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A new species of *Trachelomonas* (Euglenaceae, Euglenophyceae) from China

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Abstract: A new species of *Trachelomonas* was collected in China. We analysed the specimen based on its morphological and molecular data. The loricae morphological were similar to two other species, *T. lefevrei* and *T. planctonica*. However, the new species formed a sister group with *T. bernadinensis*, *T. lefevrei* and *T. planctonica* in the phylogenetic tree. These results suggest it to be a new species, *Trachelomonas subplanctonica* sp. nov.

Key words: China, Euglenophyta, phylogeny, *Trachelomonas*

Introduction

Trachelomonas Ehrenberg 1834 is characterized by free–swimming, solitary, Euglena–like cells and external envelopes with diverse morphology. Deflandre (1926) improved the taxonomic system of this genus based on the size, shape and ornamentation of the loricae. Since then, the classification of *Trachelomonas* were heavily depended on the loricae morphology (Deflandre 1930; Huber–Pestalozzi 1955; Bourrelly (1970); Hager 1979; Starmach (1983); Dunlap et al. 1986; Tell & Conforti 1986; Conforti et al. 1994; Wang et al. 2003; Brosnan et al. 2005). In comparison, Pringsheim (1953) classified the species of *Trachelomonas* based on the number of chloroplast and the presence or absence of pyrenoid observed in the cultured cells. Pringsheim (1953) reported the variety of envelope in culture in the same species. Many of the named species and varieties thus might be synonymous (Brosnan, 2005).

With the advance of molecular biology, researchers attempted to classify *Trachelomonas* throughmolecular analysis (Moreira et al. 2001; Brosnan et al. 2003; Marin et al. 2003; Nudelman et al. 2003; Triemer et al. 2006; Ciugulea et al. 2008; Linton et al. 2010; Bennett et al. 2014; Karnkowska et al. 2014). However, the results of these studies sometimes depended on the number of taxon. There are 375 species were included in AlgaeBase (Guiry & Guiry 2021), and only 21 species had molecular data in NCBI.

Loricae morphological characteristics are often used to classify *Trachelomonas* in China. A total of 104 species in the genus have been reported from China, including nine new species (Shi et al. 1999; Wang 2001). Seven of these species have been accepted taxonomically (*T. dictyophora* Jao et Lee Yaoyin, *T. granulatopsis* Z.X.Shi, *T. magnigranulata* Q.X.Wang et Z.X.Shi, *T. quadriformis* Z.X.Shi, *T. spiricostatum* C.C.Jao et Y.Y.Lee, *T. subcoronetta* C.C.Jao et Y.Y.Lee, *T. undulaticollum* Z.X.Shi). However, the taxonomic status of *T. infundibularis* Z.X.Shi is uncertain , and *T. heteromorpha* is not included in the Algaebsae. Only four sequences of uncultured *Trachelomonas* (JN679187, JN679099, JN679075, JN679161) came from China, but these data showed that these sequences were close to uncultured bacterium.

In this study, we described a new species of the genus *Trachelomonas* from China based on lorica and cell morphology, and molecular data.

Materials and Methods

Strains and cultures. We measured more than 50 specimens for *T. subplanctonica* and *T. lefevrei* Deflandre. The strain information and accession numbers are listed in Table 1. Phytoplankton algae were collected using 20 μm plankton net from small ponds in Shanghai and Heilongjiang province. Living specimens of *Trachelomonas* were isolated with capillary under a Nikon Ts2 inverted microscope (NIKON, Tokyo, Japan). All of the strains were cultured in AF–6 medium (Watanebe & Jan 2000) and were maintained at 22–25 °C under conditions of a 14:10 light: dark cycle at 3000 lux photons from cool white fluorescent tubes. Iron citrate (0.03 $g.l^{-1}$) were added for producing loricae.

Strain identification. Cultured strains were observed and identified using an Axio Imager A2 microscope (Carl Zeiss Inc., Hallbergmoos, Germany) and photographed with microscope (BX–51, Olympus) appendant camera (DP72, Olympus, Tokyo, Japan). For SEM observation, the field and cultured samples were dropped on glass, dehydrated using a graded ethanol series, then attached to copper stubs, coated with ~15 nm gold–palladium using a sputter coater (HITACHI E–1045), and examined using a Hitachi SU 8010 SEM (2 kV) at Shanghai Normal University, Shanghai, China. Both the cleaned material and stubs were stored at the Laboratory of Algae and Environment of Shanghai Normal University.

Extraction of DNA and amplification. The total DNA of strains were extracted using Plant Genomic DNA Kit (Tiangen Biotech Co., Beijing, China). We amplified and sequenced the nuclear SSU rRNAs (~2,000 bp), plastid SSU (~1,000 bp) and plastid LSU rRNAs (~2,000 bp) from four specimens. Polymerase chain reactions (PCR) were performed using published primers (Table 2), reaction mixes and amplification conditions. The PCR products were purified using a SanPrep column DNA gel purification kit (Sangon, China), and then to BGI Tech Corporation (Shanghai, China) for sequencing on an ABI 3730XL sequencer. The sequences were submitted to the BLAST search program of the National Center for Biotechnology Information (NCBI) to find closely related sequences. All sequences were downloaded from GenBank (listed in Table S1) and aligned using the Clustal W (Thompson et al. 1997) option in the BioEdit sequence analysis software (Hall 1999). Very short sequences (< 200 bp) were excluded from the alignment. Based on the Neighbor–joining phylogenetic analyses,the identical and near identical sequences were excluded and one or a few sequences of each species were retained. Table S1 lists the 92 taxa with GenBank accession numbers used in this study, including three *Euglenaria*, one *Euglenaformis*, two *Colacium*, five *Cryptoglena*, eleven *Strombomonas*, 27 *Trachelomonas*, eight *Monomorphina* strains, fourteen Phacaceaee strains. Combined nuclear SSU rRNAs, plastid SSU and plastid LSU rRNAs sequence, a set of 4,214 characters (nulear SSU = 1,218, plastid SSU = 1,106, plastid LSU = 1890) was generated for phylogenetic analysis. The sequences of Phacaceaee were used as outgroup taxa to root the trees. Untrimmed bases on both ends were deleted to produce an identical length alignment. Then nuclear SSU rRNAs, plastid SSU and plastid LSU rRNAs alignments of all taxa were concatenated by Sequence Matrix (Vaidya et al. 2011).

The optimal substitution model for each marker was generated using Modeltest with related results listed in Table 3 (Posada & Buckley 2004). We used PHYML software to generate Maximum likelihood trees and the bootstrap analysis was conducted using 1,000 replicates (Guindon & Gascuel 2003; Felsenstein 1981). Bayesian analyses were using MrBayes version 3.1.2 (Ronquist & Huelsenbeck 2003). A Markov chain Monte Carlo (MCMC) algorithm running three hot Markov chains simultaneously and one cold Markov chains simultaneously was used to estimate the posterior probability of phylogenetic trees. The Markov chains were started from a random tree and run for 2,000,000 generations sampling every 1000 generations for a total of 2,000 samples for each run. Figtree 1.4.2 was used to edit all resulting phylogenetic trees. The sequences were submitted to GenBank.

Table 1. List of strains examined in this study and their GenBank accession numbers.

Strain	Sample location	pH	Collection of date	Coordinates	Sample type	GenBank accession number		
						Nuclear SSU	Plastid SSU	Plastid LSU
T. subplanctonica strain GLGY202017–C2	Guilin Park, Shanghai	8.82	July 14, 2020	121°42'494"E 31°17'173"N	Phytoplankton	MZ323680	MZ323687	MZ328716
T. subplanctonica strain GLGY202017–D4		8.82	July 14, 2020	121°42'494"E 31°17'173"N	Phytoplankton	MZ323681	MZ323686	MZ328717
T. lefevrei strain HLJ2020731–B2	Binshui Avenue, Liye street, Hulan District, Harbin City, Heilongjiang Province	7.49	July 31, 2020	26°41'510"E 45°54'179"N	Phytoplankton	MZ323679	MZ323685	MZ328714
T. lefevrei strain HLJ2020731–B3		7.49	July 31, 2020	26°41'510"E, 45°54'179"N	Phytoplankton	MZ323678	MZ323684	MZ328715

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Results

Combining morphological characteristics and molecular data, we identified two species, *T. subplanctonica* sp. nov. and *T. lefevrei*.

***Trachelomonas subplanctonica* Jiang et Pang sp. nov. (Figs 1–9, 13–19)**
Description: loricae ellipsoidal (Figs 1–6), surface punctured (Fig 18, 19), length 27–30 µm, width 22–24.5 µm, hyaline to dark brown; collar cylindrical, with toothed edge (Figs 14, 15, 17), length 2.5–5 µm, width 3.8–5.5 µm; cells ellipsoidal with metabolic movements (Figs 7–9), length 23–34 µm, width 19–30 µm; chloroplasts discoid with pyrenoids protruding towards the interior and covered by cup–shaped paramylon grains, range 10–20; paramylon grains spherical, numerous; stigma obvious; contractile vacuole below the stigma; a visible process at the posterior end or not.

Holotype: SHTU–GLGY202017–C2, fixed material, collected 3 April 2021, deposited in Biology Department Herbarium, Shanghai Normal University, Shanghai, China.
Reference strain: Deposited as FACHB–3318 in the Freshwater Algae Culture Collection at the Institute of Hydrobiology Chinese Academy of Science, Wuhan, Hubei Province, China.
Type locality: 12º142'494"'E, 31º17'173"N; phytoplankton, Shanghai.
Habitat: plankton.
Etymology: The epithet of the new species refers to the morphological structures of the loricae, which similar to *T. planctonica*.

***Trachelomonas lefevrei* Deflandre (Figs 10–12)**
Description: loricae cylindrical to ellipsoidal, surface punctured, length 27–31 µm, width 20–24 µm, hyaline to dark brown; collar cylindrical, with toothed edge, length 2.5–3 µm, width 5–6 µm; cells ellipsoidal with metabolic movements, length 23–34 µm, width 19–30 µm; chloroplasts discoid with pyrenoids protruding towards the interior and covered by cup–shaped paramylon grains, range 5–15; paramylon grains spherical, numerous; stigma obvious; a visible process at the posterior end or not.

Collecting location: 26º41'510"E, 45º54'179"N; phytoplankton, Heilongjiang.

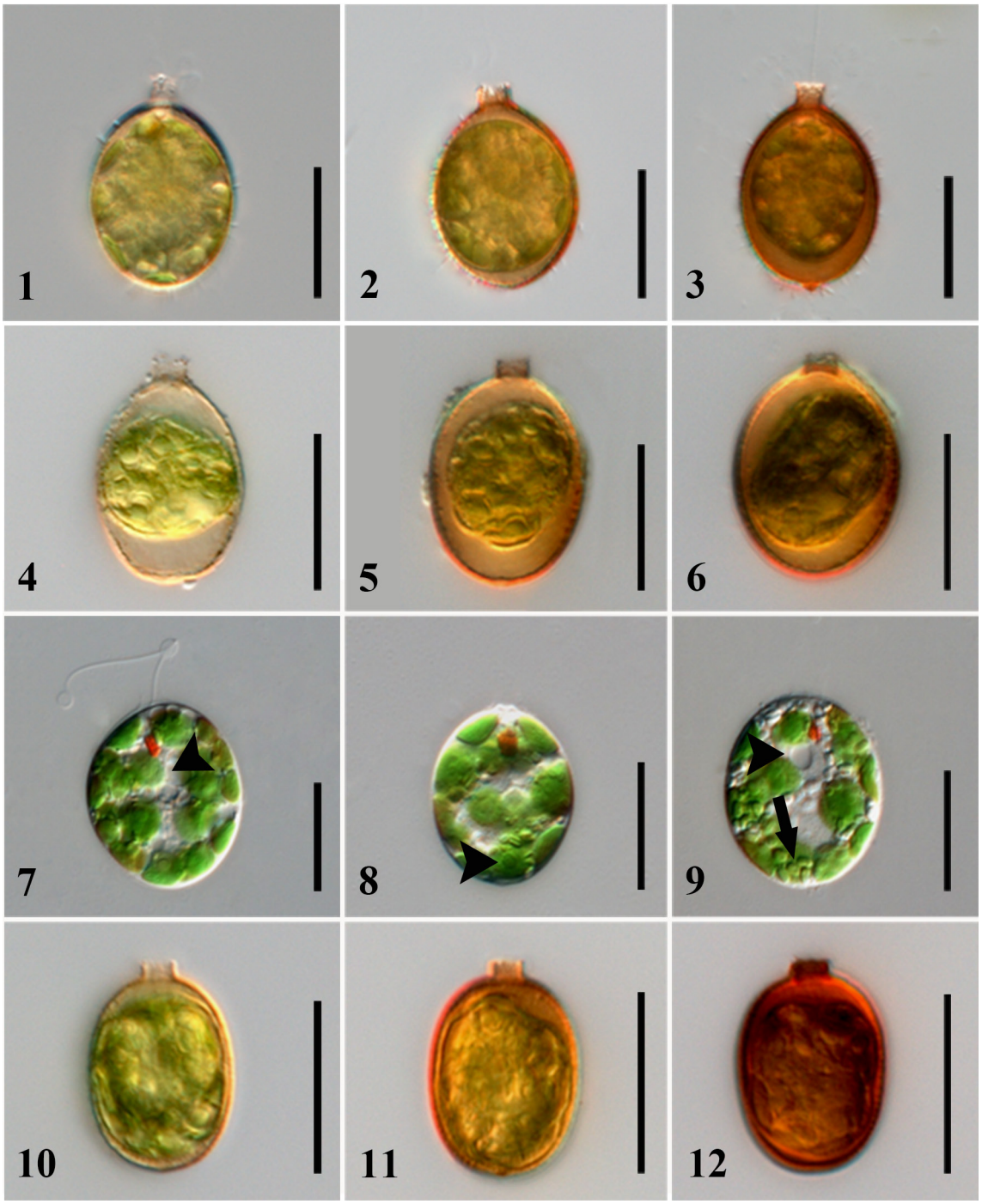
Phylogenetic analysis
We sequenced partial nuclear SSU rRNAs (~1800bp), plastid SSU (~1000bp) and plastid LSU rRNAs (~1500bp) sequence from *T. subplanctonica* and *T.*

Table 2. Primers for PCR amplification and sequencing of nuclear SSU and plastid SSU and LSU rRNA.

Gene	Primer name	Sequences (5–3)	Reference
nuclear SSU rRNAs	18S5	CAGTGGGTCTGTGAATGGCTCC	Kosmala et al. 2007
	18S3	CGACGGGCGGTGTGTACAAGT	
plastid SSU rRNAs	cpSSU–F	TTG ATC CTG GCT CAG GAT GAA CGC T	Linton & Karnkowska–Ishikawa et al. 2010
	cpSSU–R	CAA GGA GGT GAT CCA GCC GCA CCT T	
plastid LSU rRNAs	23S_AF	ATAAGCTTCATTGTCRARAGG	Kim et al. 2010
	23S_R	TATGCTTTCAGCAGTTATCCAC	

Table 3. Substitution models obtained for each gene sequence using Modeltest 3.7 analysis.

Molecular marker	Model selected	Base frequency	Rate matrix
Concatenated nuclear SSU rRNAs, plastid SSU rRNAs and plastid LSU rRNAs	GTR+I+G	freq A = 0.2713	R (a) [A–C] = 0.9135
	–lnL= 65987.2188	freq C = 0.1815	R (b) [A–G] = 3.3924
	K=10	freq G = 0.2576	R (c) [A–T] = 1.4215
	(I) = 0.1888	freq T = 0.2896	R (d) [C–G] = 0.8058
	(G)= 0.6258		R (e) [C–T] = 4.2974
			R (f) [G–T] = 1.0000



Figs 1–12. Loricae and cytological morphological details of *T. subplanctonica* sp. nov. and *T. lefevrei*: (1–3) different colors of loricae of *T. subplanctonica* sp. nov. from field samples; (4–6) different colors of loricae of *T. subplanctonica* sp. nov. from culture samples; (7) chloroplast morphology of *T. subplanctonica* sp. nov. (arrowhead); (8) pyrenoids morphology of *T. subplanctonica* sp. nov. (arrowhead); (9) paramylon grains morphology (arrows) and contractile vacuole (arrowhead) of *T. subplanctonica* sp. nov.; (10–12) loricae morphological of *T. lefevrei.* Scale bar 20 μm (1–12).

lefevrei. The topology was similar for the phylogenetic trees obtained by both Maximum likelihood and Bayesian inference, as far as the location of the analyzed strains (Fig. 20). In phylogenetic trees, all *Trachelomonas* strains are seperated into four clades (A, B, C and D), the studied strains are at D clade. Our strain HLJ2020731–B1 and strain HLJ2020731–B2 are group with *T. lefevrei* (1.00/1000).

The new species was fell into Clade D with *T. bernadinensis* Vischer, *T. lefevrei and T. planctonica* Svirenko. The pairwise distance between our strain and the *T. lefevrei* sequences was both 0.035 (plastid SSU), 0.006 (nuclear SSU rRNAs) and 0.048 (plastid LSU), corresponding to 39 base (plastid SSU), 31 base (nuclear SSU rRNAs) and 97 base (plastid LSU) pair differences.

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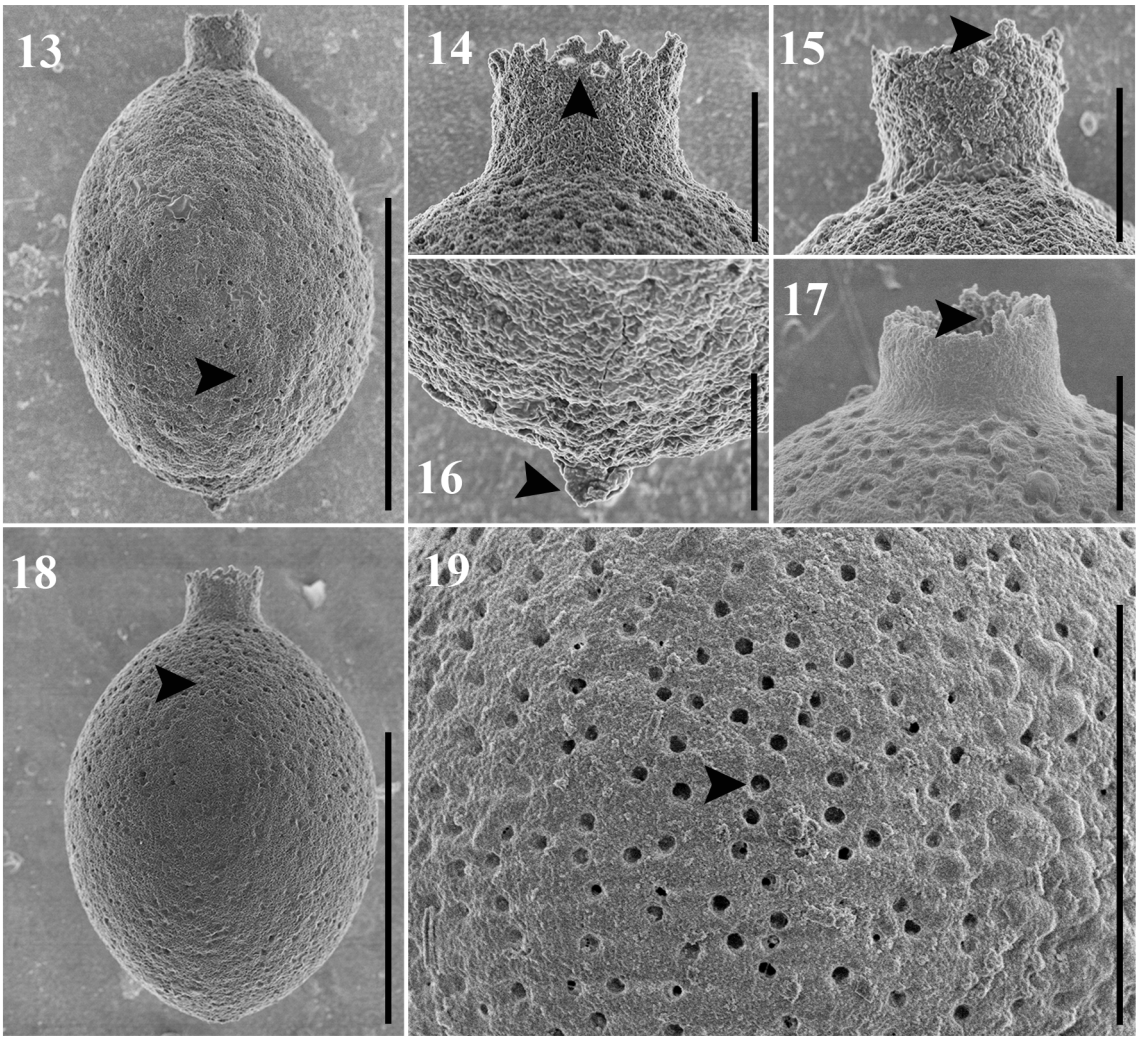

Figs 13–19. Scanning electron microscopy pictures of *T. subplanctonica* sp. nov.: (13, 18) loricae shape; (14, 15, 17) loricae collar; (16) a visible process at the posterior end; (19) loricae surface. Scale bar 20 μm (13,18), 10 μm (19), 4 μm (14–16), 3 μm (16).

Discussion

T. subplanctonica morphology is most similar to *T. planctonica,* and *T. lefevrei* and *T. caudata* (Ehrenberg) F.Stein by having punctuated ornamentation and cylindrical collar. The detail morphological data of *T. subplanctonica*, *T. planctonica*, *T. lefevrei* and *T. caudata* were listed in Table 4. The lorica of *T. planctonica* is spherical (Deflandre 1926; Shi et al. 1999; Wołowski & Hindak 2004; Wołowski & Hindak 2005; Wołowski & Walne 2007; Conforti 2010; Duangjan 2012), and not has a visible process at the posterior end. *T. subplanctonica* is different from *T. lefevrei* in terms of loricae shape and collar length. *T. lefevrei* is not typical ellipsoidal (Deflandre 1926; Shi et al. 1999; Dunlap et al. 1983; Conforti 2010; Duangjan 2012), and its collar is lower. The visible process at the posterior of *T. caudata* is longer than *T. subplanctonica*, and the loricae covered with spines or warts (Wołowski et al. 2016). The morphology of loricae changed during development (Poniewozik 2018). The cells of *T. subplanctonica* became naked and without loricae if it was cultured in AF–6 medium. The main component of loricae are Fe, Mn and Si (Dunlap et al. 1986; Conforti et al. 1994; Wang et al. 2003). When it was cultured in AF–6 medium with added Iron citrate (0.4g.l^{-1}), the loricae appeared. Its color is shallow to dark during different period of development. There was a visible process at the posterior end of the loricae (Figs 3, 16), while it was not observed in some strains. The toothed edge collar was observed in field (Figs 14, 15) and cultured samples (Fig 17), but it was relatively lower in culture. The loricae shapes of all individuals were elliptical in both field and culture samples. So loricae shape and toothed edge collar might be relatively stable for *T. subplanctonica*.

For a long time, the classification of *Trachelomonas* is entirely based on loricae characteristics (Deflandre 1926, Huber–Pestalozzi 1955, Tell and Conforti 1986). Huber–Pestalozzi (1955) divided the genus *Trachelomonas* into two main groups, Rotundatae (loricae posterior without a tailpiece) and Caudatae (loricae posterior with a tailpiece).

Table 4. Comparison of cell morphology of similar species.

Species	Loricae shape	Loricae length (µm)	Loricae width (µm)	Ornamentation	The base of loricae	Loricae collar	Collar length (µm)	Collar width (µm)	Habitat
T. subplanctonica	Ellipsoidal	27–30	22–24.5	Punctuated	A visible process at the posterior end	Collar cylindrical, with toothed edge	2.5–5	3.8–5.5	Ponds
T. lefevrei (Deflandre 1926; Shi et al. 1999; Dunlap et al. 1983; Conforti 2010; Duangjan 2012)	Cylindrical, spherical, broadly ellipsoid	22–33	20–31	Punctuated	A visible process at the posterior end	Low cylindrical collars, with toothed edge	1–3	5–6	Marsh, lake, ponds
T. planctonica (Svirenko 1914; Shi et al. 1999; Duangjan 2012)	Spherical	19–30	17–22	Punctuated	–	Collar cylindrical, irregularly toothed on edge	3–6	2.5–4	Marsh puddles, fish-ponds, ponds
T. caudata (Stein 1878; Deflandre 1926; Tell & Conforti 1986; Conforti 1993; Duque 1995; Tell 1998; Shi et al. 1999; Zalocar de Domitrovic 2014; Onuoha et al. 2014; Wołowski et al. 2016)	Broadly oval	26.1–38.3	14.1–20	Punctuated and covered with spines or warts	A visible process at the posterior end	Collar cylindrical, with toothed edge or slightly widened at the rim with shorter or longer teeth or slightly curved to one side or no collars	4–7	3–8	Lake, ponds, puddle

Rotundatae and Caudatae were seperated into five subsections (Sphaericae, Ellipticae, Ampulliformes, Saccatae and Longisetae) and three subsections (Colliferae, Obattenuatae and Speciosae) respectively based on loricae shape. But Pringheim (1953) divided fourteen *Trachelomonas* species into six groups based on cytological morphological of his observations (*T. hispida*, *T. volvocina*, *T. bernardinensis*, *T. cotispersa*, *T. deflandrei* and *T. bitlla*).

Ciugulea et al. (2008) using nuclear SSU and LSU rRNA sequences conducted phylogenetic analysis. The clade C and clade D consistent with the group 2 of Pringheim (1953). These species of group 2 had two chloroplasts with inner pyrenoids, two layered loricae, no conspicuous ornamentation on loricae (Pringheim 1953).

T. subplanctonica and *T. lefevrei* fell into clade D with *T. bernadinensis* and *T. planctonica*. Pringheim (1953) put *T. bernadinensis* and *T. lefevrei* into one group, because the two species both had discoid chloroplasts with pyrenoids protruding towards the interior and covered by cup–shaped paramylon grains. *T. subplanctonica* shares these characteristics. We also found these three species all had punctured surface and collar dentate. It was possible that besides the chloroplast and pyrenoid, some characteristics of loricae morphology were the basis of phylogenetic tree clades.

Phylogenetic tree results are related to the number and selection of taxa (Marin et al. 2003; Kim et al. 2015). The molecular data of *Trachelomonas* in previous studies involved less than 20 species (Marin et al. 2003; Triemer et al. 2006; Kim et al. 2008; Ciugulea et al. 2008; Kim et al. 2010; Kim et al. 2015). Only *T. hispida*, *T. reticulata*, *T. bernardinensis*, *T. rugulosa*, *T. volvocina*, *T. volvocinopsis*, *T. planctonica*, *T. armata* and *T. echinata* have been studied on the loricae morphology changes of during development (Brosnan et al. 2005; Poniewozik et al. 2018). We thought that there are too few taxa and genes for molecular phylogenetic analysis. At present, there is a great lack

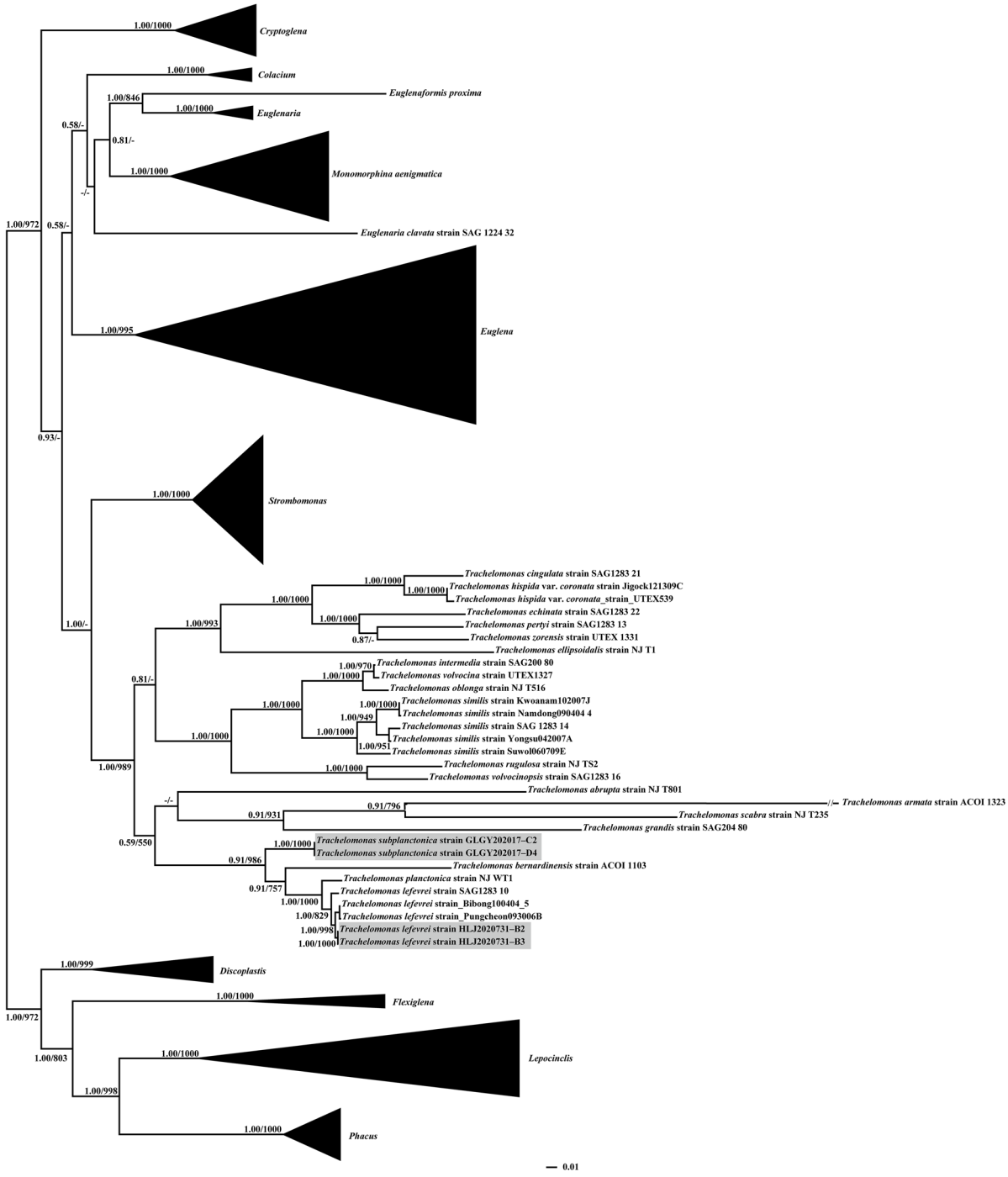

Fig 20. Bayesian phylogenetic tree based on combined nuclear SSU, plastid SSU and partial–LSU rRNA sequences. Support values >50% for all analyses are shown on branches as follows: Bayesian posterior probabilities (BA) / maximum likelihood bootstrap values (ML). '–' denotes <50% support for that analyses at that node. Sequences of our culture strains are shade in grey.

of information about the group Hispidae, Caudatae, and Scabrae, which have unusual loricae morphology. We need more molecular and stable morphological data of *Trachelomonas* for further study.

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Supplementary material

The following supplementary material is available for this article:

Table S1. Relevant sequence information downloaded from GenBank in this study.

This material is available as part of the online article (http://fottea.czechphycology.cz/contents)

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