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ORIGINAL ARTICLE

Morphology and Molecular Phylogeny of Three Cyrtophorid Ciliates (Protozoa, Ciliophora) from China, Including Two New Species, *Chilodonella parauncinata* sp. n. and *Chlamydonella irregularis* sp. n.

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Chlamydodontida; ciliate; infraciliature; small subunit rRNA gene; taxonomy.

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ABSTRACT

This study investigated the morphology and molecular characteristics of three interesting free-living cyrtophorid ciliates, including two new species, isolated from China: *Chilodonella parauncinata* sp. n. can be identified by its elongated body shape, with a sharp protrusion in the left anterior part, cell size ca. 60 × 25 μm in vivo, five right and 6–7 left kineties with kinetosomes densely arranged, and a curved cyrtos. *Chlamydonella irregularis* sp. n. differs from its congeners by the oval body shape, cell size 50–60 × 25–40 μm in vivo, irregular shape of macronucleus, 30–40 club-shaped ventral protuberances, and 17 somatic kineties. Two isolates of *Chlamydonella derouxi* Song, 2003, collected from an intertidal area in Shandong and a mangrove wetland in Guangdong respectively, correspond well with two previous descriptions, but differ in comprising more basal bodies in left and right equatorial fragments and in having more finger-like protuberances on the ventral side. Phylogenetic analyses based on the small subunit rRNA gene sequences showed that *C. parauncinata* sp. n. clustered with *Chilodonella uncinata*, but was a well-outlined species of the genus, and *C. irregularis* sp. n. and *C. derouxi* grouped in the family Lynchellidae with their congeners to form the monophyletic genus *Chlamydonella*.

CYRTOPHORID ciliates are a highly specialised and divergent group of ciliates, which include more than 150 nominal morphotypes so far, with most of these being marine free-living species (Chen et al. 2011; Deroux 1976a,b,c; Dragesco 1966; Fauré-Fremiet 1965; Foissner 1979a; Gao et al. 2012; Gong and Song 2006a,b, 2009; Gong et al. 2005, 2007, 2008; Kahl 1931; Pan et al. 2011, 2012, 2013a,b). Because there are few reliable morphological features in vivo which can be used to distinguish different species, accurate identification of cyrtophorids must rely on infraciliature information. In previous studies, however, many species in this group have only been described based on live observation, and this has resulted both in inefficiency in identifying species and, inevitably, many problems with synonyms (Gong and Song 2009). Descriptions based on infraciliature are therefore needed to clarify species circumference.

During the past decade, there has been extensive investigation of cyrtophorid ciliates in China, with more than 40 species reported. In particular, the most recent studies tend to suggest that the species richness of this group in China is even higher than previously thought, with various new taxa being successively reported (Chen et al. 2012; Gong and Song 2004; Pan et al. 2012, 2013a,b; Song et al. 2009).

This paper presents morphological descriptions of two new species, *Chilodonella parauncinata* sp. n. and *Chlamydonella irregularis* sp. n., and two populations of *Chlamydonella derouxi* Song 2003, particularly with

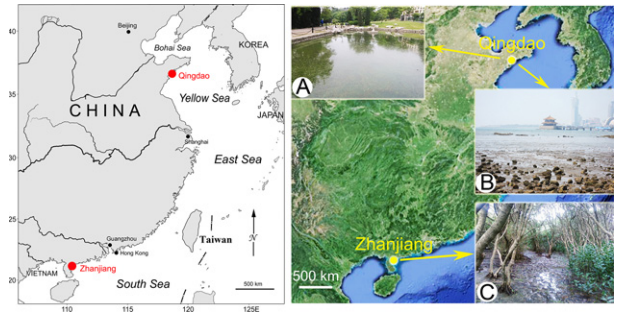

Figure 1 Sample stations. **A.** A freshwater lake in Baihuayuan park in Qingdao, Shandong province. **B.** An intertidal area near Zhanqiao in Qingdao, Shandong province. **C.** A mangrove wetland in Donghai island in Zhanjiang, Guangdong province.

respect to their morphological features and variations among different populations. In addition, the phylogenetic positions of these three species are inferred based on small subunit (SSU) rRNA gene trees.

MATERIALS AND METHODS

Sample collection and identification

Chilodonella parauncinata sp. n. was isolated from a freshwater lake in Baihuayuan Park in Qingdao (36°08′N; 120°43′E, Fig. 1A), China, on May 23, 2013, with the water temperature ca. 19.7 °C and pH ca. 7.2. *Chlamydonella irregularis* sp. n. was collected on December 2, 2008 from a sea cucumber farming pond in Qingdao, with the water temperature ca. 17 °C and the salinity ca. 30. The Qingdao population of *C. derouxi* was collected from intertidal area near Zhanqiao (Fig. 1B), on November 12, 2008, with the water temperature ca. 16 °C, pH ca. 7.9 and salinity ca. 30; while the Zhanjiang population was isolated from a mangrove wetland in Huguang (21°16′N, 110°21′E, Fig. 1C) on November 14, 2013, with the water temperature ca. 23.9 °C, pH ca. 7.1 and salinity ca. 15.

Ciliates were examined with bright field and differential interference contrast microscopy. The protargol silver

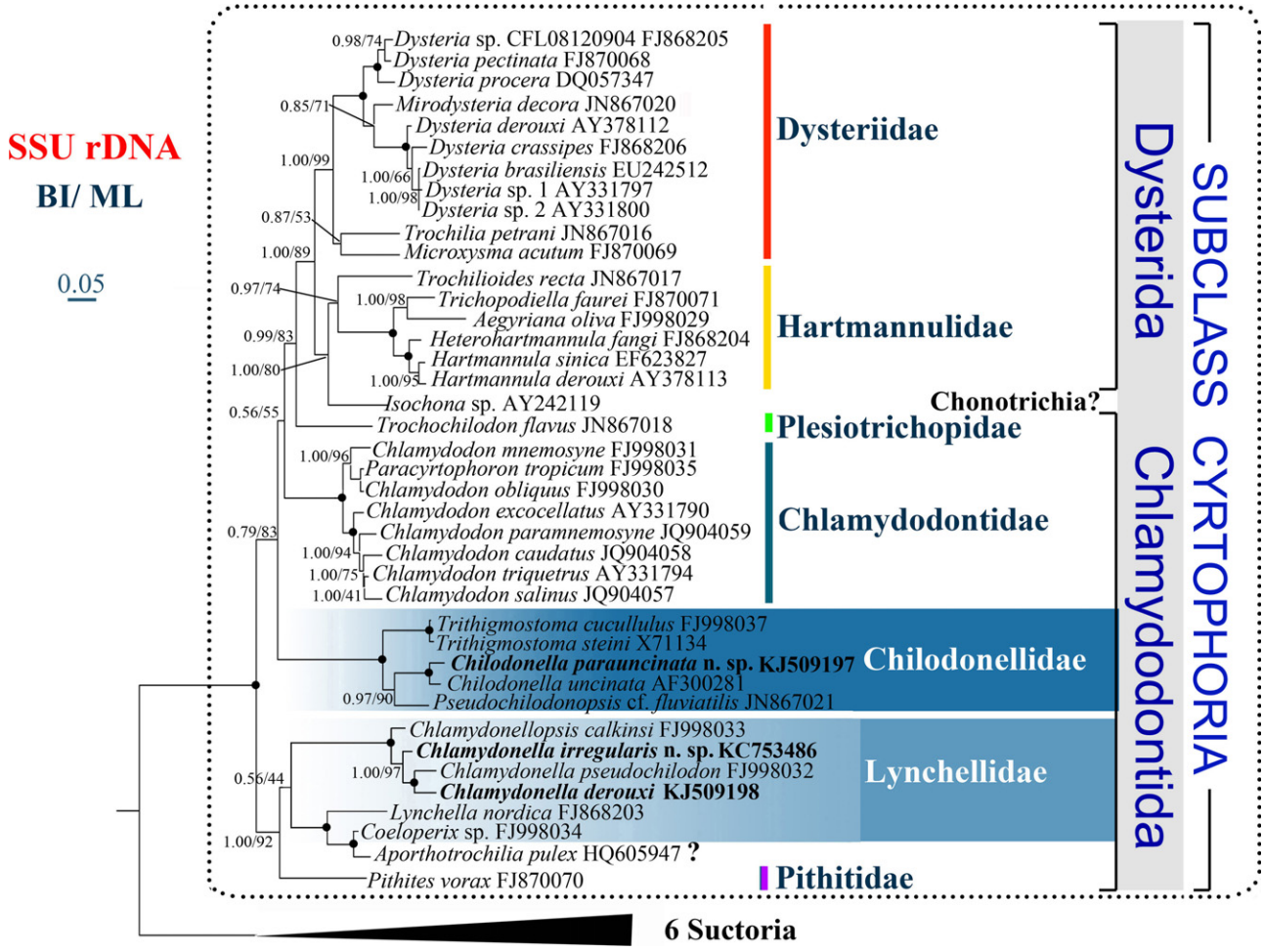

Figure 2 Phylogenetic trees (BI/ML) of small subunit ribosomal RNA genes. Sequences of the species in this study are marked in bold. Numbers at nodes represent the Bayesian posterior probability value and the bootstrap values of maximum likelihood. Solid circles represent full bootstrap support from both algorithms.

staining method was used to reveal the infraciliature (Wilbert 1975).

Measurements and counts of stained specimens were performed at a magnification of 1,250X. Terminology and classification are according to Gong and Song (2006b) and Lynn (2008) respectively.

Phylogenetic analyses

A few cells of *C. parauncinata* sp. n., *C. irregularis* sp. n., and the Guangdong population of *C. derouxi* Song 2003 were used for DNA extraction. DNA extraction, PCR amplification, cloning, and sequencing of small subunit ribosomal RNA (SSU rRNA) gene were carried out according to Zhang et al. (2012). The universal eukaryotic primers used for SSU rRNA gene amplification were Euk A and Euk B (Medlin et al. 1988).

Three new SSU rRNA gene sequences of *C. parauncinata* sp. n., *C. irregularis* sp. n., and *C. derouxi* Song 2003 were deposited in the GenBank database with the accession numbers of KJ509197, KC753486, and KJ509198 respectively. Other sequences used for phylogenetic analyses in present study were obtained from the GenBank database (accession numbers see Fig. 2).

The SSU rRNA gene sequences were aligned and manually modified using CLUSTAL W, as implemented in BioEdit v.7.0.5 (Hall 1999). A Bayesian inference (BI) analysis was performed with MrBayes 3.2.2 (Ronquist and Huelsenbeck 2003) using the GTR + I + G model which was selected as the best model by MrModeltest v.2.0 (Nylander 2004), via the CIPRES Science Gateway website (CIPRES Portals: http://www.phylo.org/sub_sections/portal). The maximum likelihood (ML) analysis was conducted using RAxML-HPC2 on XSEDE (8.0.0) (Stamatakis 2006; Stamatakis et al. 2008) via the CIPRES Science Gateway. The reliability of internal branches was estimated by bootstrapping with 1,000 replicates.

RESULTS

Morphological description of *C. parauncinata* sp. n. (Table 1 and Fig. 3, 7A)

Cell size 35–85 × 25–35 μm in vivo, usually ca. 60 × 25 μm. Body elongate ellipsoid with the anterior part

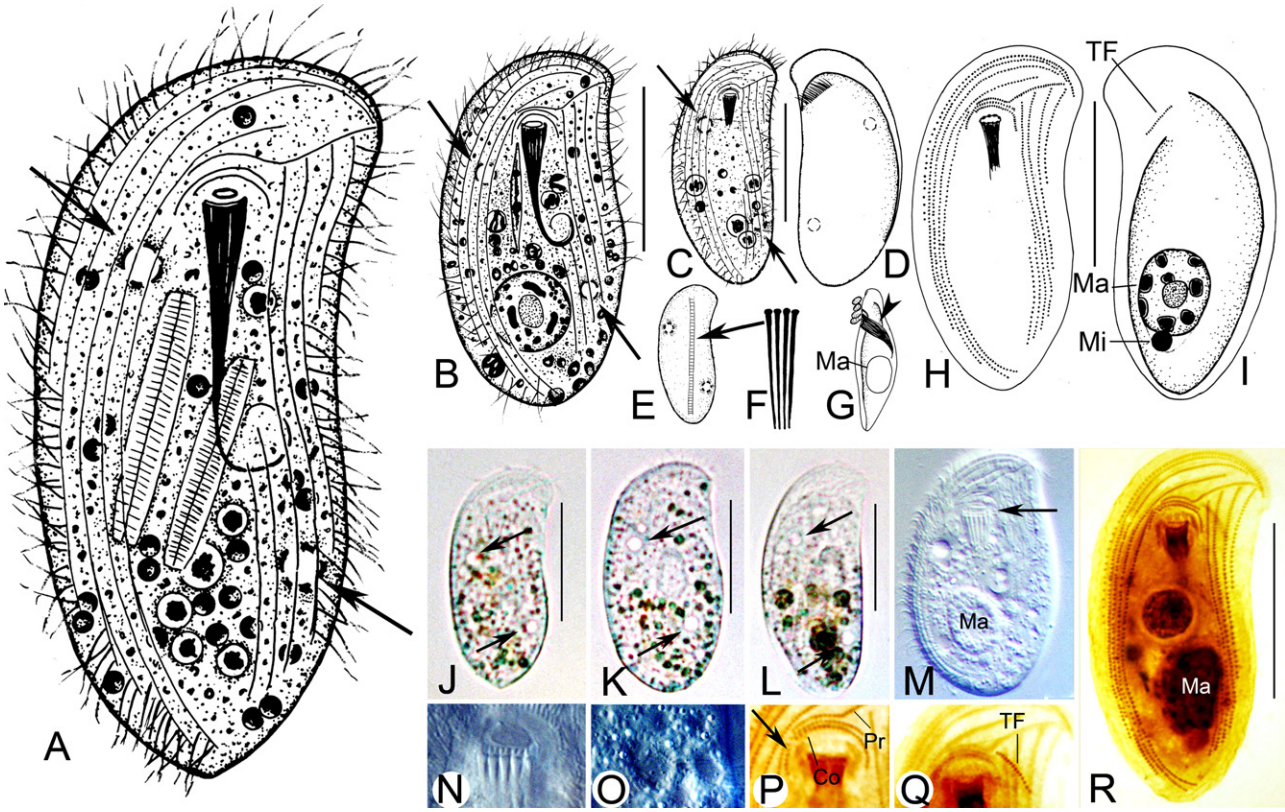

Figure 3 *Chilodonella parauncinata* sp. n. from life (**A**–**G**, **J**–**O**) and after protargol impregnation (**H**, **I**, **P**–**R**). **A.** Ventral view of a typical individual, arrows mark the contractile vacuoles. **B, C.** Different body shapes, arrows point to the contractile vacuoles. **D.** Dorsal view of the cell. **E.** Individual with filamentous cyanobacteria, arrow marks the filamentous cyanobacteria. **F.** Nematodesmal rods. **G.** Lateral view, arrowhead shows the cyrtos. **H, I.** Ventral and dorsal views to show infraciliature. **J.** View of a typical individual, arrows mark the contractile vacuoles. **K, L.** Different body shapes, arrows point to the contractile vacuoles. **M, N.** Cytostome. **O.** Terminal fragment. **P.** Oral infraciliature, arrow points to the short fragment from the outer circumoral kinety. **Q.** Terminal fragment. **R.** Ventral view. Co = circumoral kineties; Ma = macronucleus; Pr = preoral kinety; TF = terminal fragment. Bars: 30 μm.

Table 1. Morphometric characterisations of *Chilodonella parauncinata* sp. n. from protargol-impregnated specimens. Measurement in μm

Characters	Min	Max	Mean	SD	CV	*n*
Body length	36	66	54.9	7.77	14.1	25
Body width	17	28	23.6	2.93	12.4	25
Number of somatic kineties	11	12	11.0	0.20	1.8	25
Number of right kineties	5	5	5.0	0.00	0.0	25
Number of left kineties	6	7	6.0	0.20	3.3	25
Number of frontoventral kineties	4	4	4.0	0.00	0.0	25
Number of nematodesmal rods	13	14	13.2	0.43	3.3	13
Number of basal bodies in terminal fragment	10	16	12.0	1.64	13.7	21
Number of basal bodies in equatorial fragment	3	22	13.0	4.92	37.8	12
Length of macronucleus	10	32	17.2	4.22	24.6	25
Width of macronucleus	8	16	10.4	1.68	16.2	25

Min = minimum; Max = maximum; Mean = arithmetic mean; SD = standard deviation; CV = coefficient of variation in %; *n* = number of individuals examined.

protruding to left and posterior end generally narrow and rounded (sometimes slightly tapering, Fig. 3A–D, J–L). Well-fed individuals somewhat oval (Fig. 3B, K), while starved cells much more slender (Fig. 3C, E, L). Ventral side flat and dorsal side vaulted, with width:thickness ratio ca. 2:1 (Fig. 3G). Cytostome transverse ellipse-shaped, 6 μm in width, located in the anterior fourth of the cell (Fig. 3M, N). Cyrtos curved in a circle posteriorly, and composed of 13–14 toothed nematodesmal rods which about 20-μm long (Fig. 3A, B, G, M, N). Two contractile vacuoles, 5 μm in diam., diagonally located at anterior and posterior third of body underneath ventral cortex, pulsating at an interval of 5–8 s (Fig. 3A–E, J–M). Macronucleus centrally heteromerous, 25 × 15 μm in vivo, ellipsoid, and located in the centre to posterior fourth of body (Fig. 3A, B, I, M, R). Cortex soft and barely contractile. Dot-like cortex granules colourless, about 1 μm across and sparsely distributed (Fig. 4O). Cytoplasm colourless and contains many undigested food vacuoles, including yellow or green algae (3–8 μm across), diatoms (25 μm long), or filamentous cyanobacteria (as long as body length), and 1–3-μm-sized lipid droplets, which render cell yellowish or greenish at low magnification (40X, Fig. 3A–C, J–M). Movement by slowly gliding on substrate or on interface between air and water.

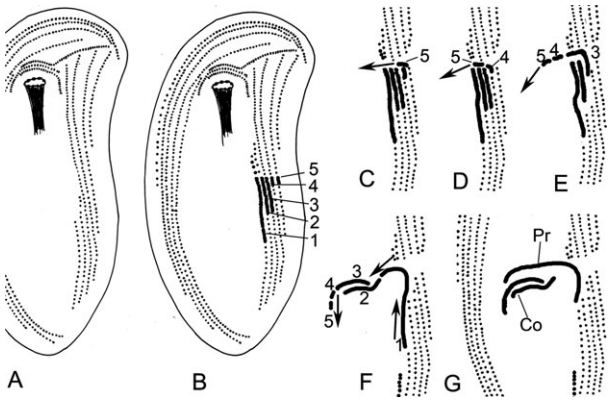

Figure 4 Stomatogenesis of *Chilodonella parauncinata* sp. n. **A.** Individual before stomatogenesis. **B.** Anlagen of stomatogenesis: kinetofragments 1–5. **C–E.** Kinetofragment 5 migrates anticlockwise, kinetofragments 4, 3 migrate afterwards. **F.** Kinetofragments 2, 1 migrate to the central part of the cell after kinetofragments 5, 4, 3. **G.** The final state of stomatogenesis. Co = circumoral kineties; Pr = preoral kinety.

Generally, 11 (rarely 12, only one of 25 specimens observed) somatic kineties, of which five and six (rarely seven, only one of 25 specimens observed) rows positioned on right and left respectively (Fig. 3H, R). Two innermost right kineties extending to posterior end of body, while remaining three successively shortened from left to right. The four rightmost kineties anteriorly extending beyond level of cytostome and bending to left, forming a suture with left kineties. Inner two left kineties shortened anteriorly (below the mid-body) and extending to subterminal portion of body. Remaining four (rarely five, only one of 25 specimens observed) left kineties starting at same level anteriorly, with leftmost one terminating at anterior third. Basal bodies densely spaced (ca. 66 in the rightmost kinety; Fig. 3H). Equatorial fragment hard to detect. Terminal fragment containing 10–16 basal bodies, positioned at anterior 1/5 on dorsal side (Fig. 3I, O, Q).

Two circumoral kineties parallel to each other, with outer one slightly longer than inner one (Fig. 3H, P, R). Occasionally, a fragment separated from main part of outer circumoral kinety (Fig. 3P). Preoral kinety encircling circumoral kineties at about halfway point and extending to anterior-left of cell (Fig. 3H, P, R).

Morphogenesis of *C. parauncinata* sp. n. (Fig. 4)

Several individuals at divisional stages were observed. As to its congeners, the stomatogenetic field was situated in the left part of the somatic ciliature. The basal bodies of the five inner left kineties became densely spaced, and were cut into two parts each at the middle of the cell. The posterior five fragments which were numbered from right to left, segmented and developed one kinetofragment each. Kinetofragment 1 was the anlage of the preoral kinety, kinetofragment 2 was the anlage of the inner circumoral kinety, and kinetofragments 3–5 together were the anlagen of the outer circumoral kinety. The

kinetofragments migrated to the oral area of opisthe by an anticlockwise circular movement which started from kinetofragments 5 to 1 (Fig. 4C–F). Finally, kinetofragments 3–5 together formed the new outer circumoral kinety, kinetofragment 2 formed the inner circumoral kinety, and kinetofragment 1 became the preoral kinety (Fig. 4G). The stages of the morphogenesis of the innermost left kinety of opisthe were not detected.

Morphological description of *C. irregularis* sp. n. (Table 2 and Fig. 5)

Cell size 50–60 × 25–40 μm in vivo. Body roughly oval in outline, with both ends evenly rounded (Fig. 5A, E, F). Cell dorsoventrally flattened, with ratio of width to thickness ca. 5: 1. Ventral side flat, while dorsal side slightly vaulted. Cytostome conspicuous in vivo, located in anterior fourth, and surrounded by 14–19 nematodesmal rods (Fig. 5A, E). A total of 30–40 club-shaped protuberances, each ca. 4 μm in length, densely distributed on ventral side (Fig. 5A, E). Two contractile vacuoles, about 5 μm across, diagonally located in anterior and posterior third respectively (Fig. 5A, F, G). A single macronucleus about 14 × 10 μm in vivo, irregularly shaped, located in body centre (Fig. 5A, C, G, H). Colourless cortical granules (< 1 μm) sparsely distributed on dorsal side. Movement by slowly gliding on substrate.

Seventeen somatic kineties on ventral side. Rightmost four kineties surpassing cytostome and bending to left, with inner two or three cut into two fragments by oral kineties (Fig. 5B, H). Left equatorial fragment composed of 2–9 basal bodies, and right equatorial fragment consisting of 1–6 basal bodies (Fig. 5B, K, L). Terminal fragment composed of about nine basal bodies (Fig. 5D, G, J).

Oral kineties Y-shaped, with usually three pairs of basal bodies separated from main part on right side (Fig. 5B, H).

Table 2. Morphometric characterisations *Chlamydonella irregularis* sp. n. (upper line), Zhanjiang population of *C. derouxi* (middle line), and Qingdao population of *C. derouxi* (lower line) from protargol-impregnated specimens

Characters	Min	Max	Mean	SD	CV	*n*
Body length (μm)	39	62	50.8	6.95	13.7	21
	16	28	22.0	3.15	14.3	25
	27	46	33.5	4.53	13.5	25
Body width (μm)	26	50	37.5	6.79	18.1	21
	13	20	14.7	2.25	15.3	25
	16	36	23.1	3.99	17.3	25
Number of somatic kineties	17	19	17.9	0.62	3.5	21
	12	12	12	0.00	0.0	25
	12	15	12.5	0.87	69.7	25
Number of frontoventral kineties	4	4	4.0	0.00	0.0	21
	4	4	4	0.00	0.0	25
	4	4	4.0	0.00	0.0	25
Number of nematodesmal rods	14	19	16.6	1.39	8.4	20
	13	15	13.6	0.62	4.6	18
	12	14	12.4	0.71	5.7	25
Number of basal bodies in TF	9	10	9.4	0.51	5.4	14
	4	6	4.8	0.47	9.8	25
	4	9	5.9	1.54	26.2	24
Number of basal bodies in REF	1	6	2.6	1.23	47.3	20
	1	5	2.0	1.28	65.7	21
	1	8	4.2	2.46	58.9	17
Number of basal bodies in LEF	2	9	4.4	2.14	48.4	19
	2	9	4.9	2.40	48.7	25
	2	7	4.6	1.53	33.4	24
Length of macronucleus (μm)	10	22	15.5	3.82	24.6	20
	5	10	7.4	1.58	21.4	25
	7	15	10.4	1.91	18.3	24
Width of macronucleus (μm)	7	15	10.9	2.45	22.5	20
	4	8	4.5	1.05	23.3	25
	4	9	7.2	1.24	17.3	24

Min = minimum; Max = maximum; Mean = arithmetic mean; SD = standard deviation; CV = coefficient of variation in %; *n* = number of individuals examined; TF = terminal fragment; REF = right equatorial fragment; LEF = left equatorial fragment.

Morphological description of Qingdao and Zhanjiang populations of *C. derouxi* Song 2003 (Tables 2, 3 and Fig. 6)

Cell size 16–45×15–30 μm in vivo. Body oval in outline, with anterior end slightly protruding to left (Fig. 6A, B, F–I, M, N). Cytoplasm colourless to greyish at low magnifications (40X). Cytostome located in anterior fourth, elliptical in shape with a width of 4–6 μm (Fig. 6A, B, F, I, N). Cyrtos composed of 12–14 nematodesmal rods measuring about 12 μm in length (Fig. 6A, B). Seven to 14 club-shaped protuberances, about 3–4-μm long, distributed in 3–4 longitudinal rows on ventral side (Fig. 6A, B, F, H, I, T). Two contractile vacuoles, each about 3 μm in diam., diagonally located in anterior and posterior third, contracting at an interval of about 5–8 s (Fig. 6A, B, G, N). Single macronucleus juxtaposed heteromerously, about 10 × 6 μm in vivo. Colourless cortical granules (about 0.5 μm across) irregularly distributed on dorsal side (Fig. 6K, L). Cilia about 7 μm in length. Movement by gliding on substrate or on interface between air and water.

A total of 12–15 somatic kineties, four of which were frontoventral kineties (Fig. 6C, P, Q). The left and right equatorial fragments composed of 2–9 and 1–8 basal bodies respectively (Fig. 6C, P, Q). The terminal fragment apically positioned on dorsal side, consisting of 4–9 basal bodies (Fig. 6E, R). One to two micronucleus (Fig. 6C).

Oral kineties Y-shaped, with a fragment of 3–6 pairs of basal bodies usually separated from main part on right (Fig. 6C, P, Q, S).

SSU rRNA gene sequences and phylogenetic analyses (Table 4 and Fig. 2)

The length, G + C content, and accession numbers of the SSU rRNA gene sequences in this study are as follows: *C. parauncinata* sp. n., 1,709 bp, 44.70 mol.%, KJ509197; *C. irregularis* sp. n., 1,650 bp, 45.58 mol.%, KC753486; and *C. derouxi* Song 2003, 1,677 bp, 44.36 mol.%, KJ509198.

Pairwise comparison of SSU rRNA gene sequences reveals 55 nucleotide differences between *C. parauncinata*

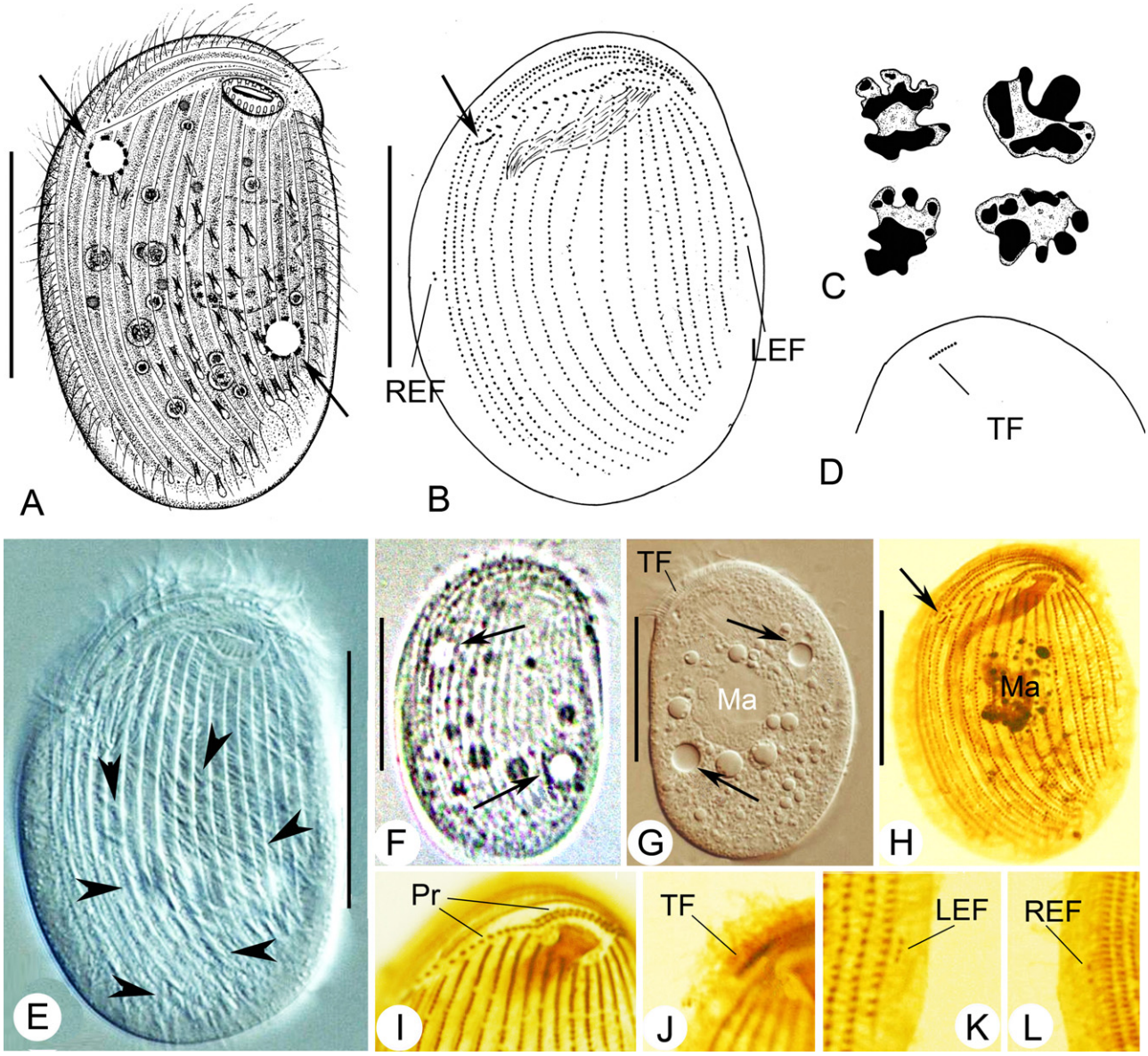

Figure 5 *Chlamydonella irregularis* sp. n. from life (**A**, **E**–**G**) and after protargol impregnation (**B**–**D**, **H**–**L**). **A, E, F.** Ventral views, arrowheads show the protuberances on the ventral side, arrows mark the contractile vacuoles. **B, H.** Ventral views, arrows mark the fragment separated from preoral kinety on the right. **C.** Variable shapes of macronucleus after protargol impregnation. **D, J.** Dorsal view, showing the terminal fragment. **I.** Shows the oral kineties. **K, L.** Shows the left and right equatorial fragments. LEF = left equatorial fragment; Ma = macronucleus; Pr = preoral kinety; REF = right equatorial fragment; TF = terminal fragment. Bars: 30 μm.

sp. n. and *C. uncinata*, with a sequence identity of 96.5%. The SSU rRNA gene sequences of *C. derouxi*, *C. irregularis* sp. n., and *C. pseudochilodon* differ from each other in 79–88 nucleotides with sequence identities ranging from 94.4% to 95.1% (see Table 4).

Maximum likelihood and BI trees have similar topologies, and were therefore combined into a single tree (Fig. 2). The monophyly of the Subclass Cyrtophorida is fully supported. As shown in Fig. 2, *C. parauncinata* sp. n. is sister to *C. uncinata* with full support, and is positioned inside the monophyletic family Chilodonellidae. *Chlamydonella* is a monophyletic genus: *C. derouxi* clusters with the *Chlamydonella pseudochilodon* with full support values, forming a parallel clade to *C. irregularis* sp. n. with high support values (BI/ML, 1.00/97).

DISCUSSION

Comparison of *C. parauncinata* sp. n. with congeners (Table 5 and Fig. 7, 8A–H)

Kahl (1931) recorded more than 30 nominal *Chilodonella* species in his epic work. After that, only four new species have been suggested (Jankowski 2007; Kidder and Sum-

Table 3. List of different populations of *Chlamydonella derouxi* Song 2003

Characters	Pop. 1	Pop. 2	Pop. 3	Pop. 4
Length in vivo in μm	20–30	20–30	16–35	25–45
Number of somatic kineties	12	12–13	12	12–15
Number of ventral protuberances	1	3	10–12	7–11
Number of nematodesmal rods	13–16	11–13	13–15	12–14
Number of basal bodies in terminal fragment	5–7	4–6	4–6	4–9
Number of basal bodies in right equatorial fragment	1	1–4	1–5	1–8
Number of basal bodies in left equatorial fragment	1	1–3	2–9	2–7

Pop. 1 = population in Song (2003); Pop. 2 = population in Gong and Song (2006b); Pop. 3 = population of Zhanjiang (this work); Pop. 4 = population of Qingdao (this work).

mers 1935; Lepsi 1947; Marcus 1943; Tucolesco 1962). With more extensive in vivo observations and the application of the Chatton–Lowff and other silver staining methods, many nominal species were recognised as synonyms (Gong et al. 2005; Jankowski 2007; Kahl 1931; Pan et al. 2013a), and some were transferred to other genera, e.g. *Odontochlamys* Certes, 1891, *Trithigmostoma* Jankowski 1967, *Thigmogaster* Deroux, 1976, and *Pseudochilodonopsis* Foissner, 1979 (Blatterer and Foissner 1990; Foissner et al. 1991; Jankowski 1967). Currently, this genus contains 13 valid species (Foissner et al. 1991; Jankowski 2007; Kahl 1931), with infraciliature data available for only four of these, i.e. *Chilodonella acuta* Kahl 1931, *Chilodonella hexasticha* Kiernik, 1909, *Chilodonella piscicola* Zacharias, 1894, and *C. uncinata* Ehrenberg, 1838.

Chilodonella parauncinata sp. n. is very similar to *C. uncinata* (Fig. 7B–F, 8A, B), especially in the number of somatic kineties and the stomatogenesis, but differs from the latter by: (i) larger body size (ca. 60 μm after protargol impregnation vs. 28–50 μm) and (ii) densely spaced basal bodies in the kineties (ca. 66 in the rightmost kineties vs. 30–42); (iii) the posterior position of the two inner left kineties (subterminal vs. posterior 1/5–1/4); (iv) the length of the outmost left kinety (extending to 1/3 to 1/2 of the body length vs. 1/6–1/5; Foissner 1979a, 1981, 1988; Song 1997; Song and Wilbert 1989).

Chilodonella hexasticha (Fig. 8D) and *C. piscicola* (Fig. 8C, known as *Chilodonella cyprini*, which is a synonym of *C. piscicola*; Urawa and Yamao 1992) are parasitic species (vs. free-living), and possess many more kineties (14–17 and 15–29 kineties respectively vs. 11–12), thus can be distinguished from our isolate. In addition, although *C. piscicola* has similar stomatogenesis with our species, the morphological differences make the latter a distinct species (Hofmann 1987; Pádua et al. 2013; Rydlo and Foissner 1986).

Chilodonella acuta (Fig. 8G, H) has seven left and five right kineties, which resembles our isolate, but it can be clearly distinguished from the later by a tail-like spine at posterior end (vs. absent; Fan et al. 2014; Kahl 1931).

Considering the body shape and cell size, *Chilodonella aplanata* Kahl 1931 (Fig. 8E) and *C. capucina* (Penard 1922) Kahl 1931 (Fig. 8F) should be compared with our isolate, but the two species differ from the latter by broad rounded posterior end (vs. slightly rounded or tampering sometimes) and fewer kineties (7 and 10 respectively from living observation vs. 11; Kahl 1931). In addition, our species differs from *C. capucina* in habitat (free-living vs. parasitic; Kahl 1931; Penard 1922).

Based on the differences above, we suggest a new *Chilodonella* species here.

Comparison of *C. irregularis* sp. n. with congeners (Fig. 5, 8I, J)

Deroux (1970) established the genus *Chlamydonella*, but did not fix the type species. Petz et al. (1995) then reported an Antarctic population of *C. pseudochilodon* and fixed it as the type. Up to now, there are six valid species in the genus (Deroux 1970, 1976b; Foissner 1979b; Gong and Song 2006b; Petz et al. 1995; Song 2003).

Considering the body size, contractile vacuoles and infraciliature, the new species closely resembles *C. pseudochilodon* Deroux 1970 (Fig. 8I, J), but there are some differences: (i) the body shape (broadly wide vs. slender); (ii) the shape of the macronucleus (irregular-shaped vs. oval or ellipsoidal to elongate) and (iii) the distribution pattern of ventral protuberances (densely distributed on the ventral side vs. distributed along the perimeter of the ventral surface in *C. pseudochilodon*; Deroux 1970; Gong and Song 2006b; Petz et al. 1995). Thus, our isolate can be separated from *C. pseudochilodon*.

Chlamydonella derouxi Song 2003 has a smaller size (20–30 μm vs. 50–60 μm) and fewer kineties (12–13 vs. 17), which can be easily distinguished from the species described here (Song 2003).

Chlamydonella galeata Deroux 1970 is a relatively larger species (65–80 μm vs. 50–60 μm) with a considerably higher number of somatic kineties (ca. 25 vs. 17) and many micronuclei (vs. one). It can also, therefore, be clearly separated from the species introduced here (Deroux 1970).

The new species clearly differs from *Chlamydonella rostrata* Vuxanovici, 1963, *C. minuta* Pätsch 1974, and *Chlamydonella alpestris* Foissner, 1979 in the combination of larger size, higher number of kineties, the presence of the ventral protuberances (vs. absence), and the habitat (marine vs. freshwater; Foissner 1979b; Pätsch 1974; Song and Wilbert 1989).

On the basis of the differences above, we suggest that this isolate represents a new *Chlamydonella* species.

Identification of Qingdao and Zhanjiang populations of *C. derouxi* Song 2003 (Table 3 and Fig. 5)

The two isolates correspond well with the original description by Song (2003; Fig. 6J, O) and redescription by Gong

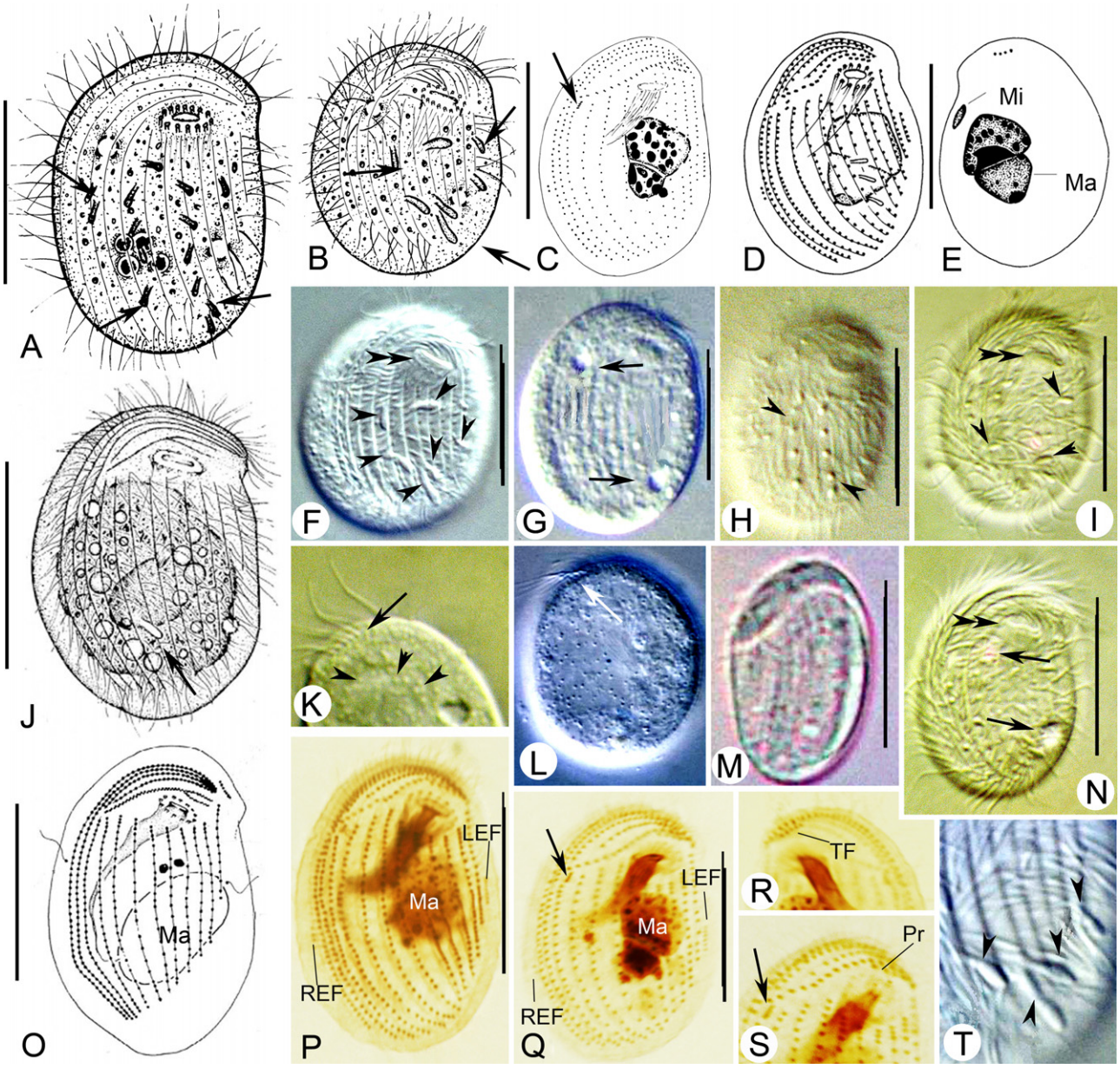

Figure 6 *Chlamydonella derouxi* Song 2003 from life (**A**, **B**, **F**–**N**, **T**) and after protargol impregnation (**C**–**E**, **O**–**S**). **A, B.** Ventral views, A. Guangdong population. B. Qingdao population, arrows show the protuberances on ventral side. **C, D, E, O–S.** Ventral views, arrows point to the fragment separated from the main part of the preoral kinety, arrowhead marks micronucleus. **F–I, M, N.** Ventral views, double arrowheads show the cytostome, arrowheads mark the protuberances, arrows point to the contractile vacuoles. **J.** Ventral view from Song (2003). **K, L.** Dorsal view of Guangdong population, arrow marks the terminal fragment, arrowheads show the cortical granules. **T.** Shows the protuberances. LEF = left equatorial fragment; Ma = macronucleus; Mi = micronucleus. Pr = preoral kinety; REF = right equatorial fragment; TF = terminal fragment. Bars: 10 μm. D, E from Gong and Song (2006b); F, G from Song (2003).

and Song (2006b; Fig. 6D, E) in both living morphology (body size/shape, number of nematodesmal rods, and position of contractile vacuoles) and the infraciliature. So, we are confident that the identification of the two isolates is correct. Notwithstanding, (i) the number of basal bodies in the equatorial fragments and (ii) the number of ventral protuberances are different (see Table 3). As the variation in the number of basal bodies in the equatorial fragments is common among different populations or even in the same population (see Tables 2, 3), it is not a reliable criteria for species identification. The consistency of the living morphology and the infraciliature between our isolates and previous populations suggests that slight variation in the number of ventral protuberances among the three

Table 4. The numbers of unmatched nucleotides (upper right) and sequences similarities (lower left) of *Chlamydonella irregularis* sp. n. (KC753486), *C. derouxi* (KJ509198), and *C. pseudochilodon* (FJ998032)

Species	1	2	3
1. *C. irregularis*	–	79 bp	88 bp
2 *C. derouxi*	95.0%	–	79 bp
3. *C. pseudochilodon*	94.4%	95.1%	–

C. derouxi populations is intraspecific variation. In addition, the original population of Song (2003) has two micronuclei and the population of Gong and Song (2006b) has one micronucleus, while both the two isolates in present work have one to two micronuclei, which is consistent with the previous studies.

Phylogenetic positions of *C. parauncinata* sp. n., *C. irregularis* sp. n., and *C. derouxi* Song 2003

Our results support the monophyly of the Subclass Cyrtophorida and are generally consistent with the topologies proposed by previous researchers (Fan et al. 2014; Gao et al. 2012; Pan et al. 2012, 2013a; Zhang et al. 2014). Furthermore, *C. parauncinata* sp. n. groups with its congener *C. uncinata* with full support values, which, along with their morphological and sequence comparisons, indicates that *C. parauncinata* is a well-outlined species of the genus. Three *Chlamydonella* species, *C. irregularis* sp. n. clusters with *C. derouxi* and *C. pseudochilodon*, group together with high support values to form the monophyletic genus, which also, together with the morphological and sequence differences, confirms the validity of the establishment of the new *Chlamydonella* species.

TAXONOMIC SUMMARY

Order Chlamydodontida Deroux, 1976
Family Chilodonellidae Deroux 1970
Genus *Chilodonella* Strand, 1928

Chilodonella parauncinata sp. n

Diagnosis. Freshwater *Chilodonella* species, cell size 35–85 × 25–35 μm in vivo; elongate ellipsoid shaped, anterior part protrusion to left; five right and 6–7 left kineties with two right innermost kineties extending to posterior portion of body; basal bodies of somatic kineties densely distributed; cyrtos curved posteriorly; two contractile vacuoles diagonally located.
Type locality. A freshwater lake in Baihuayuan Park in Qingdao (36°08′N; 120°43′E), China.
Deposition of type materials. A protargol slide with the holotype specimen (marked with a black circle) and two slides with paratype specimens are deposited in the Laboratory of Protozoology, Ocean University of China, with registration numbers of QZS2013052301-1, QZS20130 52301-2 and QZS2013052301-3 respectively.
Etymology. The species-group name *parauncinata* is a composite of the Greek adjective *para-* (beside) and the species-group name *uncinata*, meaning a ciliate similar to *Chilodonella uncinata*.

Family Lynchellidae Jankowski, 1968
Genus *Chlamydonella* Petz, Song and Wilbert, 1995

Chlamydonella irregularis sp. n

Diagnosis. Marine *Chlamydonella* with oval body shape, 50–60 × 25–40 μm in vivo; 30–40 club-shaped protuberances densely distributed on ventral side; two contractile vacuoles diagonally positioned; in total 17 ventral kineties; terminal fragment consisting of ca. 9 basal bodies; one irregularly shaped macronucleus, and one micronuclei; 14–19 nematodesmal rods.
Type locality. A sea cucumber farming pond in Qingdao (36°08′N; 120°43′E), China.
Deposition of type materials. A protargol slide with the holotype specimen (marked with a black circle) and another slide with paratype specimens are deposited in the Laboratory of Protozoology, Ocean University of China, with registration numbers of PHB2008120201-1 and PHB200 8120201-2 respectively.

Table 5. Comparison of morphometric characterisations of *Chilodonella parauncinata* sp. n. and *C. uncinata*

Characters	*C. uncinata*	*C. uncinata*	*C. uncinata*	*C. uncinata*	*C. uncinata*	*C. parauncinata* sp. n
Body size in vivo (μm)	28–36	ca. 30	30–50	30–50	40–50	ca. 60
Number of basal bodies in the rightmost kinety	ca. 37	ca. 34	29–32	ca. 42	ca. 30	ca. 66
Number of basal bodies in the innermost right kinety	ca. 45	ca. 39	45–49	ca. 41	ca. 37	75–88
Number of basal bodies in the leftmost kinety	ca. 8	ca. 6	3–4	ca. 7	ca. 7	ca. 25
Number of basal bodies in the innermost left kinety	ca. 6	ca. 9	ca. 17	ca. 14	ca. 14	ca. 22
Source	Foissner (1981)	Song (1997)	Song et al. (2009)	Pan, H., unpubl. work	Katz, L. A., unpubl. work	Original

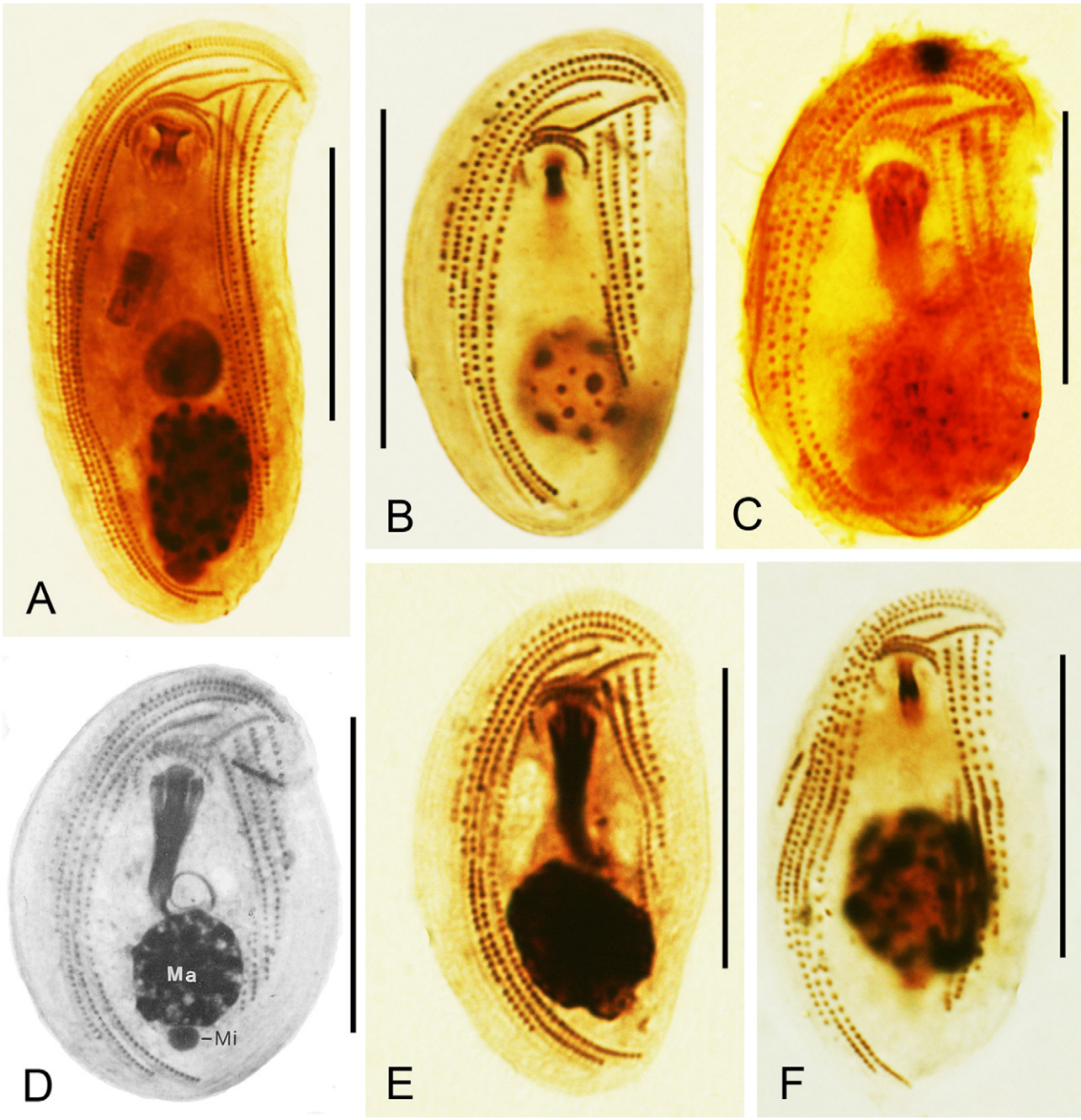

Figure 7 Microphotographs of *Chilodonella parauncinata* sp. n. (**A**) and *C. uncinata* (**B–F**). **A.** Original; **B.** Qingdao population 1 (Song 1997); **C.** From the materials of American population (by courtesy of Prof. Laura A. Katz); **D.** A population from Foissner (1981); **E.** Qingdao population 2 (Song et al. 2009); **F.** Qingdao population 3 (from Hongbo Pan, unpublished). Bars: 20 μm.

Etymology. The specific epithet *irregularis* is a Latin word for "irregular", which refers to the fact that the shape of the macronucleus is irregular in this species.

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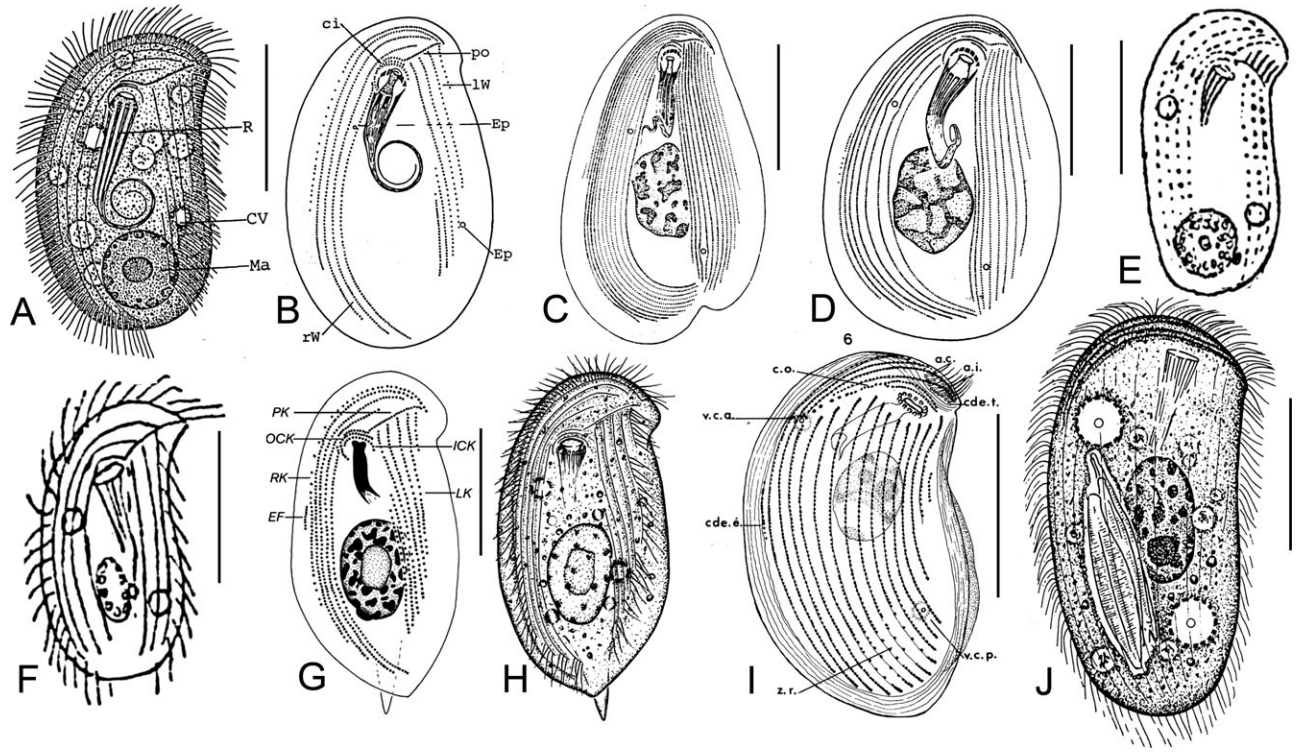

Figure 8 Morphology of related *Chilodonella* and *Chlamydonella* species. **A, B.** *Chilodonella uncinata* (from Foissner et al. 1991). **C.** *Chilodonella piscicola* (from Rydlo and Foissner 1986). **D.** *Chilodonella hexasticha* (from Rydlo and Foissner 1986). **E.** *Chilodonella aplanata* (from Kahl 1931). **F.** *Chilodonella capucina* (from Kahl 1931). **G, H.** *Chilodonella acuta* (from Fan et al. 2014). **I.** *Chlamydonella pseudochilodon* (from Deroux 1970). **J.** *Chlamydonella pseudochilodon* (from Petz et al. 1995). Bars: 20 μm.

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