non-parametric bootstrap method with 1,000 replicates.

RESULTS AND DISCUSSION

Frontonia guangdongensis spec. nov. (Fig. 1; Tables 1, 2)

Diagnosis: Brackish water *Frontonia*, about 160 × 35 μm *in vivo*, elongated body shape with right margin depressed in anterior third of body; length to width ratio about 4:1 to 5:1; small buccal field about 10 to 12% of body length; 62–75 somatic kineties; three or four vestibular kineties, four or five postoral kineties; peniculi 1 and 2 each with four rows, peniculus 3 with two rows; macronucleus ellipsoidal and located in central region of body; one contractile vacuole in mid-body region right of cell median.

Type locality: A coastal shrimp-culturing area in Nansha (23°43′N, 113°33′E), Guangdong province.

Deposition of type slides: One slide containing the holotype (registration no. LWW-08110901-01) and several paratype slides (registration no. LWW-

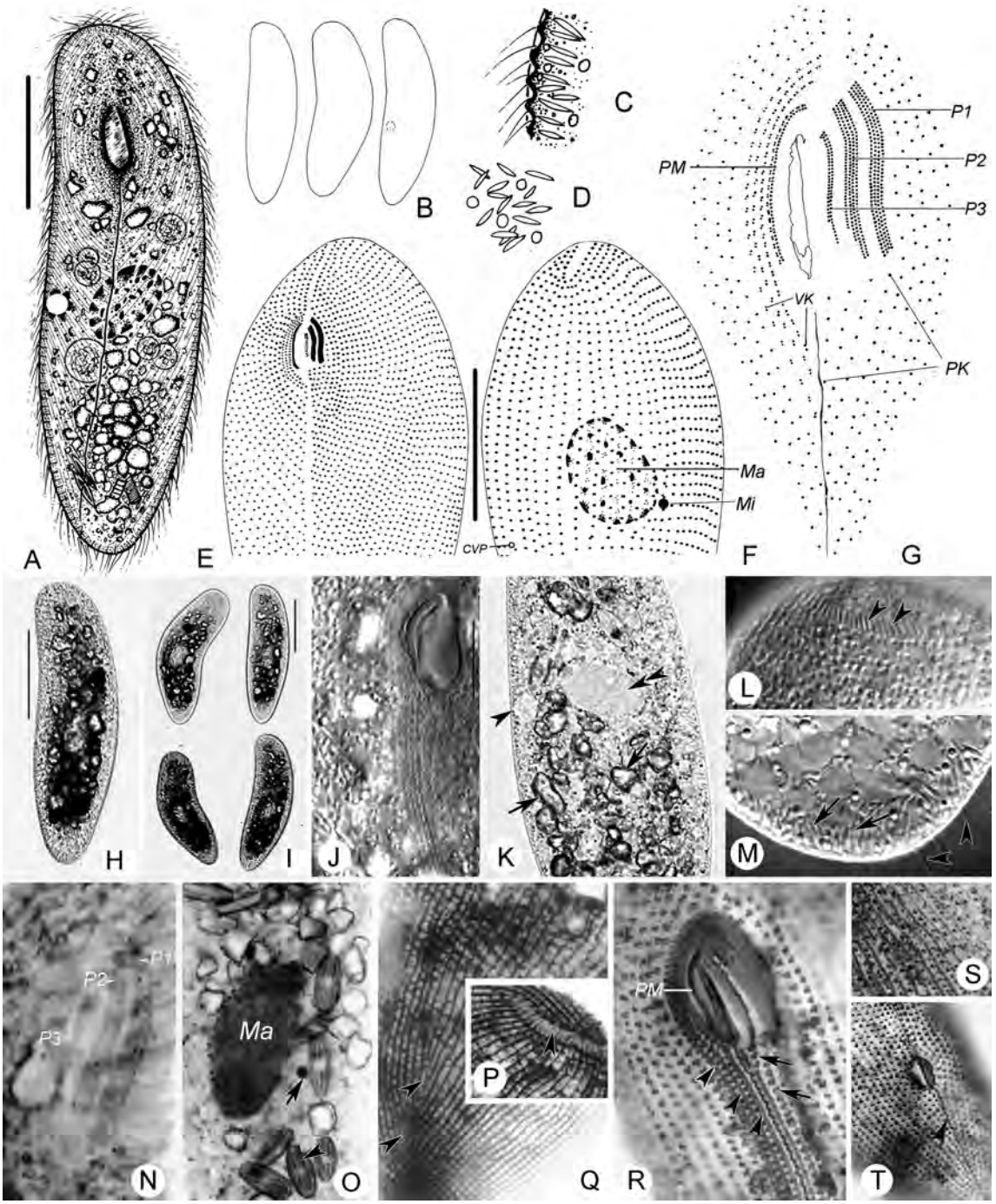

Fig. 1. *Frontonia guangdongensis* spec. nov. *in vivo* (A–D, H–M) , after protargol (E–G, N–Q, S) and silver nitrate (R, T) impregnation. **A**, **H** – ventral view of a typical individual; **B**, **I** – different body shapes; **C** – part of a pellicle, to show extrusomes; **D** – extrusomes; **E**, **F** – infraciliature in ventral and dorsal views, macronucleus, and contractile vacuole pore of a holotype specimen; **G** – infraciliature of the buccal area; **J** – buccal area; **K** – ventral view, arrows show polygonal crystal granules, double arrowhead marks the single macronucleus, arrowhead exhibits the contractile vacuole; **L** – anterior end of cell, arrowheads mark the anterior suture; **M** – posterior part of the cell, arrowheads mark the caudal cilia, arrows show extrusomes; **N**, **R** – structure of the buccal region, arrows on R depict postoral kineties and arrowheads mark vestibular kineties; **O** – macronucleus and micronucleus, arrow marks micronucleus and arrowhead shows ingested algae; **P**, **Q**, **T** – anterior suture (arrowhead in P) and postoral suture (arrowheads in Q, T); **S** – somatic kineties. CVP – contractile vacuole pore; Ma – macronucleus; Mi – micronucleus; P1–P3 – peniculi 1, 2, 3; PK – postoral kineties, PM – paroral membrane; VK – vestibular kineties. Scale bars: A = 60 μm, E, F = 40 μm, H, I = 70 μm, J = 20 μm.

Table 1. Morphometric data of *Frontonia guangdongensis* spec. nov. (upper row, bold font), *F. ocularis* Bullington, 1939 (middle row) and *F. schaefferi* Bullington, 1939 (lower row). Data according to protargol-impregnated specimens. All measurements in μm. Abbreviations: CV – coefficient of variation in %, n – number of specimens measured, SD – standard deviation.

Character	Min	Max	Mean	SD	CV	n
Body length	**152**	**175**	**164.9**	**11.1**	**7.6**	**23**
	88	110	95.5	4.8	4.9	24
	74	101	88.7	7.1	7.9	20
Body width	**45**	**58**	**49.8**	**7.4**	**13.4**	**23**
	45	55	47.9	2.9	6.3	24
	34	60	44.3	6.1	13.5	20
Number of somatic kineties	**52**	**65**	**61.4**	**5.8**	**7.9**	**19**
	93	107	95.7	9.6	12.8	18
	59	80	69.2	12.1	13.8	18
Number of vestibular kineties	**3**	**4**	**3.1**	**0.3**	**8.7**	**15**
	3	3	3	0	0	14
	3	3	3	0	0	14
Number of postoral kineties	**4**	**5**	**4.1**	**5.8**	**7.9**	**14**
	3	4	3.5	0.5	14.6	13
	5	5	5	0	0	12
Number of ciliary rows in peniculus 1	**4**	**4**	**4**	**0**	**0**	**15**
	4	4	4	0	0	16
	4	4	4	0	0	15
Number of ciliary rows in peniculus 2	**4**	**4**	**4**	**0**	**0**	**15**
	4	4	4	0	0	16
	4	4	4	0	0	15
Number of ciliary rows in peniculus 3	**2**	**2**	**2**	**0**	**0**	**14**
	2	2	2	0	0	14
	2	2	2	0	0	15

08110901-02) with protargol-impregnated specimens are deposited in the Laboratory of Protozoology, Ocean University of China, China.

Etymology: The species name '*guangdongensis*' refers to the location where this organism was first isolated.

Description: Cell *in vivo* distinctly elongate, usually about 150–170 × 35–40 μm, with ratio of length to width about 4:1 to 5:1 (Fig. 1A, H). Right margin slightly depressed in anterior third of body (Fig. 1A, B, H, I). Dorsoventrally flattened about 5:4. Buccal cavity small and shallow, elliptical to triangular in outline, about 20 × 11 μm in size, 10 to 12% of body length (Fig. 1G). Cytoplasm grayish with many large (6–10 μm across), black, polygonal crystal granules. Food vacuoles (10–12 μm across) and ingested algae distributed randomly in cytoplasm (Fig. 1A, K). Macronucleus ellipsoidal, about 20 μm × 15 μm, located in mid-region of body (Fig. 1A, O). Single micronucleus located near end of macronucleus, spherical, about 5 μm in diameter (Fig. 1O). Single contractile vacuole in mid-body region right of cell median, about 7 μm in diameter, contracting at about one minute intervals (Fig. 1A, K); no collecting canals observed; one contractile vacuole pore located on right-dorsal surface (Fig. 1F). Two types of extrusomes, spindle (about 8 μm long) and round (2 μm across), densely arranged beneath pellicle (Fig. 1C, D, M); somatic cilia generally about 6 μm long, cilia in caudal region being longer than others at approximately 10 μm long (Fig. 1M). Locomotion by gliding on sub-

Table 2. Comparison of marine or brackish *Frontonia* species isolated in China.

Species	Body length *in vivo* (μm)	Number of					Data sources
		somatic kineties	ciliary rows in peniculi 1, 2, and 3	vestibular kineties	postoral kineties	contractile vacuole	
F. subtropica Pan *et al*., 2012	180–230	104–114	4, 4, 4	5	5	1	Pan *et al.* (2013)
F. magna Fan *et al*., 2011	200–410	165–216	4, 4, 4	5–6	4–7	1–2	Pan *et al.* (2013)
F. canadensis Roque and Puytorac, 1972	120–150	77–88	4, 4, 4	3–4	5	1	Pan *et al.* (2012)
F. sinica Fan *et al*., 2013	100–200	100–119	4, 4, 2	5–6	3–5	1	Fan *et al.* (2013)
F. pusilla Fan *et al*., 2013	70–100	70–77	4, 4, 2	4	3	2	Fan *et al.* (2013)
F. mengi Fan *et al*., 2011	150–242	48–60	5, 5, 2	3	5	1	Fan *et al.* (2011)
F. tchibisovae Burkovsky, 1970	80–180	110–130	4, 4, 4	4	7	1	Long *et al.* (2008)
F. lynni Long *et al*., 2005	100–210	71–83	4, 4, 5	3	5	1	Long *et al*. (2005)
F. didieri Long *et al*., 2008	100–150	61–71	4, 4, 3	3	3–5	1	Long *et al.* (2008)
F. multinucleata Long *et al*., 2008	70–120	58–67	4, 4, 4	3	4–5	1	Long *et al*. (2008)
F. elegans Fan et al., 2013	75–90	69–78	4, 4, 3	4	3	2	Fan *et al*. (2013)
***F. guangdongensis* spec. nov.**	**150–170**	**52–65**	**4, 4, 2**	**3–4**	**4–5**	**1**	**present work**
***F. ocularis* Bullington, 1939**	**115–140**	**93–107**	**4, 4, 2**	**3**	**3–4**	**2**	**present work**
***F. schaefferi* Bullington, 1939**	**95–100**	**59–80**	**4, 4, 2**	**3**	**5**	**1**	**present work**

strate or by swimming while rotating about long body axis.

Somatic ciliature as shown in Fig. 1E, F, G, P–T. About 52 to 65 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, P, S); posterior part of somatic kineties terminating below posterior region of oral apparatus forming the postoral suture (Fig. 1G, Q, T). Three to four vestibular kineties with close-set dikinetids (Fig. 1G, R). Four or five postoral kineties left of postoral suture, beginning anteriorly below buccal cavity and gradually shortening from left to right (Fig. 1G, R).

Buccal apparatus as shown in Fig. 1G, N, R. Three conspicuous peniculi (P1–3) located on left wall of shallow buccal cavity, slightly curved to right at anterior end. Peniculi 1 and 2 about equally long, parallel to each other, and each composed of four rows of kinetosomes. Peniculus 3 composed of two kineties, right one of which extends entire length of buccal cavity, left one extending only to about anterior 4/5 of cavity length (Fig. 1G, N). Double-rowed paroral membrane on right side of buccal cavity: inner row composed of densely arranged monokinetids, outer row composed of loosely arranged dikinetids (Fig. 1G, R).

Comparison and remarks: There are three species that closely resemble *Frontonia guangdongensis* spec. nov. in terms of the conspicuously elongated body shape: *F. mengi* Fan, 2011, *F. pallida* Czapik, 1979 and *F. microstoma* Kahl, 1931.

Frontonia mengi differs from *F. guangdongensis* in three respects. Firstly, its body size *in vivo* is larger (250 × 45 μm vs. 160 × 35 μm); secondly, it has more kinety rows in peniculi 1 and 2 (each row has five in *F. mengi* vs. four in *F. guangdongensis*): thirdly, the contractile vacuole of *F. mengi* is located in the posterior third of the cell, whereas in *F. guangdongensis* it is located in the mid-body region (Fan *et al.* 2011a).

Frontonia pallida can be clearly distinguished from *F. guangdongensis* by the following combination of characteristics: *F. pallida* has three kinety rows in peniculus 2, whereas *F. guangdongensis* has four; *F. pallida* has a funnel-shaped cytopharynx (vs. absent in *F. guangongensis*); *F. pallida*'s contractile vacuole is to the left of the cell median, whereas it is to the right in *F. guangdongensis* (Czapik 1979, Dragesco and Dragesco-Kernéis 1986).

Frontonia guangdongensis spec. nov. can also be easily separated from *F. microstoma* Kahl, 1931 in having only a single contractile vacuole with no canals whereas the latter has two contractile vacuoles with

associated canals. *Frontonia guangdongensis* also has fewer somatic kineties (52–65 vs. 110–120 in *F. microstoma*) and fewer kinety rows in peniculus 3 (2 vs. 3 or 4) (Roque 1961, Carey 1992).

Frontonia ocularis Bullington, 1939 (Figs 2, 3; Tables 1, 2)

The original definition of this organism was based only on live specimens and no information about its infraciliature was previously available (Bullington 1939). Based on data from both the present and previous studies, an improved diagnosis is presented here, including a description of its infraciliature.

Improved diagnosis: Marine or brackish *Frontonia*, about 115–140 × 50–75 μm *in vivo*, reniform with anterior end broad and posterior end slightly narrowed, right margin depressed in anterior 1/3 of body; length to width ratio about 2:1; small buccal field about 15 to 20% of body length; a prominent pigment on right side of anterior extremity; about 93–107 somatic kineties; three vestibular and three or four postoral kineties; peniculi 1 and 2 each with four rows, peniculus 3 with two rows; macronucleus ellipsoidal, located in central region of body; two contractile vacuoles located right-ventral, one in anterior half and the other in posterior half of body.

Description of Zhuhai population: Body 115–120 × 50–60 μm *in vivo*, reniform with anterior end broad and posterior end slightly narrowed, right margin depressed in anterior 1/3 of body (Figs 2A, C, 3A, C). Ratio of length to width between 2:1 and 3:1 (Figs 2A, 3A). Dorsoventrally flattened about 3:2. Buccal cav-

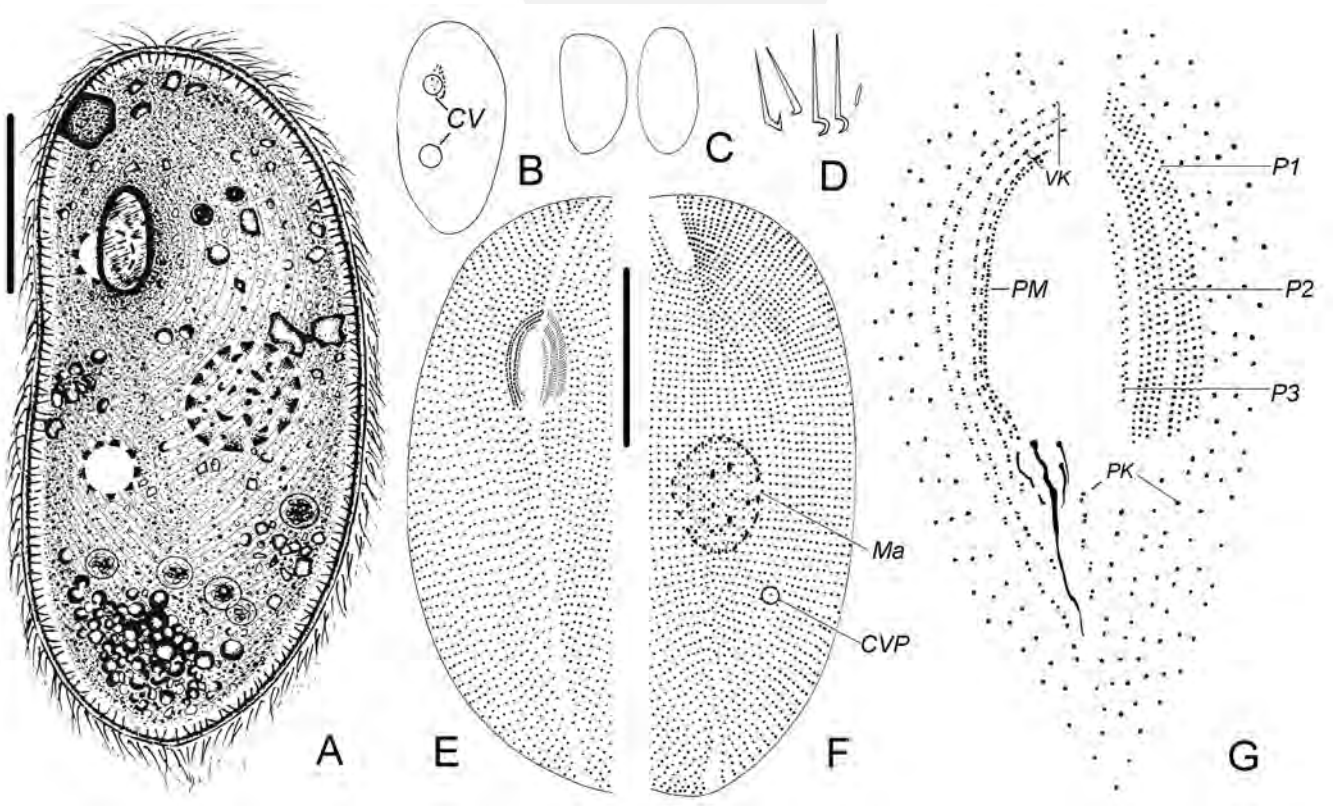

Fig. 2. *Frontonia ocularis in vivo* (A–D) and after protargol impregnation (E–G). **A** – ventral view of a typical individual; **B** – ventral view showing position of contractile vacuoles; **C** – different body shapes; **D** – extruded extrusomes; **E**, **F** – infraciliature in ventral and dorsal views, macronucleus, and contractile vacuole pore; **G** – infraciliature of the buccal area. CV – contractile vacuole, CVP – contractile vacuole pore, Ma – macronucleus, P1–P3 – peniculi 1, 2, 3, PM – paroral membrane, PK – postoral kineties, VK – vestibular kineties. Scale bars: A = 60 μm, E, F = 40 μm.

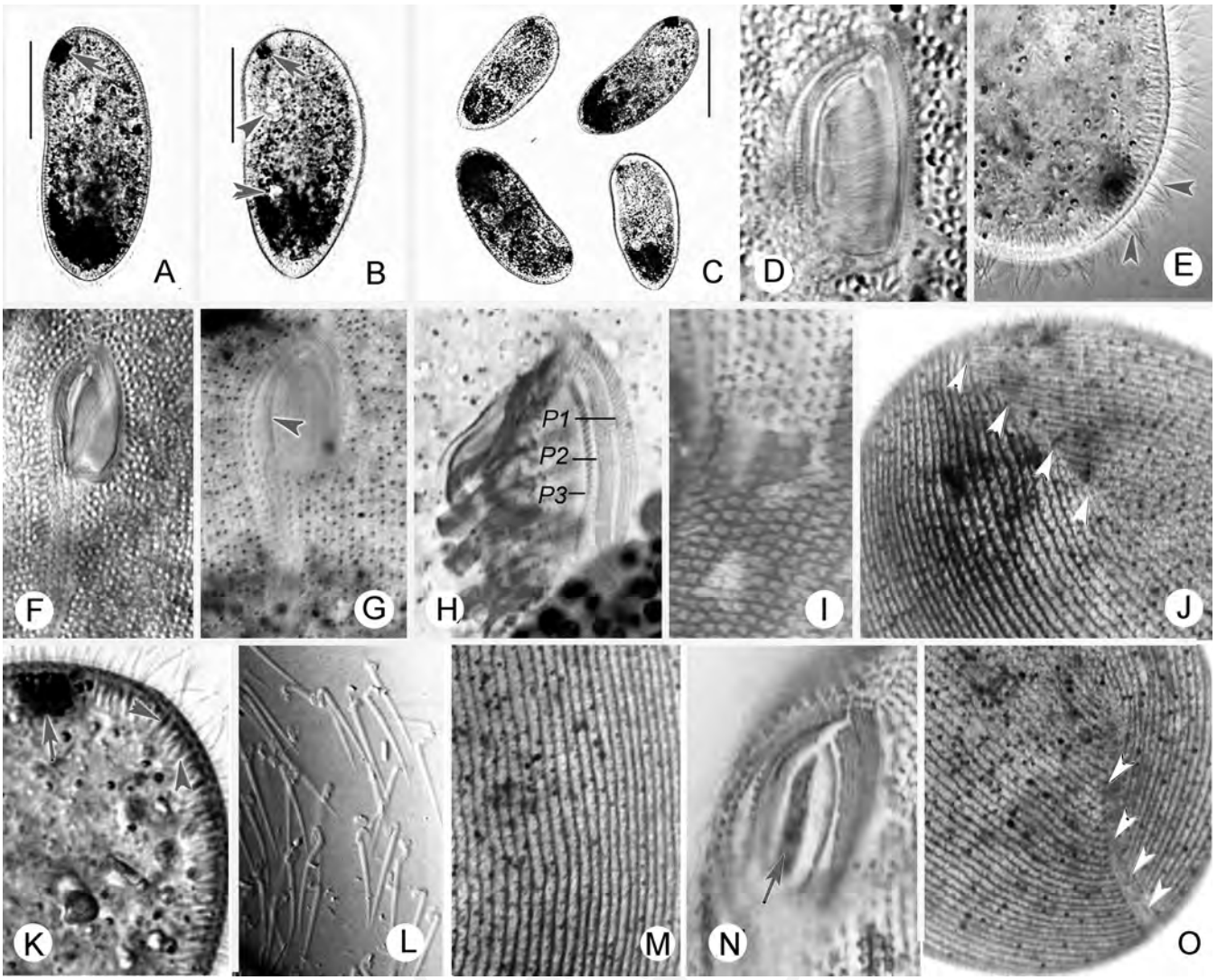

Fig. 3. *Frontonia ocularis in vivo* (A–F, K, L) and after silver carbonate (I, N) and protargol impregnation (G, H, J, M, O). **A** – ventral view of a typical individual, arrow shows the black brown pigment spot; **B** – ventral view, to show two contractile vacuoles (arrowheads), arrow shows the prominent brown pigment spot; **C** – different body shapes; **D**, **F** – buccal area; **E** – posterior part of cell, arrowheads mark caudal cilia; **G**, **H**, **N** – detailed structure of buccal area, arrowhead (G) marks paroral membrane, arrow (N) shows argentophilic line; **I** – part of argyrome; **J**, **O** – anterior suture (arrowheads in J) and postoral suture (arrowheads in O); **K** – extrusomes (arrowheads) forming distinct seam underneath cortex, arrow shows the black brown spot; **L** – extruded extrusomes; **M** – ventral view to show closely arranged somatic kineties. P1–P3 – peniculus 1, 2, 3. Scale bars: A, B = 50 μm, C = 80 μm.

ity about 20 × 10 μm, occupying 15 to 20% of body length (Fig. 3D, F). Cytoplasm slightly grayish, often with many large (8–10 μm across), black granules at posterior end of body (Fig. 2A). Many dark-green food vacuoles and small, blue crystal granules (1–2 μm) distributed randomly in cytoplasm (Fig. 2A). Brown-black pigment spot usually present on extreme right border near anterior end of body (Figs 2A, 3A, B, K). Extrusomes spindle-shaped, about 4 μm long but, when extruded, about 15 μm long and rod-shaped with one end curved (Figs 2D, 3K). Macronucleus ellipsoidal, about 25 × 15 μm and located in mid-region of body (Fig. 2A). No micronucleus observed. Two contractile vacuoles 7–10 μm in diameter, located right-ventrally, one each in anterior and posterior 1/3 of body respectively (Figs 2B, 3B). Somatic cilia about 6 μm long (Fig. 3E);

cilia in caudal end longer than others, about 8 μm long. Locomotion by revolving moderately rapidly on substrate or by swimming in water while rotating clockwise about the long body axis.

Somatic ciliature as shown in Figs 2E, F, G, 3G, I, J, M, O. About 93 to 107 somatic kineties shortened progressively both anteriorly and posteriorly from lateral side to oral area, forming conspicuous anterior and posterior sutures that extend from anterior end of buccal cavity over apical end of cell and onto dorsal side. Buccal structure as shown in Figs 2G, 3H, O. Three conspicuous peniculi located on left wall of buccal cavity: peniculi 1 and 2 about equal in length, positioned close to each other parallel to edge of left vestibular wall, slightly curved to right at anterior ends and each composed of four rows of kinetosomes (Figs 2G, 3H). Peniculus 3 composed of two kinety rows which are about equal in length. Paroral membrane double-rowed, located on right side of buccal cavity (Figs 2G, 3H). Three vestibular kineties extending along the paroral membrane, each composed of densely arranged dikinetids. Three or four postoral kineties (Fig. 2G). Several argentophilic lines left of paroral membrane (Fig. 3N).

Comparison and remarks: The population studied here corresponds very well with the original description (Bullington 1939) in terms of body shape, the prominent pigment spot in the anterior border, the number and location of the contractile vacuoles, and the manner of spiralling when swimming forwards. Hence, the identification of this species is not in doubt. Compared with previous descriptions of *F. ocularis*, our population has a smaller body size (about 120 × 55 μm vs. about 141 × 76 μm) and the prominent pigment spot is brown-black, whereas it is cited as being reddish-brown in earlier descriptions (Bullington 1939). However, we consider these dissimilarities to be population-dependent.

SSU rRNA gene sequence: The SSU rRNA gene sequence of *Frontonia ocularis* Bullington, 1939 has been deposited in the GenBank database with accession number, length, and G+C content as follows: FJ868198, 1746 bp, 44.73%.

Frontonia schaefferi Bullington, 1939 (Fig. 4; Tables 1, 2)

When this species was first described by Bullington (1939), neither the details of its living morphology, nor a clearly-outlined diagnosis was given. Hence, an improved diagnosis based on both previous and present studies is supplied here.

Improved diagnosis: Marine or brackish *Frontonia*, about 95–120 × 50–65 μm *in vivo*, elliptical or reniform with anterior end broad and posterior end slightly narrowed; small buccal field about 20% of body length; about 59–80 somatic kineties; three vestibular and five postoral kineties; peniculi 1 and 2 each with four rows, peniculus 3 with two rows; macronucleus ellipsoidal, located in central region of body; one contractile vacuole, positioned on right-dorsal side at about 3/5 distance down body length and with about eight, long collecting canals.

Description of Shenzhen population: Size *in vivo* about 95–100 × 50–55 μm. Elliptical or reniform in outline with anterior end broad and posterior end slightly narrowed (Fig. 4A, B, H, I, K). Dorsoventrally flattened about 3:2 (Fig. 4M). Buccal cavity small and shallow, elliptical to triangular in outline, about 20 μm × 10 μm, which corresponds to about 20% of body length (Fig. 4J). Cytoplasm colourless to slightly grayish, often with many small black granules (3–4 μm across) at anterior end of body (Fig. 4A, H, I). A few small blue crystal granules (1–3 μm in diameter) distributed randomly in cytoplasm (Fig. 4A, H, I). Macronucleus ellipsoidal, about 25 × 15 μm, located in body centre (Fig. 4A). No micronucleus observed. One contractile vacuole, about 10 μm in diameter, positioned on right-dorsal side at about three-fifths distance down body length, with about eight long collecting canals (Fig. 4A, H): one contractile vacuole pore located on right-ventral surface (Fig. 4F). Extrusomes spindle-shaped, about 5 μm long, densely arranged beneath pellicle (Fig. 4L), approximately 20 μm long when extruded (Fig. 4N). Somatic cilia approximately 6 μm long. Locomotion by crawling slowly on substrate or by swimming in water with clockwise rotation about long body axis.

General infraciliature as shown in Fig. 4E–G, O–Q. Anterior and postoral sutures conspicuous, both extending onto dorsal side (Fig. 4E, F). About 59 to 80 somatic kineties. Five postoral kineties with dikinetids, beginning below the oral and terminating on the postoral suture (Fig. 4E, F). Three vestibular kineties with densely arranged kinetosomes extending from anterior vertex of buccal cavity and terminating at postoral suture (Fig. 4G, O). Three conspicuous peniculi, about half of the length of the buccal cavity: peniculi 1 and 2 about equal in length, each composed of four rows of kinetosomes, about half length of buccal cavity; peniculus 3 slightly curved to right at anterior end, composed of two shorter kineties which are about equal in length

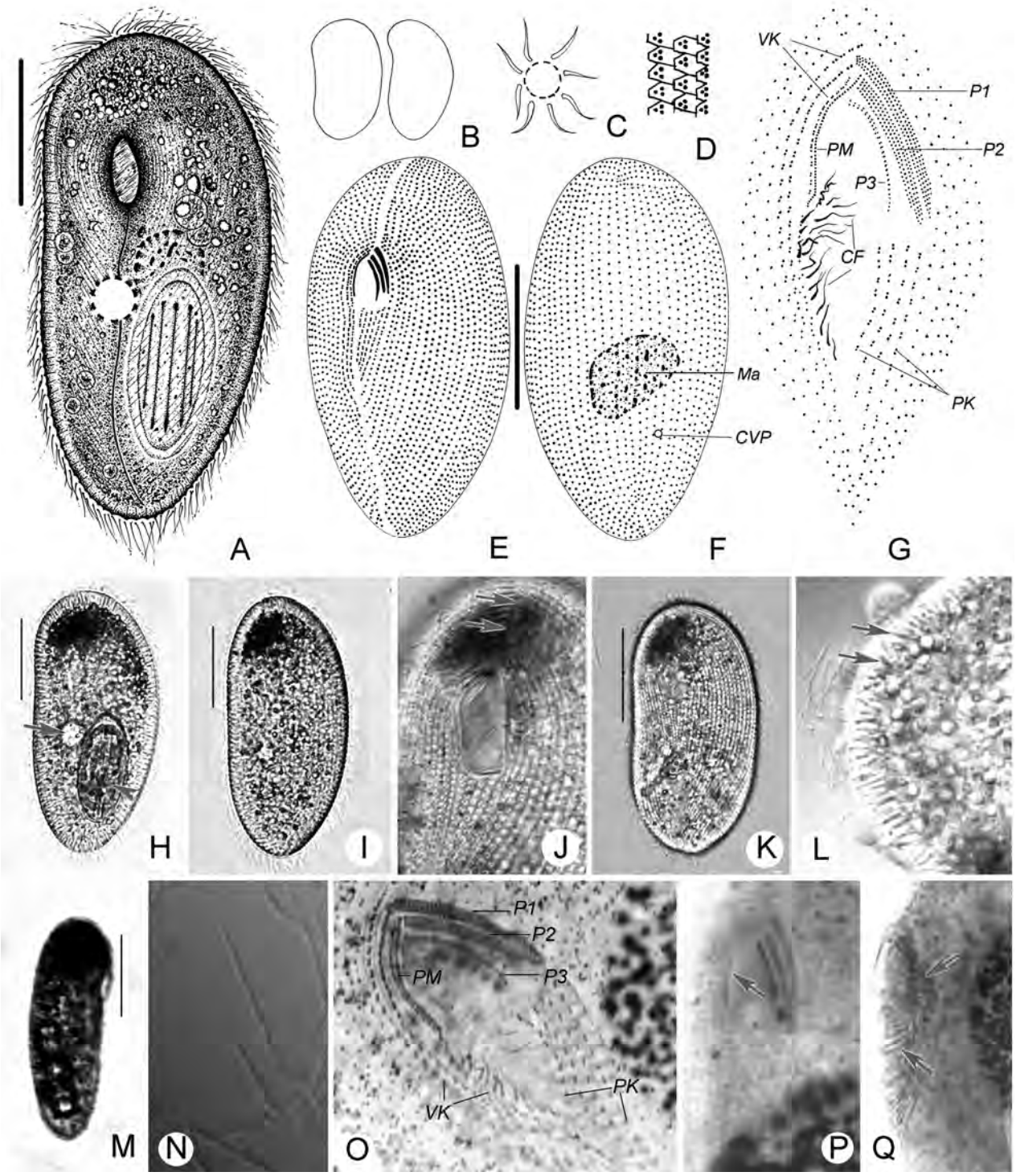

Fig. 4. *Frontonia schaefferi in vivo* (A–C, H–N) and after protargol (E–G, O–Q) and silver nitrate (D) impregnation. **A**, **H** – ventral view of a typical individual, arrow shows the single contractile vacuole and arrowhead shows a large algal cell; **B** – different body shapes; **C** – single contractile vacuole with about 8 long collecting canals; **D** – part of argyrome; **E**, **F** – infraciliature in ventral and dorsal views, macronucleus, and contractile vacuole pore; **G** – infraciliature of the buccal area; **I**, **K** – ventral view of two individuals showing different shapes; **J** – buccal area, arrows show anterior suture; **L** – extrusomes (arrows) beneath pellicle; **M** – lateral view; **N** – extruded extrusomes; **O**-**Q** – structure of buccal region, arrow on P depicts paroral membrane, arrows on Q depict cytopharyngeal fibers. CF – cytopharyngeal fibres; CVP – contractile vacuole pore; Ma – macronucleus; P1–P3 – peniculi 1, 2, 3; PM – paroral membrane; PK – postoral kineties; VK – vestibular kineties. Scale bars: A = 40 μm, E, F = 50 μm, H, I, K, M = 70 μm.

(Fig. 4G, O). Single-rowed paroral membrane on right edge of buccal cavity running from anterior to posterior edge of buccal overture, with well-developed oral pharyngeal fibres in the buccal area (Fig. 4G, Q).

Comparison and remarks: *Frontonia schaefferi* Bullington, 1939 was originally reported by Bullington (1939) with only its infraciliature described. The Shenzhen population closely resembles that described by Bullington (1939) except for a minor difference in the body size *in vivo* (120 × 65 μm vs. 100 × 55 μm in Shenzhen population) which we believe is population-dependent.

Key to the marine and brackish *Frontonia* species isolated in China (Figs 5, 6; Table 2)

Based on the data obtained, a key to fourteen nominal marine or brackish morphotypes isolated from Chinese coastal waters is provided here.

1	Single macronucleus	2
–	More than one macronucleus	*F. multinucleata*
2	Body length usually more than 300 μm	3
–	Body length usually less than 300 μm	4
3	More than 150 somatic kineties	*F. magna*
–	About 50 somatic kineties	*F. mengi*
4	Two contractile vacuoles	5
–	Single contractile vacuole	6
5	Foot-shaped; pigment spot absent	*F. pusilla*
–	Body reniform; pigment spot present	*F. ocularis*
6	Peniculus 3 two-rowed	7
–	Peniculus 3 composed of more than two rows	10
7	Peniculi 1 and 2 five rows each	*F. lynni*
–	Peniculi 1 and 2 four rows each	8
8	Five or six vestibular kineties	*F. sinica*
–	Fewer than five vestibular kineties	9
9	Ratio of length to width 4:1 to 5:1; 4 or 5 vestibular kineties	*F. guangdongensis*
–	Ratio of length to width about 2:1; constantly 3 vestibular kineties	*F. schaefferi*
10	Peniculus 3 three-rowed	11
–	Peniculus 3 four-rowed	12
11	Two contractile vacuoles	*F. elegans*
–	Single contractile vacuole	*F. didieri*
12	Five vestibular kineties	*F. subtropica*
–	Fewer than five vestibular kineties	13
13	Five postoral kineties; about 80 somatic kineties	*F. canadensis*
–	Seven postoral kineties; about 120 somatic kineties	*F. tchibisovae*

Phylogenetic analysis

The topologies of the SSU rRNA gene trees constructed using Bayesian inference and maximum-likelihood analyses were similar, therefore only the BI tree is shown (Fig. 7). The fourteen *Frontonia* species are divided into two distinct clades, supporting the viewpoint that *Frontonia* is not monophyletic (Fokin *et al.* 2006; Gao *et al.* 2008; Fan *et al.* 2011, 2013). The clade containing *F. ocularis*, *F. pusilla*, *F. elegans* and *F. didieri* clusters with members of the genus *Paramecium* and *Apofrontonia dohrni*, and then groups with a highly supported clade (1.00 BI, 87% ML) that includes the other ten *Frontonia* species. Number of contractile vacuoles and kinety rows in peniculus 3 could generally match the molecular cluster patterns within the genus *Frontonia*. *F. ocularis*, *F. pusilla* and *F. elegans*, having two contractile vacuoles and two or three kinety rows in peniculus 3 (Fan *et al.* 2013), fall into a clade. Similarly, the members with four or five kinety rows in peniculus 3 and one contractile vacuole (Bullington 1939;

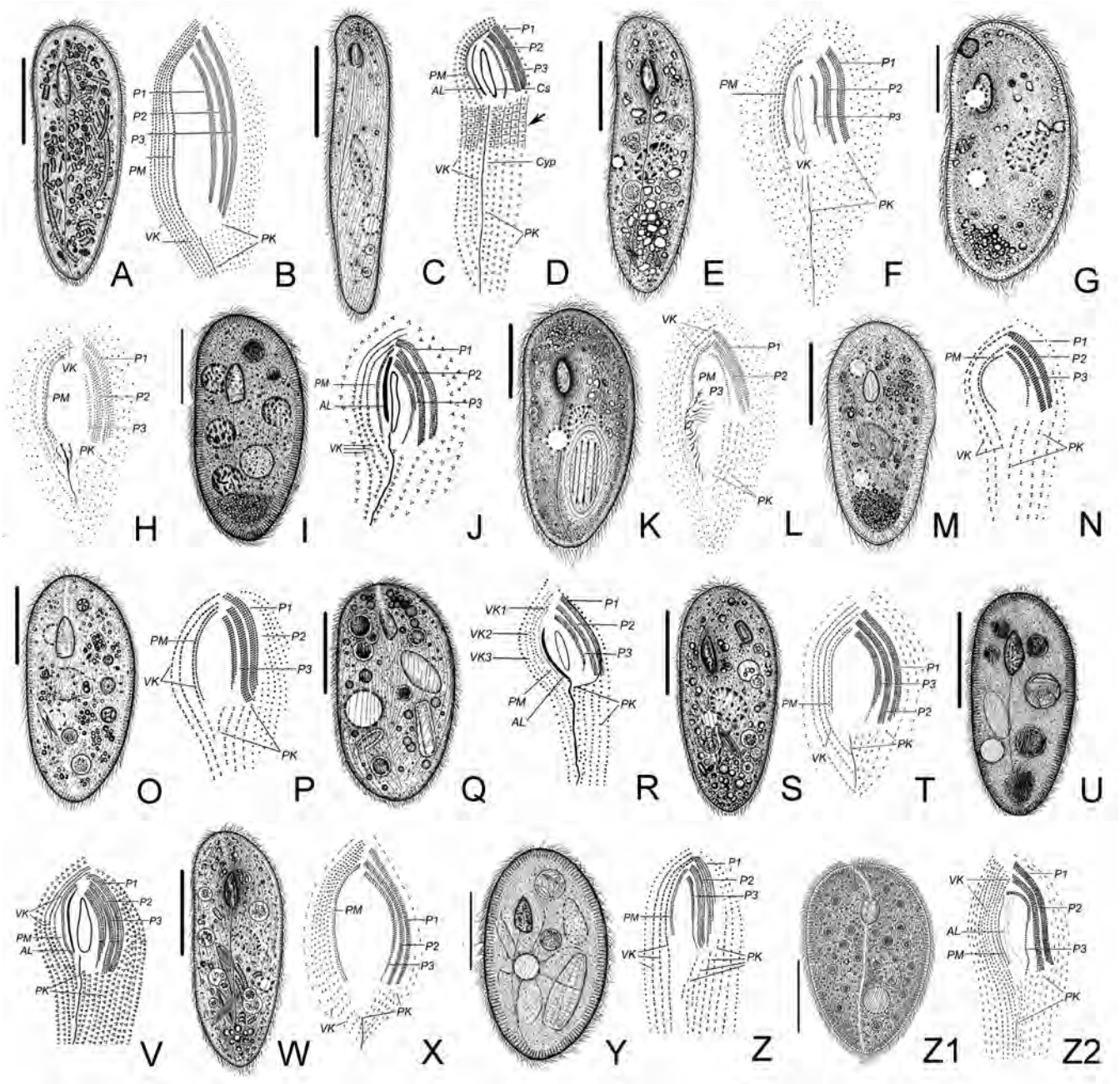

Fig. 5. The Chinese populations of fourteen *Frontonia* species *in vivo* (A, C, E, G, I, K, M, O, Q, S, U, W, Y, Z1), after protargol impregnation (B, F, H, L, N, P, T, X, Z, Z2) and Chatton-Lwoff silver nitrate impregnation (D, J, R, V). **A**, **B** – *Frontonia magna* Fan *et al.*, 2011 (from Fan *et al.* 2011a); **C**, **D** – *F. mengi* Fan *et al.*, 2011 (from Fan *et al.* 2011a); **E**, **F** – *F. guangdongensis* spec. nov. (from present work); **G**, **H** – *F. ocularis* Bullington, 1939 (from present work); **I**, **J** – *F. multinucleata* Long *et al.*, 2008 (from Long *et al.* 2008); **K**, **L** – *F. schaefferi* Bullington, 1939 (from present work); **M**, **N** – *F. pusilla* Fan *et al.*, 2013 (from Fan *et al.* 2013); **O**, **P** – *F. elegans* Fan *et al.*, 2013 (from Fan *et al.* 2013); **Q**, **R** – *F. lynni* Long *et al.*, 2005 (from Long *et al.* 2005); **S**, **T** – *F. canadensis* Roque and Puytorac, 1972 (from Pan *et al.* 2013); **U**, **V** – *F. tchibisovae* Burkovsky, 1970 (from Long *et al.* 2008); **W**, **X** – *F. subtropica* Pan *et al.*, 2013 (from Pan *et al.* 2013); **Y**, **Z** – *F. didieri* Long *et al.*, 2008 (from Long *et al.* 2008); **Z1**, **Z2** – *F. sinica* Fan *et al.*, 2013 (from Fan *et al.* 2013). P1–P3 – peniculi 1, 2, 3, PM – paroral membrane, PK – postoral kineties, VK – vestibular kineties. Scale bars: A = 150 μm, C, Q, S, U = 80 μm, E, G, Y, Z1 = 60 μm, I, K, O = 40 μm, M = 50 μm, W = 100 μm.

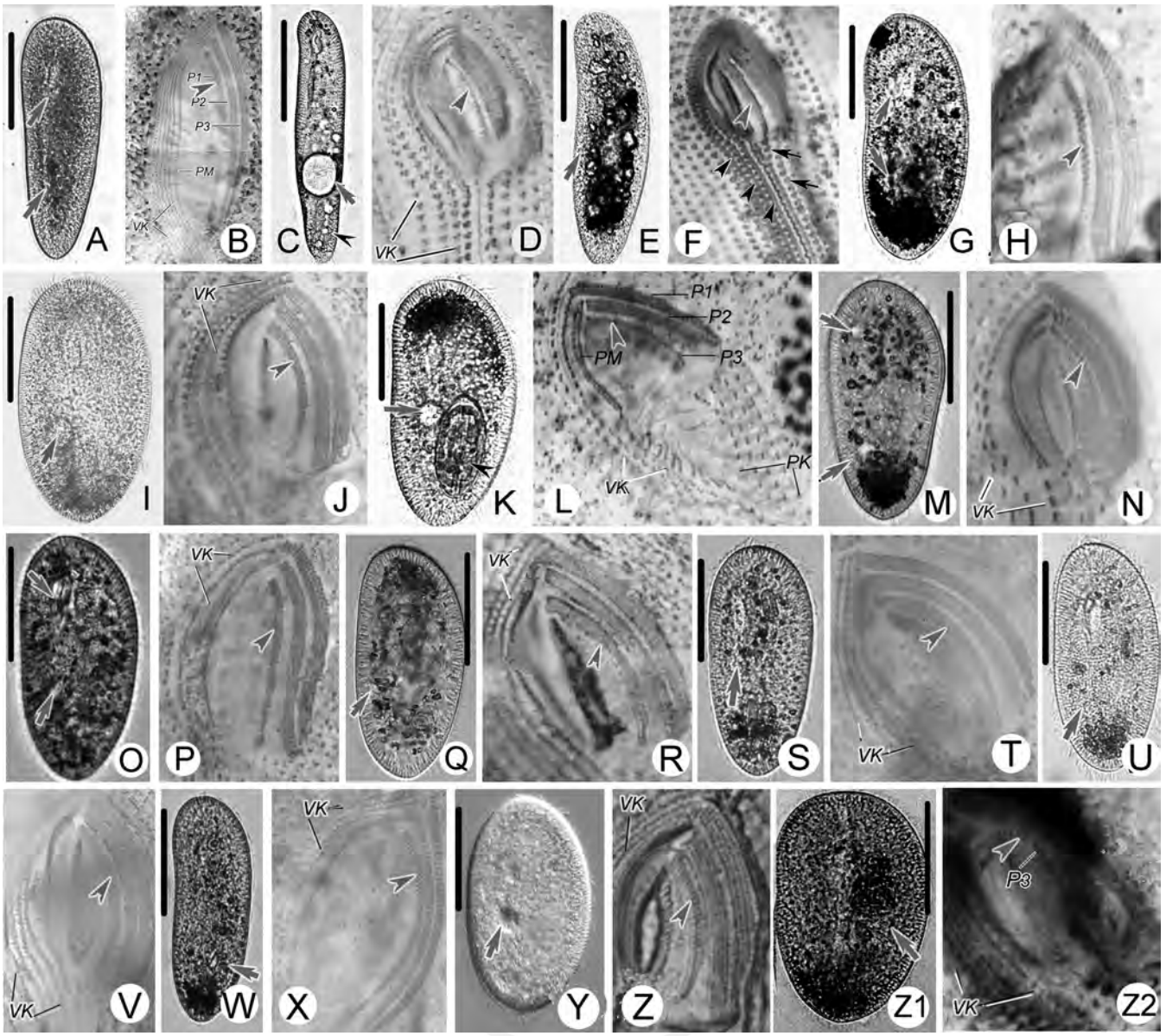

Fig. 6. Photomicrographs of the Chinese populations of fourteen *Frontonia* species *in vivo* (A, C, E, G, I, K, M, O, Q, S, U, W, Y, Z1), after protargol impregnation (B, F, H, L, N, P, T, X, Z, Z2) and Chatton-Lwoff silver nitrate impregnation (D, J, R, V). **A**, **B** – *Frontonia magna* Fan *et al.*, 2011 (from Fan *et al.* 2011a); **C**, **D** – *F. mengi* Fan *et al.*, 2011 (from Fan *et al.* 2011a); **E**, **F** – *F. guangdongensis* spec. nov. (from present work); **G**, **H** – *F. ocularis* Bullington, 1939 (from present work); **I**, **J** – *F. multinucleata* Long *et al.*, 2008 (from Long *et al.* 2008); **K**, **L** – *F. schaefferi* Bullington, 1939 (from present work); **M**, **N** – *F. pusilla* Fan *et al.*, 2013 (from Fan *et al.* 2013); **O**, **P** – *F. elegans* Fan *et al.*, 2013 (from Fan *et al.* 2013); **Q**, **R** – *F. lynni* Long *et al.*, 2005 (from Long *et al.* 2005); **S**, **T** – *F. canadensis* Roque and Puytorac, 1972 (from Pan *et al.* 2013); **U**, **V** – *F. tchibisovae* Burkovsky, 1970 (from Long *et al.* 2008); **W**, **X** – *F. subtropica* Pan *et al.*, 2013 (from Pan *et al.* 2013); **Y**, **Z** – *F. didieri* Long *et al.*, 2008 (from Long *et al.* 2008); **Z1**, **Z2** – *F. sinica* Fan *et al.*, 2013 (from Fan *et al.* 2013). Larger arrows mark contractile vacuoles, larger arrowheads show peniculus 3. P1–P3 – peniculi 1, 2, 3, PM – paroral membrane, PK – postoral kineties, VK – vestibular kineties. Scale bars: A = 150 μm, C, Q, S, U = 80 μm, E, G, Y, Z1 = 60 μm, I, K, O = 40 μm, M = 50 μm, W = 100 μm.

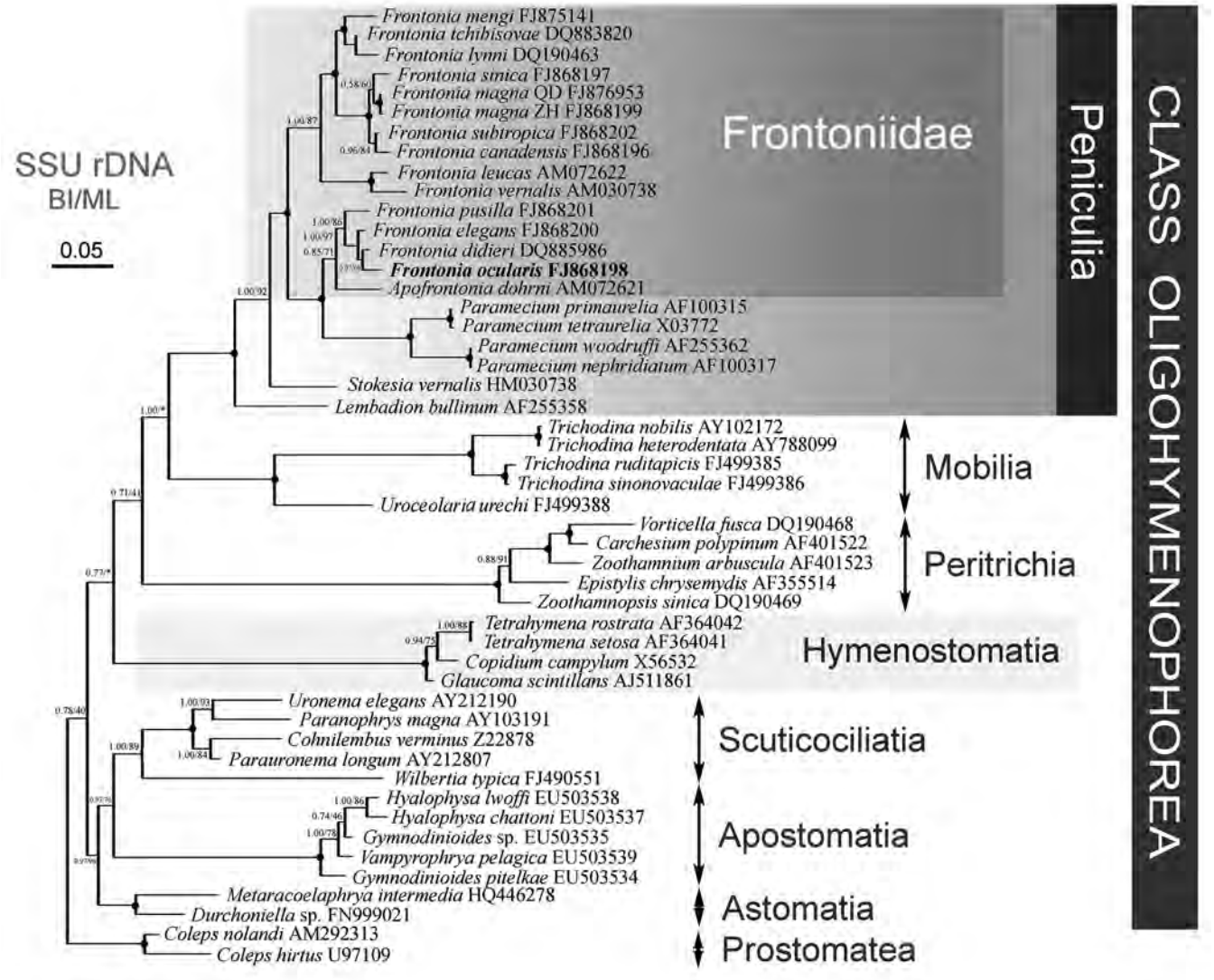

Fig. 7. BI tree inferred from small subunit rRNA gene sequences. Numbers near the branches represent BI posterior probabilities and non-parametric maximum likelihood bootstrap values. All branches are drawn to scale. The scale bar corresponds to 5 substitutions per 100 nucleotide position. The subclass Peniculia is highlighted in gray. The species newly sequenced in this work is in bold. ZH – Zhuhai population of *F. magna*, QD – Qingdao population of *F. magna.*

Fan *et al.* 2011a; Foissner *et al.* 1994; Long *et al.* 2005, 2008; Pan *et al.* 2013), i.e. *F. magna*, *F. subtropica*, *F. canadensis*, *F. leucas*, *F. vernalis*, *F. mengi*, *F. tchibisovae*, and *F. lynni*, are sister to each other. However, *F. didieri* and *F. sinica*, with one contractile vacuole and two rows in peniculus 3 (Fan *et al.* 2013, Long *et al.* 2008), do not cluster together.

Acknowledgements. This work was supported by the Natural Science Foundation of China (project numbers: 31201696, 31201708, 41006098, 31222050), specialized Research Fund for the Doctoral Program of Higher Education (project number: 20104407120006) and Research Group Project (No. RGP-VPP-083), King Saud University Deanship of Scientific Research. Many thanks are due to Prof. Weibo Song (OUC) and Dr. Alan Warren (Natural History Museum, London), who kindly read the draft and gave some helpful suggestions.

REFERENCES

Borror A. C. (1963) Morphology and ecology of the benthic ciliated protozoa of Alligator Harbor, Florida. *Arch. Protistenk.* **106:** 465–534

Bullington W. E. (1939) A study of spiralling in the ciliate *Frontonia* with a review of the genus and description of two new species. *Arch. Protistenk.* **92:** 10–66

Burkovsky I. V. (1970) The ciliates of the mesopsammon of the Kandalaksha Gulf (White Sea) I. *Acta Protozool*. **7:** 475–489

Carey P. G. (1992) Marine Interstitial Ciliates. An Illustrated Key. Chapman and Hall, London

Corliss J. O. (1979) The ciliated protozoa: characterization, classification and guide to the literature. 2nd ed. Oxford, New York, Toronto, Sydney, Paris, Frankfurt

Czapik A. (1979) *Frontonia pallida* sp. n. un nouveau cilié psammophile (Hymenostomata, Peniculina). *Acta Protozool*. **18:** 527–530

Dragesco J., Dragesco-Kernéis A. (1986) Ciliés libres de l'Afrique intertropicale. *Faune Tropicale* **26:** 1–559

Fan X., Chen X., Song W., Al-Rasheid K. A. S., Warren A. (2010) Two new marine scuticociliates, *Sathrophilus planus* n. sp. and *Pseudoplatynematum dengi* n. sp., with improved definition of *Pseudoplatynematum* (Ciliophora, Oligohymenophora). *Eur. J. Protistol.* **46:** 212–220

Fan X., Chen X., Song W., Al-Rasheid K. A. S., Warren A. (2011a) Two novel marine *Frontonia* species, *F. mengi* spec. nov. and *F. magna* spec. nov. (Protozoa, Ciliophora), with notes on their phylogeny based on small-subunit rRNA gene sequence data. *Int. J. Syst. Evol. Microbiol.* **61:** 1476–1486

Fan X., Hu X., Al-Farraj S., Clamp J. C., Song W. (2011b) Morphological description of three marine ciliates (Ciliophora, Scuticociliatia), with establishment of a new genus and two new species. *Eur. J. Protistol.* **47:** 186–196

Fan X., Lin X., Al-Rasheid K. A. S., Warren A., Song W. (2011c) The diversity of scuticociliates (Protozoa, Ciliophora): a report on eight marine forms found in coastal waters of China, with a description of one new species. *Acta Protozool.* **50:** 219–234

Fan X., Lin X., Liu W., Xu Y., Al-Farraj S., Al-Rasheid K. A. S., Warren A. (2013) Morphology and phylogeny of three new marine *Frontonia* species (Ciliophora, Peniculia) from coastal waters of China. *Eur. J. Protistol.* (in press)

Foissner W., Berger H., Kohmann F. (1994) Taxonomische und ökologische Revision der Ciliaten des Saprobiensystems – Band III: *Hymenostomata, Prostomatida, Nassulida*. *Informationsberichte des Bayerischen: Landesamtes für Wasserwirtschaft* **1/94:** 1–548

Fokin S., Andreoli I., Verni F., Petroni G. (2006) *Apofrontonia dohrni* sp. n. and the phylogenetic relationships within Peniculia (Protista, Ciliophora, Oligohymenophorea). *J. Eukaryot. Microbiol.* **35:** 289–300

Gao F., Strüder-Kypke M., Yi Z., Miao M., Al-Farraj S., Song W. (2012a) Phylogenetic analysis and taxonomic distinction of six genera of pathogenic scuticociliates (Protozoa, Ciliophora) inferred from small-subunit rRNA gene sequences. *Int. J. Syst. Evol. Microbiol.* **62:** 246–256

Gao F., Laura A., Song W. (2012b) Insights into the phylogenetic and taxonomy of *Philasterid* ciliates (Protozoa, Ciliophora, Scutiocociliatia) based on analyses of multiple molecular markers. *Mol. Phylog. Evol.* **64:** 308–317

Gao S., Chen Z., Shao C., Long H., Al-Rasheid K. A. S., Song W. (2008) Reconsideration of the phylogenetic position of *Frontonia*-related Peniculia (Ciliophora, Protozoa) inferred from the small subunit ribosomal RNA gene sequences. *Acta Protozool.* **47:** 47–54

Guindon S., Gascuel O. (2003) A simple, fast and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst. Biol.* **52:** 696–704

Hall T. A. (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids *Symp. Ser.* **41:** 95–98

Kahl A. (1931) Urtiere order Protozoa I: Wimpertiere order Ciliata (Infusoria) 2. Holotricha. *Tierwelt. Dtl.* **21:** 181–398

Liu W., Yi Z., Lin X., Warren A., Song W. (2012) Phylogeny of three choreotrich genera (Protozoa, Ciliophora, Spirotrichea), with morphological, morphogenetic and molecular investigations on three strobilidiid species. *Zool. Scr.* **41:** 417–434

Long H., Song W., Gong J., Hu X., Ma H., Zhu M., Wang M. (2005) *Frontonia lynni* n. sp., a new marine ciliate (Protozoa, Ciliophora, Hymenostomatida) from Qingdao, China. *Zootaxa* **1003:** 57–64

Long H., Song W., Al-Rasheid K. A. S., Wang Y., Yi Z., Al-Quraishy S., Lin X., Al-Farraj S. (2008) Taxonomic studies on three marine species of *Frontonia* from northern China: *F. didieri* n. sp., *F. multinucleata* n. sp. and *F. tchibisovae* Burkovsky, 1970 (Ciliophora: Peniculida). *Zootaxa* **1687:** 35–50

Ma H., Choi J., Song W. (2003) An improved silver carbonate impregnation for marine ciliated protozoa. *Acta Protozool.* **42:** 161–164

Medlin L., Elwood H. J., Stickel S., Sogin M. L. (1988) The characterization of enzymatically amplified eukaryotic 16s-like rRNA-coding regions. *Gene* **71:** 491–499

Miao M.., Shao C., Chen X., Song W. (2011) Evolution of discocephalid ciliates: Molecular, morphological and ontogenetic data support a sister group of discocephalids and pseudoamphisiellids (Protozoa, Ciliophora) with establishment of a new suborder Pseudoamphisiellina subord. n. *Sci. China (Life Sci.)* **54:** 634–641

Nylander J. A. (2004) MrModeltest version 2. Distributed by the author. Department of Systematic Zoology, Evolutionary Biology Centre, Uppsala University

Pan H., Huang J., Hu X., Fan X., Al-Rasheid K. A. S., Song W. (2010) Morphology and SSU rRNA gene sequences of three marine ciliates from Yellow Sea, China, including one new species, *Uronema heteromarinum* nov. spec. (Ciliophora, Scuticociliatida). *Acta Protozool.* **49:** 45–59

Pan X., Gao F., Liu W., Fan X., Warren A., Song W. (2013) Morphology and SSU rRNA gene sequences of three *Frontonia* species, including a description of *F. subtropica* spec. nov. (Ciliophora, Peniculida). *Eur. J. Protistol*. (in press)

Pan X., Shao C., Ma H., Fan X., Al-Rasheid K. A. S., Al-Farraj S., Hu X. (2011) Redescriptions of two marine scuticociliates from China, with notes on stomatogenesis in *Parauronema longum* (Ciliophora, Scuticociliatida). *Acta Protozool*. **50:** 301–310

Petz W., Song W., Wilbert N. (1995) Taxonomy and ecology of the ciliate fauna (Protozoa, Ciliophora) in the endopagial and pelagial of the Weddell Sea, Antarctica. *Protistologica* **40:** 1–223

Posada D., Crandall K. A. (1998) Modeltest: testing the model of DNA substitution. *Bioinformatics* **14:** 817–818

Ronquist F., Huelsenbeck J. P. (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19:** 1572–1574

Roque M. (1961) *Frontonia microstoma*, Kahl. *J. Protozool.* **8:** 334–341

Roque M., de Puytorac P. (1972) *Frontonia canadensis* sp. nov. (Cilié Hyménostome Péniculien). *Natural. Canad.* **99:** 411–416

Song W., Wilbert N. (1989) Taxonomische Untersuchungen an Aufwuchsciliaten (Protozoa, Ciliophora) im Poppelsdorfer Weiher. *Bonn. Lauterbornia* **3:** 2–221

Wilbert N. (1975) Eine verbesserte Technik der Protargolimprägnation für Ciliaten. *Mikrokosmos* **64:** 171–179

Wilbert N., Song W. (2008) A further study on littoral ciliates (Protozoa, Ciliophora) near King George Island, Antarctica, with description of a new genus and seven new species. *J. Nat. His.* **42:** 979–1012

Zhang Q., Miao M., Strüder-Kypke M. C., Al-Rasheid K. A. S., Al-Farraj S., Song W. (2011) Molecular evolution of *Cinetochilum* and *Sathrophilus* (Protozoa, Ciliophora, Oligohymenophorea), two genera of ciliates with morphological affinities to scuticociliates. *Zool. Scr.* **40:** 317–325

Received on 22nd October, 2012; revised on 5th December, 2012; accepted on 15th December, 2012