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Phylogeny of *Trachelomonas* and *Strombomonas* (Euglenaceae) Based on Morphological and Molecular Data

Xiaodie Jiang [1,2], Xi Chen [1], Wanting Pang [1,*] and Quanxi Wang [1,*]

[1] College of Life Sciences, Shanghai Normal University, Shanghai 200234, China
[2] School of Environmental and Geographical Sciences, Shanghai Normal University, Shanghai 200234, China
* Correspondence: pangwt@shnu.edu.cn (W.P.); wangqx@shnu.edu.cn (Q.W.)

Abstract: The classification of *Trachelomonas* and *Strombomonas* is based on the morphology of loricae, which may not reflect phylogenetic relationships. There are different views on the relationship between the two genera. It is thus important for researchers to classify the two genera based on more characteristics besides loricae, such as protoplast and molecular data, and to establish a natural classification system. In this study, 29 strains (13 species) of *Trachelomonas* and 12 strains (eight species) of *Strombomonas* were collected from various locations in China. The morphological characteristics of *Trachelomonas* and *Strombomonas* were observed in the field and culture conditions, and the sequences of nuclear SSU rDNA, nuclear LSU rDNA, plastid-encoded LSU rDNA and plastid-encoded LSU rDNA of strains were obtained. We constructed a phylogenetic tree by combining four gene sequences with maximum likelihood and Bayesian methods. Based on the development of the loricae, it was found that the shape of the loricae and the presence or absence of the collar were relatively stable in culture, while the length of the collar presence or absence of spines and the color of the loricae changed. The phylogenetic tree showed that *Trachelomonas* and *Strombomonas* were sister branches, which supported *Trachelomonas* and *Strombomonas* as independent genera. Taxa in the genus *Strombomonas* sorted into two clades and *Trachelomonas* sorted into five strongly supported clades. Key morphological features could be attributed to each of the clades.

Keywords: euglenoids; morphological characteristics; protoplast; phylogenetic; diversity

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1. Introduction

In 1833, Ehrenberg established the genus *Trachelomonas* based on cells surrounded by a lorica [1]. Deflandre (1926) improved the taxonomic system of the genus based on the lorica shape and ornamentation [2]. This system included two sections (Rotundatae and Caudatae) and seven subsections (Sphaericae, Ellipticae, Ampulliformes, Saccatae, Longisetae, Colliferae, Accuminatae). Later, Deflandre (1930) separated the subsection Saccatae, which has distinctive collar, possession of a tailpiece, lack of ornamentation, and the ability aggregate particles on the surface of the lorica from the genus *Trachelomonas* and established a new genus, *Strombomonas*. However, some authors suggested the taxonomic positioning of organisms that are presently classified as *Strombomonas* be reconsidered [3]. The classification of *Trachelomonas* and *Strombomonas* has been heavily dependent on the morphology of loricae. Only Pringsheim classified 13 *Trachelomonas* species into 6 groups by combining the morphology of loricae and protoplasts [4]. Singh [5], Rosowski et al. [6], Barnes et al. [7], and Wang Quanxi et al. [8] suggested both loricae and protoplasts characteristics be considered in the systematic classification of *Trachelomonas* and *Strombomonas*.

With the development of molecular biology, the understanding of the phylogeny relationships between *Trachelomonas* and *Strombomonas* also changed. The monophyly of these two lorica-bearing genera could not be confirmed initially [9]. A single monophyletic loricate clade was supported based on SSU and LSU rDNA data of three species. Marin et al. (2003) revised the classification of euglenoids and reclassified *Strombomonas* into

Trachelomonas [10]. However, only thirty *Trachelomonas* strains and one *Strombomonas* strain were used for the SSU rDNA analysis. Subsequently, Triemer et al. (2006) sequenced SSU and LSU rDNA of eight *Strombomonas* and 25 *Trachelomonas* strains, and combined them in a multigene phylogenetic analysis. *Trachelomonas* and *Strombomonas* were confirmed to be two independent genera [11]. This was also supported by other studies [12–17]. These analyses added 11 definite species and a few undetermined species (*T.* sp.). However, the position of many taxa on the tree depends on the number of species and the genes used [18].

It is difficult to construct phylogenetic studies of loricate genera only based on loricae morphology. In addition, many features of loricae are unstable, which also causes difficulties for the classification of *Trachelomonas* and *Strombomonas* [12]. At present, it is necessary to find out stable morphological characteristics of the two genera. In AlgaeBase, there are 374 accepted species of *Trachelomonas*, but molecular data are available for only 22 species in NCBI [19]. For *Strombomonas*, molecular data are available for eight of 89 species. This data gap makes it difficult to establish phylogenetic relationships between these two genera and within each of them. Previous Chinese studies on *Trachelomonas* and *Strombomonas* were based only on morphology. A total of 106 species of *Trachelomonas* and 30 species of *Strombomonas* have been reported from China [20,21]. Shi et al. divided *Trachelomonas* into nine groups (Volvocinae, Curtae, Oblongae, Cylindricae, Ovoideae, Hispidae, Pyriformes, Caudatae, Scabrae) based on the shape and ornamentation of loricae for facilitating identification. Almost no molecular data from Chinese strains have been published so far except for the taxa *T. subplanctonica*, *S. borystheniensis*, *S. fluviatilis*, *S. gibberosa*, *S. maxima* and *S. triquetra* [21,22]. A total of 13 species of *Trachelomonas* and eight species of *Strombomonas* were collected from China. Lorica and protoplast of isolated and cultured strains were observed and measured to explore the stability of traits. The nuclear SSU rDNA (nSSU rDNA), nuclear LSU rDNA (nLSU rDNA), plastid-encoded SSU rDNA (cpSSU rDNA) and plastid-encoded LSU rDNA (cpLSU rDNA) of each species were obtained and combined in a multigene phylogenetic analysis. A phylogenetic tree was constructed by combining four genes and using maximum likelihood and Bayesian methods to assess the relationship between the two genera and clarify the phylogenetic characteristics of each lineage.

2. Materials and Methods

2.1. Strains Collection, Cultivation and Morphological Study

During 2019–2021, we collected 13 *Trachelomonas* species and eight *Strombomonas* species from various sites in China (Table S1). Phytoplankton algae were collected with a 20 μm plankton net. Individual euglenoid cells were isolated with capillary pipettes under a Nikon Ts2 inverted microscope (NIKON, Tokyo, Japan). All strains were cultured in AF–6 medium [23] 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. Species (environmental samples and laboratory cultures of strains) were observed and identified using an Axio Imager A2 microscope (Carl Zeiss Inc., Hallbergmoos, Germany) and photographed with a microscope appendant camera (DP72, Olympus, Tokyo, Japan). More than 50 specimens for each strain were measured with ImageJ software [24]. The measurement results were analyzed by constructing box plot with SPSS v21.0 (Statistical Product and Service Solutions, Chicago, IL, USA) [25]. Excel 2016 was used to conduct one-way analysis of variance for the measured data.

2.2. DNA Extraction, Amplification and Sequencing

The total DNA of strains was extracted using Plant Genomic DNA Kit (Tiangen Biotech Co., Beijing, China). We amplified and sequenced the nSSU rDNA, nLSU rDNA, cpSSU rDNA and cpLSU rDNA from our strains. Polymerase chain reactions (PCR) were performed using published primers, reaction mixes and amplification conditions [13,26–28]. The PCR products were purified using a SanPrep column DNA gel purification kit (Sangon, Shanghai, China), and and sequenced in BGI Tech Corporation (Shanghai, China).

2.3. Phylogenetic Analyses

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 and aligned using the Clustal W [29] option in the BioEdit v7.2.1 (BioEdit Sequence Alignment Editor, Scotts Valley, CA, USA) sequence analysis software [30]. Very short sequences (<200 bp) were excluded from the alignment. Based on Neighbor–joining phylogenetic analyses, the identical and near identical sequences were excluded and one or a few sequences of each species were retained. Table S2 lists the 73 taxa with GenBank accession numbers used in this study. Sequences of the *Colacium* genus were used to root the trees. Sequences of four genes were aligned separately. A combined alignment was generated (5431 aligned sites; cpSSU Rdna = 1–854; nSSU rDNA = 855–2713; cpLSU rDNA = 2714–3758; nLSU rDNA = 3759–5431) by using PhyloSuite v1.2.2 (A desktop platform for streamlined molecular sequence data management and state of the art evolutionary phylogenetics studies, Wuhan, China) [31]. The best-fit model for the concatenated alignments were selected using ModelFinder v1.6.8 (Fast and Accurate Model Selection, Canberra, Australia) [32] with all algorithm and AIC criterion (for ML: cpSSU rDNA, nSSU rDNA = GTR + F + R4; nLSU rDNA = TIM2 + F + R4; cpLSU rDNA = TVM + F + I + G4; for BI: cpSSU rDNA, nSSU rDNA, cpLSU rDNA, nLSU rDNA = GTR + F + I + G4). ML phylogenies were inferred using IQ-TREE v1.6.8 (Efficient Tree Reconstruction, Canberra, Australia) [33] with 5000 ultrafast bootstraps (under Edge-linked partition model), as well as the Shimodaira-Hasegawa-like approximate likelihood-ratio test [34]. Bayesian Inference phylogenies were inferred using MrBayes v3.2.6 (Bayesian Inference of Phylogeny, Stockholm, Sweden) [35] with 2 parallel runs, 10,000,000 generations, of which the initial 25% of sampled data were discarded as burn-in (under partition model). Figtree v1.4.2 (Produce Figures of Phylogenetic Trees, Edinburgh, UK, Britain) was used to edit all resulting phylogenetic trees.

3. Result

3.1. Morphological Characteristics

The morphological characteristics of 13 species of *Trachelomonas* and eight species of *Strombomonas* varied (Figures 1–4 and Table 1). Among these species, *T. cervicula* is a new record for China.

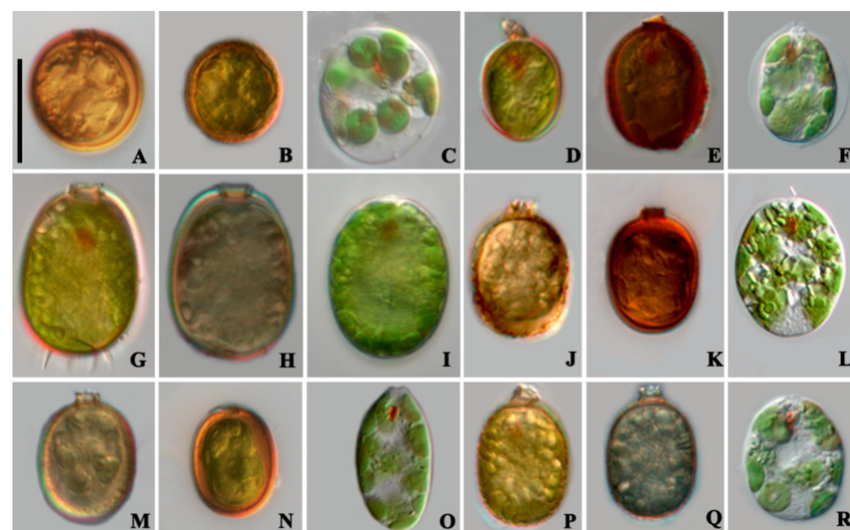

Figure 1. Morphology of *Trachelomonas* strains in this study. Each species includes: morphology of lorica in the field (left), morphology of lorica in culture (middle), Protoplast morphology (right). (**A**–**C**) *T. cervicula* strain SHNUS39D1; (**D**–**F**) *T. playfairii* strain SHNUN14B1; (**G**–**I**) *T. armata* strain SHNUS50B3; (**J**–**L**) *T. lefevrei* strain SHNUCC; (**M**–**O**) *T. planctonica* var. *oblonga* strain SHNUS2C1; (**P**–**R**) *T. similis* strain SHNUS41C3. Species are all in the same scale, scale bar 20 μm.

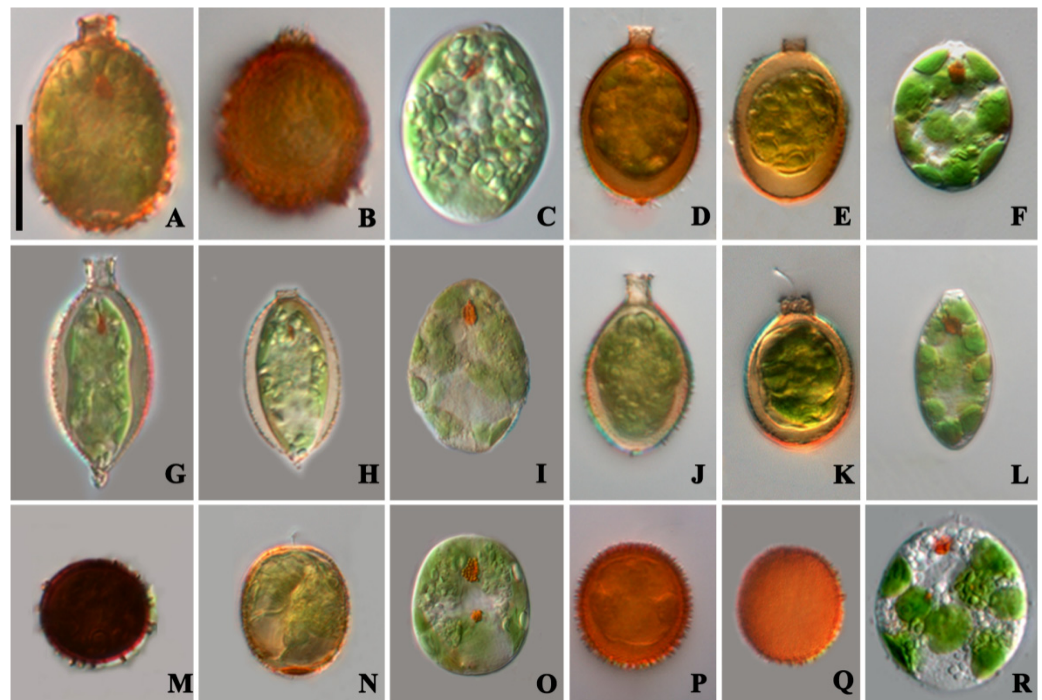

Figure 2. Morphology of *Trachelomonas* strains in this study. Each species includes: the morphology of lorica in the field (left), morphology of lorica in culture (middle), protoplast morphology (right); (**A**–**C**) *T.* cf. *crebea* strain SHNUS39B2; (**D**–**F**) *T. subplanctonica* strain SHNUS17C2; (**G**–**I**) *T. bernardinensis* strain SHNUWeijian; (**J**–**L**) *T. undulaticollum* strain SHNUS35D5; (**M**–**O**) *Trachelomonas* sp. strain SHNUC4C3; and (**P**–**R**) *T.* cf. *bacillifera* var. *minima* strain SHNUQ4B2. Species are all in the same scale, scale bar 20 µm.

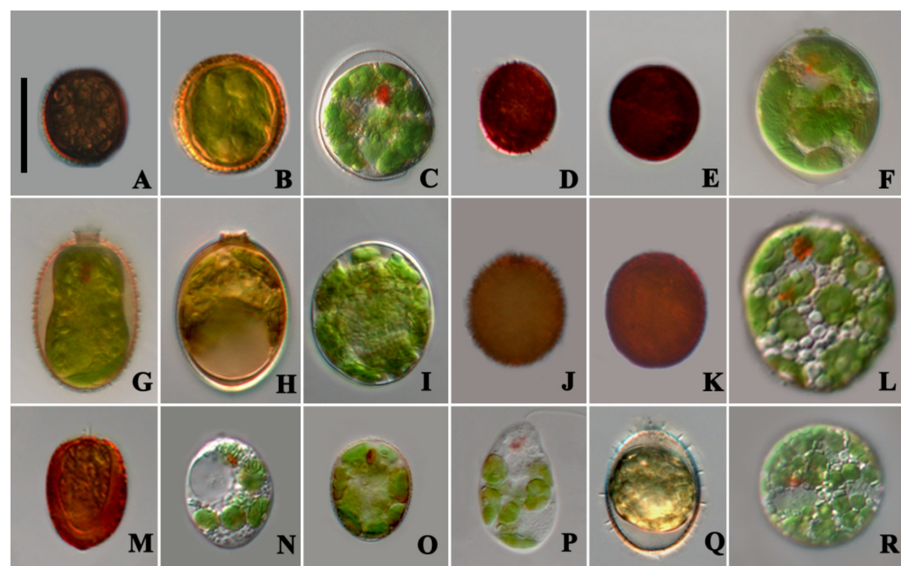

Figure 3. Morphology of *Trachelomonas* strains in this study: (**A**–**L**) each species includes: morphology of lorica in the field (left), morphology of lorica in culture (middle), protoplast morphology (right); (**A**–**C**) *Trachelomonas* sp. strain SHNUS12C2; (**D**–**F**) *Trachelomonas* sp. strain SHNUS26A3; (**G**–**I**) *T. hispida* strain SHNUQ1D6; (**J**–**L**) *T.* cf. *sydneyensis* var. *minima* strain SHNUC1D1; (**M**,**N**) Morphology of *Trachelomonas* sp. strain SHNUS25D1 in culture; (**O**,**P**) morphology of *Trachelomonas* sp. strain SHNUN16B1 in culture; (**Q**) morphology of *Trachelomonas* sp. strain SHNUS30A1 in culture; and (**R**) protoplast morphology of *Trachelomonas* sp.strain SHNUC2D. Species are all in the same scale, scale bar 20 µm.

Figure 4. Morphology of *Strombomonas* strains in this study: (**A**–**I**) each species includes: morphology of lorica in the field (left), morphology of lorica in culture (middle), protoplast morphology (right); (**A**–**C**) *S. maxima* strain SHNUS6A3; (**D**–**F**) *S. fluviatilis* strain SHNUQ6B2; (**G**–**I**) *S. borystheniensis* strain SHNUQ6C2; (**J**,**K**) Morphology of *S. verrucosa* strain SHNUS28C1; (**L**) protoplast morphology of *S. ovalis* strain SHNUS49C6; (**M**–**R**) each species including: morphology of lorica in the field (left), protoplast morphology (right); (**M**,**N**) *S. gibberosa* strain SHNUQ6A1; (**O**,**P**) *S. triquetra* strain SHNUQ6A4; and (**Q**,**R**) *S.* cf. *borystheniensis* strain SHNUQ6C3. Species are all in the same scale, scale bar 20 µm.

Table 1. Morphological characters of *Trachelomonas* and *Strombomonas* strains in this study.

Taxon	Isolate/Strain	Length (µm)	Width (µm)	Number of Chloroplasts	Pyrenoid	Loricae Shape
T. armata (Ehrenberg) Stein	SHNUS50B3	32–49	15–22	10–15	Haplopyrenoid	Ovoid with short collar, posterior end with long cone thorns
T. bernardinensis Vischer	SHNUWeijian	31–58	18–29	6–10	Haplopyrenoid	Fusiform with collar, collar with toothed edge, a visible process at the posterior end
T. cervicula A.Stokes	SHNUS39D1	20–29	20–29	5–10	None	Spherical without collar, surface smooth
T. cf. *bacillifera* var. *minima*	SHNUQ4B2	24–25	20–21	8–10	Haplopyrenoid	Elliptical without collar, surface with rod spines
T. cf. *crebea*	SHNUS39B2	20–37	20–27	8–10	Diplopyrenoid	Elliptical with collar, surface with granular process
T. cf. *sydneyensis* var. *minima*	SHNUC1D1	23–26	19–20	10–12	Haplopyrenoid	Elliptical without collar, surface with short conical spines
T. hispida (Perty) Stein	SHNUQ1D6	30–33	22–24	7–10	Diplopyrenoid	Oblong without collar, surface with short cone spines
T. lefevrei Deflandre	SHNUCC	27–31	20–24	9–10	Haplopyrenoid	Elliptical with collar, collar with toothed edge
T. planctonica var. *oblonga* Svirenko	SHNUS2C1	20–23	17–20	10–20	Haplopyrenoid	Elliptical with collar, collar with toothed edge
T. similis Stokes	SHNUS41C3	20–23	16–17	5–8	Haplopyrenoid	Wide elliptical with oblique collar, surface punctured
T. playfairii Deflandre	SHNUN14B1	19–26	16–20	8–10	Haplopyrenoid	Elliptical with oblique collar, collar with toothed edge
Trachelomonas sp.	SHNUS26A3	16–19	13–16	7–10	Haplopyrenoid	Elliptical without collar, anterior end with annular thickenings, surface with short spines

Table 1. *Cont.*

Taxon	Isolate/Strain	Length (µm)	Width (µm)	Number of Chloroplasts	Pyrenoid	Loricae Shape
Trachelomonas sp.	SHNUC4C3	21–23	21–23	5–8	Diplopyrenoid	Spherical without collar, surface with short conical spines
Trachelomonas sp.	SHNUS12C2	20–22	17–20	10–12	Haplopyrenoid	Elliptical without collar, surface with fine spines
T. subplanctonica Jiang & Pang	SHNUS17C2	23–34	19–30	6–10	Haplopyrenoid	Ellipsoidal with collar, collar with toothed edge, surface punctured
T. undulaticollum Shi	SHNUS35D5	28–30	20–22	8–10	Haplopyrenoid	Elliptical with long collar
S. borystheniensis (Roll) Popova	SHNUQ6C2	22–30	19–20	6–11	Diplopyrenoid	Wide ellipsoidal with a wide short collar, without tail
S. cf. *borystheniensis*	SHNUQ6C3	27–38	18–25	5–7	Haplopyrenoid	Ellipsoidal with collar, without tail
S. gibberosa (Playfair) Deflandre	SHNUQ6A1	40–53	21–35	7–9	Haplopyrenoid	Middle wide-rhomboidal, loricae with a wide short collar and a long tail
S. fluviatilis (Lemmermann) Deflandre	SHNUQ6B2	53–71	21–28	9–13	Haplopyrenoid	Fusiform with straight collar and a long tail
S. maxima (Skvortsov) Deflandre	SHNUS6A3	70–109	28–43	8–17	Haplopyrenoid	Broadly ovate with straight collar and a long tail
S. triquetra (Playfair) Deflandre	SHNUQ6A4	34–43	20–21	6–10	Haplopyrenoid	Inverted triangle with short collar and a short caudate process

Trachelomonas cervicula **A.Stokes (Figure 1A–C)**

Description: loricae spherical without collar, surface smooth, diameter 20–29 µm; cells with metabolic movements, chloroplasts discoid without pyrenoid, range 5–10; paramylon grains rod-shaped granules, numerous; stigma obvious.

Reference strain: Deposited as FACHB-3562 in the Freshwater Algae Culture Collection at the Institute of Hydrobiology Chinese Academy of Science, Wuhan, Hubei Province, China.

Collected location: 31°14′91″ N 121°37′75″ E; phytoplankton, Shanghai.

3.2. Stability of Loricae Morphology

In measuring the length and width of the loricae of five strains of *Trachelomonas* (Figure S1), we found the length of *T. planctonica* var. *oblonga* and *T. subplanctonica* decreasing significantly before and after cultivation (Figure S1A). In comparison, there was no significant change in the loricae width of *T. hispida*, *T. lefevrei* and *T. planctonica* var. *oblonga* after culture (Figure S1B). For the length-width ratio of the loricae of five strains, only *T. subplanctonica* had a significant difference (Figure S1E).

The collar morphology in cultured specimens is different from those in the field. In cultured samples, the collars of *T. armata* (Figure 1G,H), *T. hispida* (Figure 3G–I), *T. planctonica* var. *oblonga* (Figure 1M,N) and *T. subplanctonica* (Figure 2D,E) became shorter. In measuring the length of the collar, we found that the collar length of loricae in the cultured samples was significantly reduced compared with that in the field sample (Figure S1C). There was no significant difference in the collar width of *T. hispida*, *T. planctonica* var. *oblonga*, *T. lefevrei*, but the collar width of *T. similis* and *T. subplanctonica* decreased significant (Figure S1D). Comparing the collar length-width ratio of five strains, only *T. lefevrei* had no significant difference (Figure S1F). The collar of *T. similis* became short and straight (Figure 1P,Q). And the teeth on the collar edge of *T. lefevrei* (Figure 1J,K) were not present in cultured specimens.

In the field samples, the loricae color of *T. subplanctonica* varied from colorless, yellowish brown to reddish brown. The same situation also occurred in all other strains. The spines on the loricae surface did not grow in culture for most species. The cone spines of *T. armata* (Figure 1G,H), *T. hispida* (Figure 3G–I), *T.* cf. *sydneyensis* var. *minima* (Figure 3J,K) and *Trachelomonas* sp. (Figure 2M,N and Figure 3D,E) did not grow (Figure 2M,N). The rod spines on the surface of the loricae of *T.* cf. *bacillifera* var. *minima* still grew in culture (Figure 2P,Q).

3.3. Phylogenetic Analysis and Morphological Characteristics of the Clades

We sequenced nSSU rDNA, nLSU rDNA, cpSSU rDNA and cpLSU rDNA sequences from all *Trachelomonas* and *Strombomonas* strains. The topology was similar for the phylogenetic trees obtained by both Maximum likelihood and Bayesian inference, in terms of the location of the analyzed strains (Figure 5). In the phylogenetic trees, both *Trachelomonas*

and *Strombomonas* are monophyletic groups and sister branches of each other (1.00/100). All strains are separated into seven clades (A–H).

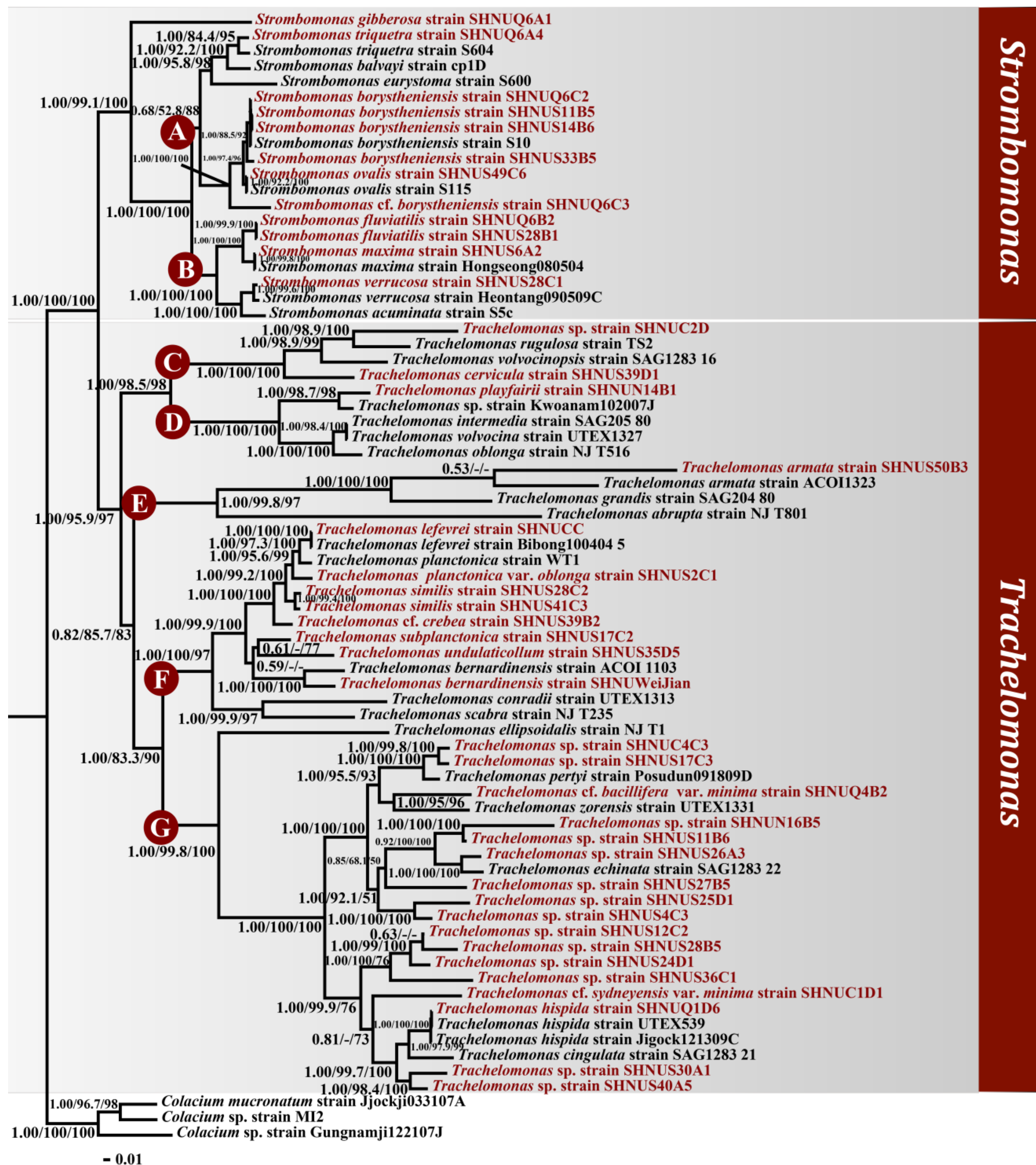

Figure 5. Bayesian phylogenetic tree based on combined nuclear SSU, nuclear LSU plastid SSU and plastid LSU rDNA sequences. Support values for all analyses are shown on branches as follows: Bayesian posterior probabilities (BA)/SH aLRT values/maximum likelihood bootstrap values (ML). The pp values < 0.50, SH aLRT values < 50 or rbs values < 50 was marked with a hyphen (-). Strain collected from China in red.

All *Strombomonas* species fall into the A-B clades. *S. gibberosa* was independent of other *Strombomonas* species. The lorica of *S. gibberosa* has long collar and stout caudal tail. The middle of the lorica is wide-rhomboidal. *S. gibberosa* has 7–9 chloroplasts with haplopyrenoids. Clade A was a relatively low supported clade (0.66/88) of six species, including *S.* cf. *borystheniensis* strain SHNUQ6C3. *S.* cf. *borystheniensis* is closely related to *S. borystheniensis* and *S. ovalis*, and clustered into a subclade (1.00/100). *S. triquetra*,

S. balvayi and *S. eurystoma* clustered into a subclade (1.00/98). Loricae of species in Clade A have short collars, the posterior end rounded or have a short caudate process. All these species have five or more chloroplasts with haplopyrenoids or diplopyrenoid. Clade B contains four species (1.00/100), with *S. fluviatilis* and *S. maxima* clustered into a subclade (1.00/100), *S. verrucosa* and *S. acuminata* clustered into another subclade (1.00/100). These four species have fusiform to broadly elliptic loricae which have long collars and long tails except *S. verrucosa*. Each species had 6–17 chloroplasts with haplopyrenoids.

All *Trachelomonas* species fall into the C-G clades. Clade C and clade D both contain four species that have strong support (1.00/100). The species of Clade C have round loricae without collars and spines. The cells have more than five chloroplasts with haplopyrenoids. Clade D consists of four species with strong support (1.00/100). All species have rectangular loricae with collar or annular thickenings and without spines. Each cell had 8–10 chloroplasts with haplopyrenoids. Clade E contains three closely related species (1.00/97). Loricae of species in clade E are oval, with annular thickenings or short collars and with spines. The cells had more than ten chloroplasts with haplopyrenoids. Clade F contains nine species (1.00/97). *T. lefevrei*, *T. planctonica*, *T. planctonica* var. *oblonga*, *T. similis* and *T.* cf. *crebea* are clustered into a subclade with strong support (1.00/100). *T. subplanctonica*, *T. bernardinensis* and *T. undulaticollum* clustered into another subclade with low support (0.59/-). *T. conradii* and *T. scabra* clustered into a subclade with strong support (1.00/97). These nine species have round or oval loricae with short collars or annular thickenings and are without spines. The cells have 7–18 chloroplasts with haplopyrenoids. Clade G contains 11 species (1.00/100). *T. ellipsoidalis* is independent of other species of *Trachelomonas*. In addition to *T. ellipsoidalis*, other species in the G clade clustered into a subclade with strong support (1.00/100). Species in clade G have the greatest morphological diversity of all the group identified herein. Loricae are rectangular to round, elliptic or nearly round, with short collars or annular thickenings, and possess spines. Each cell had chloroplasts with haplopyrenoids or diplopyrenoid.

4. Discussion

4.1. Stable Morphological Characteristics

The morphologic identification of loricate genera is based on the shape, color, ornamentation of loricae and collar shape. However, these characteristics may change under culture conditions. Poniewozik et al. found that the loricae shape of *T. hispida* changed as the loricae aged [36]. We measured lorica sizes of five *Trachlemonas* species, and found that lorica shape did not change significantly after being cultivated (Figures 1–3 and Figure S1E). The collar became shorter and smaller but was not lost. Poniewozik et al. showed that the loricae surface could not stably form spines [36]. The cone spines on the loricae surface in our cultured strains did not grow steadily. Moreover, the loricae color was light to dark during different periods of development both in culture samples and field samples [21,36]. We also found this phenomenon in this study. It might have been a result of different rates of incorporating and releasing microelements in and out of the lorica structure. [36,37]. Therefore, we thought the shape of lorica and whether the lorica has a collar are more reliable in identifying species. And spines are also important characteristic of those species in the field samples.

4.2. The Relationship between Phylogenetic Tree and Morphological Classification

Strombomonas was separated from *Trachelomonas* based on its rough and less ornamented lorica surface. Although early molecular data reclassified *Strombomonas* into *Trachelomonas* [10], subsequent studies recovered strongly supported monophyletic *Strombomonas* and *Trachelomonas* clades [11–17]. Our molecular data analysis supported the separation of these two genera. Brosnan et al. [38] also supports the separation of *Trachelomonas* and *Strombomonas* as distinct genera using two sets of morphological characters, loricae development and pellicle strip reduction. Lorica development in *Strombomonas* occurred from the anterior of the cell to the posterior. But for *Trachelomonas*, a layer of mucilage was excreted

over the entire protoplast, followed by creation of the collar at the anterior end. In our cultivation process, this phenomenon was also found. The loricae tails of cultured *Strombomonas* were often missing (Figure 3B,E,J), while those of *Trachelomonas* were intact (Figures 1–3). The protoplasts of *Trachelomonas* and *Strombomonas* species were very similar, and the chloroplasts mostly contained haplopyrenoids or diplopyrenoids. Therefore, our results supported that *Trachelomonas* and *Stromomonas* were independent based on the morphological data of loricae and protoplasts as well as molecular data. However, the systematic classification status of those species which are morphologically intermediate between the two genera need to be analyzed based on more morphology and molecular data.

The morphology of loricae is the only characteristic in the classification and identification of loricate genera [2,39,40]. Huber-Pestalozzi once divided *Trachelomonas* into two sections (Rotundatae, Caudatae) and *Strombomonas* into four sections (Rotundatae, Complanatae, Prismaticae, Intermediae) according to the morphology and ornamentations of loricae [40]. Shi et al. [20] thought that the classification of the two genera by Huber-Pestalozzi lacked protoplast characteristics and was artificial and they did not systematically classify the two genera.

The study of Ciugulea et al. has shown that the molecular phylogeny was inconsistent with Huber-Pestalozzi's classification but was consistent with Pringsheim's [4] classification. [12]. This result is only partly supported by our study. The six groups classified by Pringsheim are interlaced in our phylogenetic tree. Pringsheim placed *T. cervicula* in group six, this species fell in clade C of our study. Clade C also included *T. volvocinopsis* which was placed to group two in Pringsheim. Pringsheim placed *T. pertyi* in group three. It fell into clade G, which also contains *T. zorensis* and *T. hispida* of group one. It may be because Pringsheim [4] only cultivated 13 species, and the classification was using not only the shape of the loricae, but also the color of the loricae and the number of chloroplasts, which were not stable morphological characteristics. It was clear from the phylogeny that classification based on protoplast morphology alone is not sufficient and often misleading.

Our results on the loricate taxa were similar with Ciugulea et al. [12] and Kim et al. [18]. In our study, seven clades with well-defined phylogenetic positions were presented. For *Strombomonas*, the clade relationships and composition were more similar to the results found in the study of Ciugulea et al. [12]. All species of *Strombomonas* were divided into Clade A-B. Clade A corresponded to clade H and clade G in Ciugulea et al. [12]. Our clade B corresponded to clade F in Ciugulea et al. [12]. For *Trachelomonas*, the clade relationships and composition obtained herein were more similar with the study of Kim et al. [18]. Different from the results of Ciugulea et al., we did not recover the sister taxon relationship between clades E and G in this study. Taxa of *Trachelomonas* included in this study divided into Clade C-G. Clade C corresponded to clade D in Ciugulea et al. [12] and to a subclade of clade F2 in Kim et al. [18]. Clade C contained sequences of *T. cervicula* not present before. Clade D corresponded to clade C in Ciugulea et al. [12] and to another subclade of clade F2 in Kim et al. [18]. Clade E corresponded to clade B in Ciugulea et al. [12] and to clade F3 in Kim et al. [18] but added *T. armata* strain SHNUS50B3. Clade F corresponded to clade E in Ciugulea et al. [12] and to clade F4 in Kim et al. [18]. In clade F, there were five species (*T. planctonica* var. *oblonga*, *T. subplanctonica*, *T. undulaticollum*) not present in Ciugulea et al. [12] and Kim et al. [18]. The morphological characteristics of these species were consistent with those concluded by Ciugulea et al. [12]. Clade G corresponded to clade A in Ciugulea et al. [12] and to clade F1 in Kim et al. [18]. In this clade, *T.* cf. *bacillifera* var. *minima*, *T.* cf. *sydneyensis* var. *minima* were not present in previous studies of Ciugulea et al. [12] and Kim et al. [18]. After adding our additional species, the morphological characteristics of this clade became diversified, with the shape of loricae not only oval, but also round and rectangular to round, and the chloroplasts are with haplopyrenoids or diplopyrenoid.

The pyrenoid nucleus was not easily observed. We think that it is necessary to examine protoplasts of species, although protoplast characteristics are rare and not suitable

for observation. The phylogeny of loricate genera should be established based on the morphology of loricae and protoplast.

5. Conclusions

We collected 13 species of *Trachelomonas* and eight species of *Strombomonas* in China. We isolated and cultured specimens from field samples to observe the stability of loricae, and supplemented the protoplast characteristics of species. We extracted DNA, amplificated and sequenced four genes from the cultured samples. A phylogenetic tree was constructed based on Bayesian and maximum likelihood methods. The results showed that the shape of the lorica, whether the lorica has a collar and spines could be used to identify species. Our data support *Trachelomonas* and *Strombomonas* as separate genera. We define the phylogenetic location of each clade. Species of each clade identified with molecular methods share common morphological characteristics.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d14080623/s1, Table S1: The collection localities of strains in this study, Table S2: Relevant sequence information downloaded from GenBank in this study. Figure S1: Box plot display of loricae length, loricae width, collar length, collar width, loricae length/loricae width and collar length/collar width of field samples and cultured samples.

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