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ORIGINAL ARTICLE

Redescription of a Hymenostome Ciliate, *Tetrahymena setosa* (Protozoa, Ciliophora) Notes on its Molecular Phylogeny

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ABSTRACT

In recent years, *Tetrahymena* species have been used as model organisms for research in a wide range of fields, highlighting the need for a fuller understanding of the taxonomy of this group. It is in this context that this paper uses living observation and silver staining methods to investigate the morphology and infraciliature of one *Tetrahymena* species, *T. setosa* (Schewiakoff 1892 *Verh. Naturh. Med. Ver. Heidelb.*, 4:544) McCoy (1975) *Acta Protozool.*, 14:253; the senior subjective synonym of *T. setifera* Holz and Corliss (1956) *J. Protozool.*, 3:112; isolated from a freshwater pond in Harbin, north-eastern China. This organism can be distinguished from other described *Tetrahymena* species mainly by its single caudal cilium, which is about twice the length of the somatic ciliature. While the Harbin isolate appears similar to the population described by Holz and Corliss (1956) *J. Protozool.*, 3:112, an improved diagnosis for *T. setosa* is given based on the previous descriptions and the Harbin population. In summary, this species can be recognized mainly by the combination of the following characters: body in vivo approximately 40 μm × 25 μm, 21–26 somatic kineties, one to four contractile vacuole pores associated with meridians 6–11 and a single caudal cilium. The small subunit ribosomal (SSU) rRNA gene and the *cox1* gene sequences of Harbin population are also characterized in order to corroborate that the isolated species branches in phylogenetic trees as a *T. setosa* species. The phylogenetic analysis also indicated that sequences of populations of *Tetrahymena* species should be published with detailed morphological identifications.

IN recent years there have been numerous reports on the cytobiology, epigenetics and molecular ecology of *Tetrahymena* species (Cervantes et al. 2015; Chen et al. 2016, 2018; Czapik 1968; Gao et al. 2013; Liu et al. 2016; Mochizuki et al. 2002; Wang et al. 2017a,b,c; Xiong et al. 2016; Zhao et al. 2017). Nonetheless, many species of this "well-known" group have still not been diagnosed/defined based on taxonomic methods and thus the species names remain invalid according to the International Code of Zoological Nomenclature (Cherry and Blackburn 1985; Corliss 1971; International Commission on Zoological Nomenclature (ICZN) 1999). Furthermore, many nominal species of this group are inadequately investigated with regards to current taxonomic criteria and lack gene sequence data (Nanney 1953; Nanney and McCoy 1976; Nanney et al. 1980; Nyberg 1981). Additionally, *Tetrahymena* species have relatively simple body structures with few morphological characters for species circumscription, which makes them difficult to separate from each other (Chung and Yao 2012; Gao et al. 2016, 2017; Jerome et al. 1996; Liu et al. 2017). Consequently, further investigations of this group are needed using a combination of morphological and molecular data (Chantangsi and Lynn 2008; Hill 2012; Nanney and McCoy 1976).

Genus *Tetrahymena* was established by Furgason (1940), and Gruchy (1955) first reported the heterogeneity of *T. pyriformis* by describing eight "syngens". Holz and Corliss (1956) established the new species *T. setifera*, the specific name of which indicated the sole feature which easily separated the ciliate from most other species in the genus (e.g. the well-known type species *T. pyriformis*), and subsequently Corliss (1971) established a lectotype for this species (later the neotype for *T. setosa*). Holz and Corliss (1956), however, did not consider possible synonymy beyond the confines of the genus (Kahl 1931, 1943; Schewiakoff 1892, 1893; Vuxanovici 1960). In this regard, McCoy (1975) drew attention to Schewiakoff's (1892, 1893) description of *Glaucoma setosa* and supposed that this species should in fact be assigned to the genus *Tetrahymena*; noting that some features of *G. setosa* agree well with those of *T. setifera* as described by Holz and Corliss (1956). Accordingly, McCoy proposed that *T. setifera* and *T. setosa* were synonymous (McCoy 1975). To summarize, *T. setosa* resulted from the transfer of *G. setosa* Schewiakoff 1892 and the suppression of *T. setifera* Holz and Corliss 1956 (Corliss 1971; Furgason 1940; Gruchy 1955; Holz and Corliss 1956; McCoy 1975; Schewiakoff 1892, 1893).

Like most other species in this genus, *T. setosa* (Schewiakoff 1892) McCoy 1975 has not been investigated based on a combination of morphological and molecular data. In the present study, therefore, we provide a detailed morphological description, high quality illustrations and photomicrographs of *T. setosa*, along with molecular phylogenetic analyses based on SSU rRNA and *cox1* gene sequences.

MATERIALS AND METHODS

Sample collection and identification

Tetrahymena setosa was collected on 10 Sep 2017 from a eutrophic and saprobic freshwater pond (45°57′58″N; 126°36′48″E) at Hulan district in Harbin, Heilongjiang province, north-eastern China (water temperature 18 °C, pH 7.5). About 0.5 l of water was collected from 0.1 to 0.5 m below the surface using a sampling bottle. Ciliates were maintained in habitat water in Petri dishes as raw cultures at room temperature (ca. 25 °C) with rice grains added to enrich the growth of bacteria as food. Isolated cells were observed and photographed in vivo using differential interference contrast microscopy. Silver carbonate (Foissner 1992) and Chatton-Lwoff silver nitrate (Chatton and Lwoff 1930) staining methods were used to reveal the infraciliature and argyrome, respectively. Counts and measurements of stained specimens were performed at magnifications of 100–1,250×. Drawings were made with the help of a drawing device. Systematics and terminology are mainly according to Elliott (1973) and Lynn (2008).

DNA extraction, PCR amplification and sequencing

A *Tetrahymena setosa* cell with an obvious caudal cilium (having been morphologically identified in vivo) was washed with distilled water. Genomic DNA was extracted from the single cell using a DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. The SSU rRNA gene was amplified with the primers 82F- (5′-GAA ACT GCG AAT GGC TC-3′) and 18s-R (5′-TGA TCC TTC TGC AGG TTC ACC TAC-3′) (Jerome et al. 1996; Medlin et al. 1988). Primers F298dT (5′-TGT AAA ACG ACG GCC AGT GCN CAY GGT YTA ATN ATG GT-3′) and R1184dT (5′-CAG GAA ACA GCT ATG ACT ADA CYT CAG GGT GAC CRA AAA ATC A-3′) were used to amplify a fragment of *cox1* gene (~850 bp) according to Strüder-Kypke and Lynn (2010). Bidirectional sequencing was performed by the Shanghai Sunny Biotechnology Company (Shanghai, China).

Comparison of SSU rRNA gene and *cox1* gene sequences within several closely related *Tetrahymena* species

Seven SSU rRNA gene sequences (in the red frame in Fig. 2) were downloaded, together with that of the newly sequenced population of *T. setosa* (accession numbers are shown in Fig. 2). Sequences were aligned and trimmed at both ends using Bioedit 7.0.1 (Hall 1999). The numbers of unmatched sites were counted one by one.

The newly amplified *cox1* gene of *T. setosa* was aligned with another 17 *cox1* gene sequences (including two other sequences of *T. setosa*, 14 sequences of *T. pyriformis* and one sequence of *T. leucophrys*) using Bioedit 7.0.1 (Hall 1999), then both ends were trimmed and pairwise comparisons were made.

Phylogenetic analyses

The SSU rRNA gene sequence of *Tetrahymena setosa* was aligned with the sequences of 53 other taxa while the *cox1* gene sequence of *T. setosa* was aligned with other 73 taxa downloaded from the GenBank database using the GUIDANCE server. *Ichthyophthirius multifiliis* and *Ophryoglena catenula* were selected as the outgroup taxa for the SSU rRNA gene tree and *Paramecium* species were selected as outgroups for the *cox1* gene tree (Landan and Graur 2008; Penn et al. 2010; Sela et al. 2015). Accession numbers are shown in the phylogenetic trees after the species names. Aligned sequences were trimmed at both ends after the alignment using the program Bioedit 7.0.1 (Hall 1999). Maximum-likelihood (ML) analysis, with bootstrapping of 1000 replicates, was performed with RAxML-HPC2 on XSEDE 8.2.10 (Stamatakis 2014) via the CIPRES Science Gateway (Miller et al. 2010) with GTRGAMMA+I model. Bayesian inference (BI) analysis was carried out using MrBayes 3.2.6 (Ronquist and Huelsenbeck 2003) on the CIPRES Science Gateway with the model GTR+I+G as the optimal choice (selected by MrModeltest v2; Nylander 2004). Markov chain Monte Carlo (MCMC) simulations were run for 10,000,000 generations with a sampling frequency of 100. The first 10,000 trees were discarded as burn-in and all the remaining trees were used to calculate the posterior probabilities

(PP) with a majority rule consensus. SeaView v4.3.3 (Gouy et al. 2010) and MEGA v6.06 (Tamura et al. 2007) were used to visualize tree topologies.

RESULTS AND DISCUSSION

Morphological description of *Tetrahymena setosa* (Schewiakoff 1892) McCoy (1975)

Remarks

Schewiakoff (1892, 1893) described *Glaucoma setosa*, then Kahl (1931, 1943) and Vuxanovici (1960) redescribed it without detailed morphological information. All of these works, however, lacked morphological information investigated using silver-staining technology (Kahl 1931; Schewiakoff 1892, 1893). Holz and Corliss (1956) established the new species *T. setifera* with a brief description of living morphology and infraciliature, then Corliss (1970, 1973), Grolière (1974) and Czapik (1968) reported and redescribed *T. setifera* based on their populations, although detailed morphological or molecular information were lacked in their work. McCoy (1975) recognized that *Glaucoma setosa* should be assigned to the genus *Tetrahymena* as *T. setosa* and proposed the synonymy between *T. setifera* and *T. setosa*, since the latter shares some features with the former. The documentation for *T. setosa* so far still lacks high quality illustrations, photomicrographs or an analysis using a combination of morphological and molecular data. A redescription with detailed morphological information based on the Harbin population, along with an improved diagnosis on the basis of the previous descriptions and the data from the present work, are therefore provided herein.

Living morphology

Body 30–40 μm × 20–25 μm in vivo, oval in outline with anterior end slightly pointed and posterior end broadly truncated (Fig. 1A, H). Buccal cavity small and shallow, elliptic to triangular in outline, approximately 1/6–1/5 of body length, positioned one-quarter of the way down body from anterior end (Fig. 1A). Buccal cilia approximately 8 μm long in vivo (Fig. 1A, K). Somatic cilia densely arranged, approximately 4 μm long (Fig. 1A). Single caudal cilium approximately 8 μm long (Fig. 1A, H, J). Pellicle slightly indented at base of cilia (Fig. 1A, H). Mucocysts about 2 μm long (Fig. 1L). Cytoplasm colourless to greyish, containing several to many large (approximately 3 μm in diameter) bacteria-filled food vacuoles and variable-sized (0.5–1 μm) refringent granules, distributed randomly (Fig. 1A, H–J). One spherical to ovoidal (commonly spherical) macronucleus, approximately 10 μm across, located near body centre (Fig. 1A, D, E, M–O, S). Micronucleus not recognizable in silver preparations. Contractile vacuole subcaudally positioned, approximately 8 μm in diameter, pulsating at intervals of approximately 1 min (Fig. 1A, H, J). Locomotion by swimming moderately fast while rotating about main body axis, sometimes lying motionless or crawling slowly along bottom of Petri dish or detritus.

Infraciliature

Somatic ciliature as shown in Fig. 1C–E, M–O, R. Twenty-one or 22 (mostly 21) somatic kineties (SK) arranged longitudinally, commencing at anterior end of cell, forming a conspicuous anterior suture that extends from anterior end of buccal cavity to small glabrous areas at anterior end (Fig. 1C–E, M–O, R). Two postoral kineties (PK); PK1 commences anteriorly at level of mid-portion of paroral membrane and extends posteriorly nearly to end of cell; PK2 commences near posterior end of paroral membrane (Fig. 1C, M, N). Postoral kinety 1 and PK2 containing 19–23 and 15–18 monokinetids, respectively (Fig. 1C, M, N). Buccal apparatus as shown in Fig. 1C, D, P, exhibiting typical tetrahymenal organization. Three membranelles located on left wall of cavity. Membranelle 1 (M1) and membranelle 2 (M2) about equally long, positioned close to each other and each composed of three diagonally oriented rows of kinetids. Membranelle 3 (M3) much shorter, two- or three-rowed (Fig. 1C, D, P). Paroral membrane (PM) with paired basal bodies organized in zigzag pattern, located on right edge of the buccal cavity and extending anteriorly to anterior end of M2 (Fig. 1C, D, P).

Argyrome

Basal bodies of ciliary row connected by primary silverline meridians which produce many minute, transversely oriented cross-fibres (Fig. 1G). Secondary silverline meridians not observed (Fig. 1G). Two or three contractile vacuole pores located associated with meridians 9–11 (Fig. 1F). Three argentophilic granules encircled by a delicate argentophilic fibril located at the geometric centre of the naked posterior polar area, which is designated as the "polar basal granule-complex" (Fig. 1F).

Comparison of the Harbin population with previous populations of *Tetrahymena setosa*

Tetrahymena setosa resulted from the transfer of *G. setosa* Schewiakoff 1892 to the genus *Tetrahymena* and and the suppression of *T. setifera* Holz and Corliss 1956; and it has been described many times (Corliss 1970, 1973; Czapik 1968; Grolière 1974; Holz and Corliss 1956; Kahl 1931, 1943; McCoy 1975; Vuxanovici 1960). The Harbin isolate appears similar to the previous descriptions in body size (mostly about 40 μm ×25 μm in vivo), the pattern of infraciliature, buccal apparatus with a typical tetrahymenal organization, and the habitat (Fig. 1B; Corliss 1970, 1973; Czapik 1968; Grolière 1974; Holz and Corliss 1956; Kahl 1931, 1943; McCoy 1975; Vuxanovici 1960). The harbin population differs from the previous populations in having a different body shape (slightly slender in the present work), fewer somatic kineties (21 or 22 in the present work vs. mostly 22–24 in the previous studies) and the locations of the contractile vacuole pores associated with meridians (9–11 in the present work vs. 6–10, mostly 8 or 9 in the previous studies), which we consider to be population-dependent variations (Corliss 1970, 1973; Czapik 1968; Grolière 1974; Holz and Corliss 1956; Kahl 1931, 1943; McCoy 1975; Vuxanovici 1960).

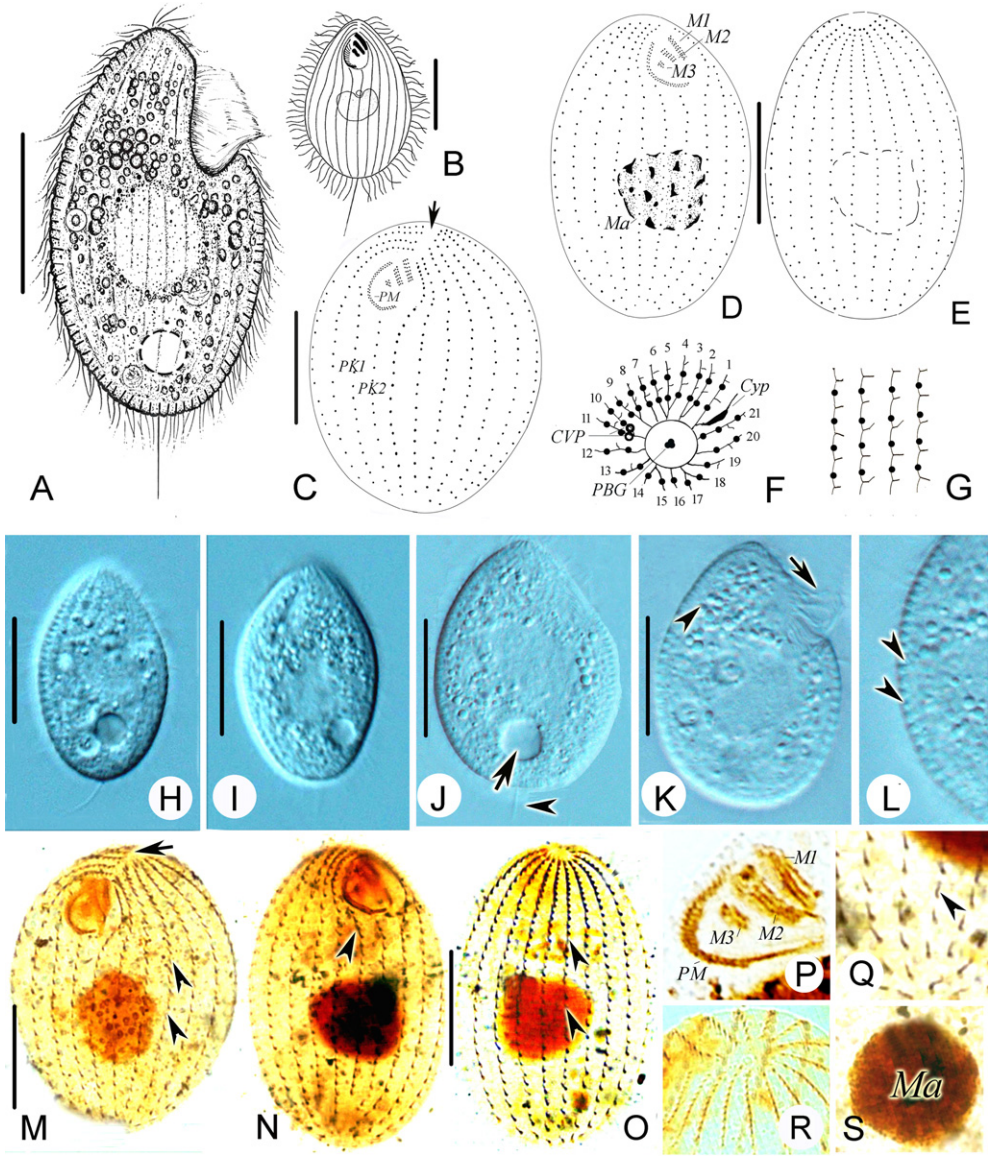

Figure 1 *Tetrahynema setosa* from life (**A**, **B**, **H**–**L**) and after carbonate- (**C**–**E**, **M**–**S**) and silver nitrate- (**F**, **G**) staining. (A, H) Right-ventral views of a representative individual. (B) Ventral view of the junior synonym *T. setifera* Holz and Corliss 1956. (C) Ventral view, to show postoral kineties and paroral membrane, arrow marks anterior suture. (D, E) Infraciliature in ventral (D) and dorsal (E) view. (F, G) Argyrome of the voucher specimen: caudal view (F) showing contractile vacuole pores and cytoproct; part of dorsal argyrome (G) showing the meridians with minute, transversely oriented cross-fibres. (I, J) Different individuals, arrow and arrowhead in (J) mark contractile vacuole and caudal cilium, respectively. (K) Ventral view, arrow and arrowhead show buccal cilia and food vacuoles, respectively. (L) Cell margin, arrowheads show mucocysts. (M–O, Q) Different individuals, arrow in (M) shows anterior suture, arrowheads in (M, O, Q) mark somatic kineties, and arrowhead in (N) denotes postoral kinety 1. (P) To show the details of the oral apparatus. (R) Anterior part of cell. (S) Macronucleus. CVP, contractile vacuole pores; CYP, cytoproct; M1–3, membranelles 1, 2 and 3; Ma, macronucleus; PBG, polar basal granule-complex; PM, paroral membrane; PK1, 2, postoral kineties 1 and 2. Bar, 15 μm.

Comparison of the Harbin population of *Tetrahymena setosa* with morphologically similar species and molecularly related species

Species of the genus *Tetrahymena* have a relatively simple body with few morphological characters for species circumscription (Borden et al. 1977; Simon et al. 2008). There are only a few nominal "caudal-ciliated" species in the genus that have a caudal cilium: *T. setosa* (Schewiakoff 1892) McCoy 1975; *T. corlissi* Thompson 1955; *T. bergeri* Roque et al. 1970; *T. rostrata* (Kahl, 1926) Corliss 1952 and *T. paravorax* Corliss 1957 (Corliss 1952, 1957; McCoy 1975; Roque et al. 1970; Stout 1956). Consequently, comparisons here are made only between the Harbin population of *T. setosa* and four other species: *T. corlissi* can be easily separated from *T. setosa* since it

has more somatic kineties (27 on average in *T. corlissi* vs. 21 on average in Harbin population of *T. setosa*) and a larger body size (approximately 50 μm × 30 μm in *T. corlissi* vs. approximately 35 μm × 20 μm in Harbin population of *T. setosa*) (Hoffman et al. 1975; Thompson 1955). *Tetrahymena rostrata* differs from *T. setosa* by having more somatic kineties (30 on average in *T. rostrata* vs. 21 on average in Harbin population of *T. setosa*) (Corliss 1952; McCoy 1975; Stout 1956). *Tetrahymena paravorax* can be easily separated from *T. setosa* by having more somatic kineties (26 on average in *T. paravorax* vs. 21 on average in Harbin population of *T. setosa*) and a much larger body length (115–200 μm in *T. paravorax* vs. approximately 35 μm in Harbin population of *T. setosa*) (Corliss 1957). *Tetrahymena bergeri* is confirmed as a valid species with a unique SSU rRNA gene sequence and life cycle; the main morphological differences with *T. setosa*, however, lie in its rostrum (vs. absent in in Harbin population of *T. setosa*) and a different location of contractile vacuole pores (associated with meridian 6 in *T. bergeri* vs. 9–11 in Harbin population of *T. setosa*) (Roque et al. 1970).

The SSU rRNA gene sequence and the results of the phylogenetic analyses suggest that *Tetrahymena setosa* has a close relationship with the *T. pyriformis* complex. As is shown in Fig. 2, four populations of *T. pyriformis*, *T. leucophrys*, *T.* aff. *pyriformis*, and two populations of *T. setosa* (including the Harbin population), are grouped in one clade. Notably, the Harbin population of *T. setosa* has no sequence difference with two of the *T. pyriformis* populations (EF070254 and EF070255), and the previous population of *T. setosa* (AF364041) also shares 100% sequence similarity with another two populations of *T. pyriformis* (X56171 and M98021). This result is consistent with several previous studies that have demonstrated that there are no nucleotide differences in the SSU rRNA gene sequences between *T. pyriformis* and *T. setosa* (Nanney et al. 1980; Preparata et al. 1989; Strüder-Kypke et al. 2001). Morphologically, when *T. setosa* is compared with those *T. pyriformis* complex species (*T. australis*, *T. shanghaiensis* and *T. empidokyrea*) that have morphological descriptions in their original reports (Corliss 1970, 1973; Feng et al. 1988; Foissner et al. 1994; Jerome et al. 1996; Liu et al. 2016), it can be easily distinguished by the presence of a caudal cilium (vs. absent in *T. australis*, *T. shanghaiensis* and *T. empidokyrea*). Furthermore, when one incorporates an analysis of sequence similarities between the Harbin population and the populations of the *T. pyriformis* complex and *T. leucophrys* based on the *cox1* gene, these are 93.3%, 92.9–97.9% and 88.9%, respectively; showing the obvious divergence between *T. setosa* and *T. pyriformis* complex.

SSU rRNA gene and the *cox1* gene sequence data

The SSU rRNA gene sequence of *Tetrahymena setosa* has been deposited in the GenBank database with the accession number, length and G+C content as follows: MH539651, 1,646 bp (not including 82F and 18SR primer sites), 43.01%.

The *cox1* gene sequence of *Tetrahymena setosa* we obtained was 841 bp in length (without including primers), with a 26.87% GC content, and the *cox1* gene sequence of *Tetrahymena setosa* has been deposited in the GenBank database with the accession number MH550658.

Phylogenetic analyses based on SSU rRNA gene sequences

The topologies of the ML and BI trees based on SSU rRNA gene sequences were basically congruent, albeit with variable support values; therefore, only the topology of the ML tree was shown with support values from both methods (Fig. 2). In line with previous studies of Tetrahymenidae based on analysis of SSU rRNA gene sequences, it is shown that: (1) the family Tetrahymenidae is monophyletic and divided into three clades; (2) excluding the two species of *Lambornella* sp. (JQ723973 and AF364303), the *Tetrahymena* genus also divided into three lineages, which are called the "borealis group", "australis group" and "riboset", respectively. The ribose contains only one species *T. caudata*, is clearly differentiated from marked clade II showed in Fig. 2 and constituts a third clade, which is slightly different from "classic" groupings (divided into two "major" groups: borealis group and australis group, *T. caudata* was placed in the australis group by NJ but in the borealis group by MB and ML) (Chantangsi and Lynn 2008).

As is shown in the ML tree based on SSU rRNA gene sequences (Fig. 2), the Harbin population of *T. setosa* clusters in the clade containing the previous population of *T. setosa* (AF364041, Strüder-Kypke et al. 2001), four populations of *T. pyriformis* (X56171, M98021, EF070254, EF070255) and other two *T.* spp. (*T. leucophrys* and *T.* aff. *pyriformis*) with a moderate support (40% ML, 0.88BI). Notably, in the red frame in Fig. 2, there are two "*pyriformis-setosa*-clustering" groups separated by *T. leucophrys* and *T.* aff. *pyriformis*, with each group containing two *T. pyriformis* populations and one *T. setosa* population. A plausible explanation for the grouping pattern of the species *T. setosa* and *T. pyriformis* is that: (1) *Tetrahymena pyriformis* (Ehrenberg, 1830) Lwoff, 1947 is now known to be a species complex, in which independent species are sometimes misidentified as morphologically similar species (Gao et al. 2016; Liu et al. 2016; Nanney and McCoy 1976; Strüder-Kypke et al. 2001); (2) concerning the upper "*pyriformis-setosa*-clustering" group in the red frame, *T. setosa* (AF364041) is probably in fact *T. pyriformis*, since Strüder-Kypke et al. (2001) did not provide any morphological information of their population of *T. setosa* thus the identification cannot be checked; (3) the two populations of *T. pyriformis* clustered with the Harbin population of *T. setosa* in the lower group in the red frame are probably *T. setosa* based on the clustering pattern of these three populations. Unfortunately, no morphological data of the two populations of *T. pyriformis* have been provided and thus their identifications cannot be checked. To sum up, the new grouping pattern for *T. setosa* and *T. pyriformis* based on SSU rRNA gene sequences is:

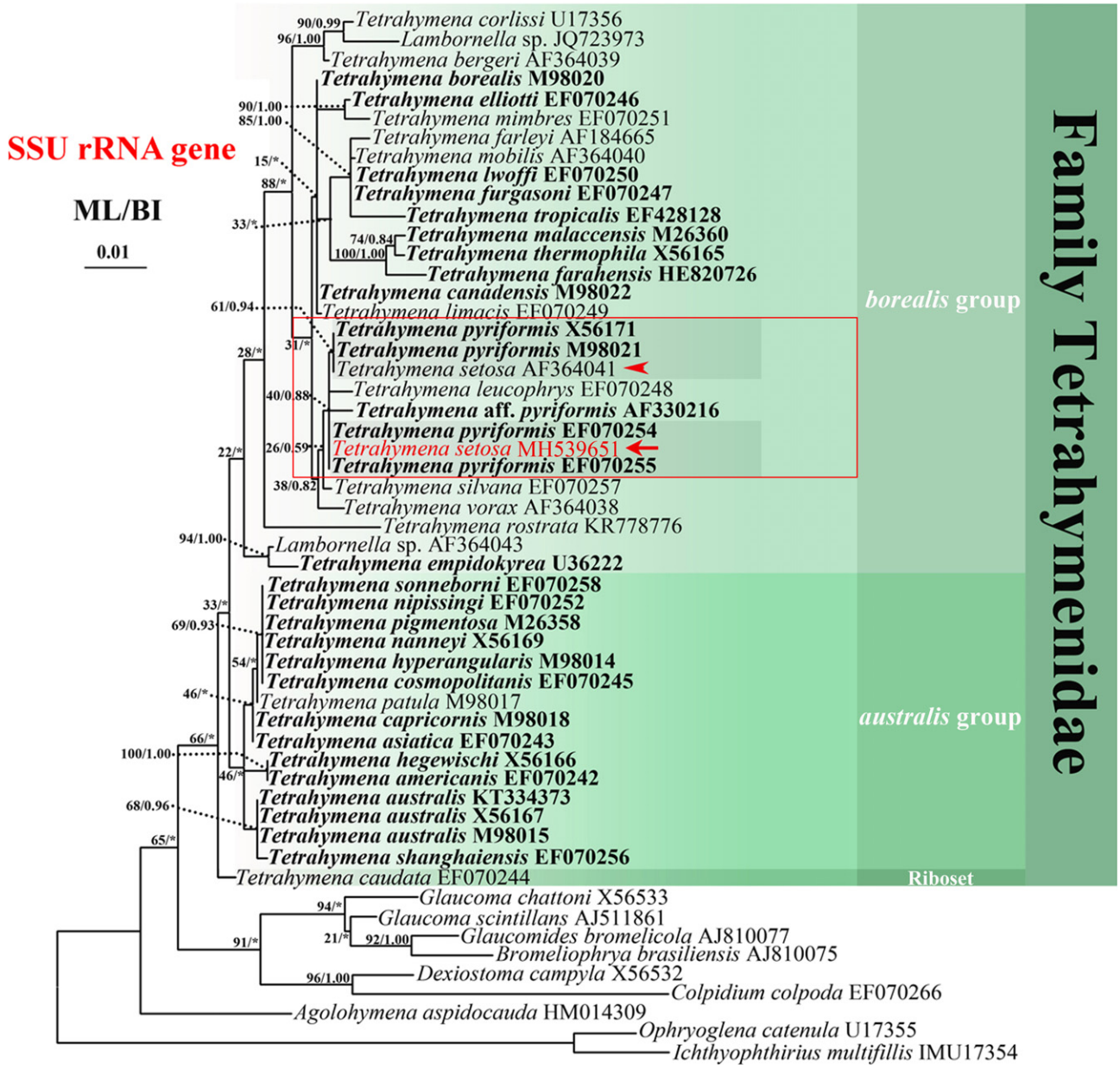

Figure 2 The Maximum likelihood (ML) tree inferred from the SSU rRNA gene sequences, showing the position of newly sequenced Harbin population of *Tetrahymena setosa* (indicated in red and marked with an arrow) and another population of *T. setosa* (arrowhead). All species belonging to *T. pyriformis* complex are in bold font. The red square frame marks the close relationship between *T. setosa* and *T. pyriformis*. Species with a red box might be *T. pyriformis*. Question marks indicate the species with no morphological data and their identifications cannot be checked. Numbers near the branches represent bootstrap values of ML and BI analyses. * indicates the disagreement in topology between ML and BI trees. All branches are drawn to scale. GenBank accession numbers are given for each species. Scale bar corresponds to one substitutions per 100 nucleotide positions.

three populations of *T. pyriformis* clustering together with a high support (61% ML, 0.94BI), while three populations of *T. setosa*, containing the Harbin population group, also clustering together, with these two groups being separated by *T. leucophrys* and *T.* aff. *pyriformis*. Besides, the two *Lambornella* species, which also assigned to the family Tetrahymenidae, both group into the *Tetrahymena* genus. These two sequences of *Lambornella* species (JQ723973 and AF364303) were also used in Dunthorn et al. (2012) and Strüder-Kypke et al. (2001). We agree with Strüder-Kypke et al. (2001) that "since we were unable to culture, stain and identify our *Lambornella* species, they might have been contaminant *Tetrahymena* from the tree-hole habitat".

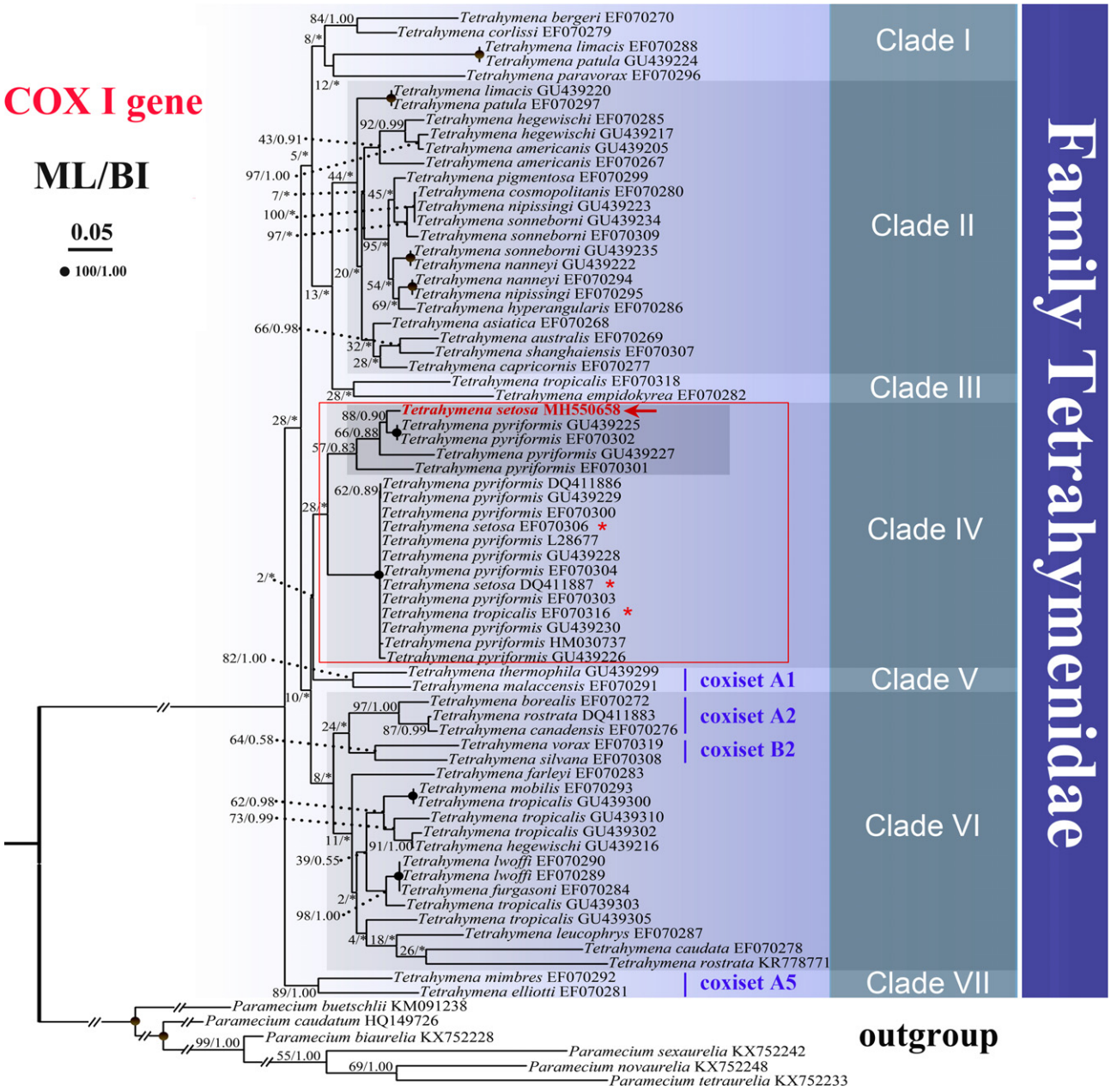

Figure 3 The Maximum likelihood (ML) tree inferred from the mitochondrial cytochrome *c* oxidase subunit I (*cox 1*) gene sequences, showing the position of newly sequenced Harbin population of *Tetrahymena setosa* (indicated in red and marked with an arrow). Numbers near the branches represent bootstrap values of ML and BI analyses. Well supported nodes (100% ML, 1.00 BI) are represented by solid circles. The red square frame marks the close relationship between *T. setosa* and *T. pyriformis*. Species with a red box might be *T. pyriformis*. Question marks indicate the species with no morphological data and their identifications cannot be checked. The blue lines show the identical coxisets which exist in Chantangsi and Lynn (2008). * in black indicates the disagreement in topology between ML and BI trees. Seven long branches have been shortened, as shown by "//", and the other branches are drawn to scale. GenBank accession numbers are given for each species. Scale bar corresponds to five substitutions per 100 nucleotide positions.

Phylogenetic analyses based on *cox1* gene sequences

The topologies of the ML and BI trees that were constructed based on the *cox1* gene sequences were also basically congruent with variable support values; therefore, only the topology of the ML tree was shown with support values from both methods (Fig. 3). *Cox1* gene sequences of the two populations of *T. setosa* both firstly cluster with two populations of *T. pyriformis,* respectively, and then group together in one clade.

We believe that the plausible reasons for the grouping pattern evident in the phylogenetic trees based on the

Table 1. Morphometric data of the Harbin population of *Tetrahynema setosa*

Character	Min	Max	Mean	*M*	SD	CV	*n*
Body length, μm	33	49	39.1	36	4.4	10.2	21
Body width, μm	22	29	25.3	25	2.7	10.3	21
Buccal field length, μm	6	8	7.2	7	1.1	15.7	15
Buccal field width, μm	4	6	4.9	5	0.2	4.2	15
Somatic kineties, number	21	22	21.2	21	1.9	9.0	15
Postoral kineties, number	2	2	2	2	0	0	15
Macronucleus length, μm	11	13	12.0	12	0.2	1.7	15
Macronucleus width, μm	11	13	12.1	12	0.3	2.5	15
Kinety rows in membranelle 1	3	3	3.0	3	0	0	11
Kinety rows in membranelle 2	3	3	3.0	3	0	0	11
Kinety rows in membranelle 3	2	3	2.8	3	0.2	6.2	11
Membranelle 1 length, μm	3	4	3.6	4	1.4	9.3	11
Membranelle 2 length, μm	3	4	3.5	3	0.1	3.3	11
Number of monokinetids in postoral kineties 1	19	23	20.2	20	2.1	10.5	14
Number of monokinetids in postoral kineties 2	15	18	17.2	18	1.4	7.8	13

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

cox1 gene sequences (Fig. 3) are that: (1) thirteen sequences with almost no nucleotide differences in the posterior part of the red frame (clade IV) are all considered to be from *T. pyriformis*, except the three forms marked with "*" (misidentified as *T. setosa* or *T. tropicalis*); (2) the five species clustered in one clade (except the Harbin population of *T. setosa*) in the anterior part of the red frame are probably not in fact *T. pyriformis* but perhaps two or three unknown independent species having affinities with *T. pyriformis*. The result revealed that sequence should be published with detailed morphological characterization of the sequenced population, and it is necessary to use a single clone for all studies (morphology and gene sequence analyses). Based on the *cox1* gene sequences, Chantangsi and Lynn (2008) divided the tetrahymenine species, including *Colpidium*, *Glaucoma*, and *Tetrahymena*, into 12 groups, the so-called coxisets. Nine of the 12 coxisets were consisted of *Tetrahymena* genus. Compared with the nine coxisets, we find that the seven clades in our study are with some different grouping and placement of taxa. Coxisets A1 and A5 are, respectively identical to clade V and VII and the coxisets A2 and B2 are located in Clade VI.

TAXONOMIC SUMMARY

Class Oligohymenophorea de Puytorac et al. 1974
Subclass Hymenostomatia Delage and Hérouard, 1896
Order Tetrahymenida Fauré-Fremiet in Corliss, 1956
Family Tetrahymenidae Corliss (1952)
Genus *Tetrahymena* Furgason (1940)
Tetrahymena setosa (Schewiakoff 1893) McCoy (1975) (Fig. 1; Table 1)

1892 *Glaucoma setosa* Schewiakoff, Verh. Naturh. Med. Ver. Heidelb., 4: 554.
1893 *Glaucoma setosa* Schewiakoff (1892), – Schewiakoff, Mem. Acad. Imper. Sci. St. Petersb. Ser., 41: 201
1931 *Glaucoma setosa* Schewiakoff (1892) – Kahl Tierwelt. Dtl., 21: 329
1943 *Glaucoma setosa* Schewiakoff (1892) – Kahl, Franckh'sche Verlagshandlung, Stuttgart, pp 46
1956 *Tetrahymena setifera* Holz and Corliss J. Protozool., 3:112–118.
1960 *Glaucoma setosa* Schewiakoff (1892) – Vuxanovici Studii Cerc. Biol, 12:363.
1968 *Tetrahymena setifera* Holz and Corliss (1956) – Czapik, Acta Protozool, 5:326.
1970 *Tetrahymena setifera* HOLZ and Corliss, 1956 – Corliss, J. Protozool., 17:202.
1973 *Tetrahymena setifera* Holz and Corliss (1956) – Corliss, Biology of *Tetrahymena*, p. 19
1974 *Tetrahymena setifera* Holz and Corliss (1956) – Grolière, Protistologica, 10:328.
1975 *Tetrahymena setosa* (Schewiakoff 1893) Mccoy, Acta Protozool., 14:253–260.

Improved diagnosis

Body in vivo approximately 40 μm × 25 μm; 21–26 somatic kineties; buccal ciliature typical of genus; contractile vacuole sub-caudally positioned; one to four contractile vacuole pores associated with meridians 6–11; single, slender caudal cilium, about twice the length of the somatic ciliature. Freshwater habitat.

Deposition of specimens

Two slides containing silver nitrate-stained voucher specimen encircled in black ink are deposited in the Laboratory of Protozoology, Harbin Normal University of China with registration numbers PMM-2017091001 -01 and -02.

Occurrence and ecology

The Harbin isolate of *Tetrahymena setosa* was collected from a eutrophic and saprobic freshwater pond (45°57′58″ N; 126°36′48″E) at Hulan district in Harbin, north-eastern China with water temperature 18 °C, and pH 7.5, while the type locality of its synonym *T. setifera* was a pond at the Jamesville Reservoir, Jamesville, New York. To date, this species has been recorded from only freshwater.

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