Taxonomy, diversity and diagnosis of Tetrahymenosis, and its recent identification measures in aquaculture inferred from infections in Northeastern China

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Short report

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Abstract

Background Tetrahymenosis caused by about ten parasitic Tetrahymena species belonging to the Phylum Ciliophora has been recognized as an emerging problem inflicting significant economic loss in aquaculture industry in the world. Increasing knowledge and identification of Tetrahymenosis are important.

Methods Four parasitic Tetrahymena species were collected from eight commercially farmed fishes in Harbin, northeastern China. Specific oligonucleotide probe and fluorescence in situ hybridization staining was designed and tested. Hoechst33342 staining methods, gene sequencing and phylogenetic analyses were also conducted.

Results Tetrahymena pyriformis, T. vorax, T. chironomi and T. bergeri are the four species responsible for Tetrahymenosis in various fishes in Harbin. Taxonomy, diagnosis, diversity, pathogenicity, and histopathology of Tetrahymenosis were supplemented, analyzed and summaried, based on the present and previous work. The term Tetrahymenosis is diagnosised as diseases affecting a number of fishes, crustaceans, mollus, beetle, dragonfly, salmon, slug, midge larvae and freshwater mussel species that caused by ciliates of the genus Tetrahymena which constitutes an abundant group inhabit various aquaculture and natural habitats. Improved classification of parasitic Tetrahymena was provided. Phylogeny of parasitic Tetrahymena species was studied, and an SSU-rDNA targeted oligonucleotide probe labeled with a fluorochrome was designed, and the FISH protocol was optimized for identification of Tetrahymena species,

Conclusions The manifestations of histopathology in fish typically include lesions on the body surface, and affected organs include the skin, musculature, viscera, eye socket and spinal cord; masses of ciliates can be detected in copious amount of mucus and between spaces in the damaged tissues. Improved classification of parasitic Tetrahymena is provided: facultatively parasitic forms (T. pyriformis , T. rostrate , T. bergeri and T. vorax), facultatively free-living forms (T. chironomi , T. corlissi, T. rotunda, T. glochidiophila and T. papula) and parasitic forms (T. stegomyiae and T. limacis).The phylogenic results indicate that Tetrahymena spp. belonging to 'borealis' group have a greater probability to become parasitism. the method of which can be used for quick and early detection of Tetrahymenosis.

Background

Tetrahymena, assigned to class Oligohymenophorea and family Tetrahymenidae, is a pyriform-shaped ciliate, with buccal cavity equipped with one paroral membrane and three serially arranged membranelles [1–15]. Occasionally, this microorganism can become parasitic and affect a wide range of hosts, including ornamental fish, edible fish, beetle, dragonfly, salmon, slug, midge larvae and freshwater mussel [16–21]. Pathogenic characteristics of infected fishes by Tetrahymena spp. are similar: possessing typical whitish lesions on the body surface, and affected organs include the skin, musculature, viscera, eye socket and spinal cord; masses of ciliates can be detected in copious amount of mucus and between spaces in the damaged tissues [16–18, 22]. Some of them are facultative parasites while others might be obligate parasites [5]. The most pathogenic species of this genus, according to published literature, is T. corlissi, which often leads to a systemic infection and well-known as "guppy killer disease" [23–25]. They are responsible for serious economic losses in commercial fish farms around the world.

While morphological method is the routine way to identify Tetrahymena spp., it has the limitations as follows: 1) the commonly used silver staining methods for reliable identification need considerable experience and staining failure is often the case, making species identification difficult, 2) parasitic Tetrahymena species are hard to control when massive infection occurs. Thus, early detection, quantification, and control of these infectious pathogens are important in aquaculture [1, 26]. FISH with specific fluorochrome-labeled oligonucleotide probes is a staining technique that allows molecular identification of targeted organisms in mixed assemblages by means of a fluorescence microscopy, this method can overcome the drawbacks of the morphological method and yield prompt detection, and has been applied successfully to pathogenic ciliates (e. g. Pseudocohnilembus persalinus, Boveria labialis and B. subcylindrica) [27–30]. However, to date, there are no general fluorochrome-labeled oligonucleotide probes for parasitic Tetrahymena spp.

Methods

Ciliates isolation, cultivation and morphological identification.

Tetrahymena pyriformis, T. vorax, T. chironomi and T. bergeri were collected from commercially farmed fishes e. g. Malapterurus electricus, Tetraodon palembangensis, Poecilia latipinna, Channa aurantimaculata, Carassius auratus, zebrafish, Phoxinus lagowskii, Pelteobagrus fulvidrac, from fish farms or markets in Harbin, northeastern China between September 2017 and November 2019. Mucus or tissue were spread on glass slides using glass slides and the fresh mounts examined under a light microscope (20X and 40X objective) to confirm infection. Slides containing ciliates were selected for silver staining and morphological description. Cells were cultured at 25 °C and maintained in a culture medium (sterilized distilled water boiled with fish meat, stored at -20 ° C). Live observations were made under a microscope with phase-contrast illumination. Silver carbonate [31] and protargol [32] staining methods were used to reveal the infraciliature. All measurements of stained specimens were made under oil immersion (1250 x).

Design and test of the specific oligonucleotide probe and FISH staining.

Probe (nucleotides, 19; GC contents, 47.37%; nucleotide-nucleotide Tm, 53.1 ^{o}C) was designed using the probe design tool as implemented in the ARB software package for the SSU-rDNA sequence of the present Tetrahymena pyriformis, T. vorax, T. chironomi and T. bergeri, and other Tetrahymena species from GenBank [30]. Generated probes were checked against the GenBank sequence collection by a standard nucleotide-nucleotide BLAST search [33]. FISH was used to visualize Tetrahymena spp. above both in field samples and a mixture of species as well as Pseudocohnilembus persalinus and Uronema marinum that frequently occurred in the same hosts as the negative controls. Cells were fixed with 50% Bouin's solution and filtered onto a 2-μm-pore-size cellulose nitrate membrane (25 mm in diameter) using low under pressure. The membrane was then washed five times with 2 ml of filtered sterile water. The basic hybridization followed the protocol of Stoeck et al. (2003) and Zhan et al. (2014).

Hoechst33342 staining methods.

Cells of parasitic Tetrahymena species were stained and fixed in 4% PFA for 20 min at room temperature, then were washed with PBS for 5 min at room temperature. Cells were then incubated with Hoechst 33342 (100 ng/mL in PBS) in the dark for 15 min at room temperature, then cells were washed with PBS for 5 min at room temperature. At last, cells were viewed using a fluorescence microscope with emission 350 nm and a blue/cyan emission filter (460 nm) (Fig. 3) [34].

Gene sequencing and Phylogenetic analyses.

Single cells of Tetrahymena pyriformis, T. vorax, T. chironomi and T. bergeri were washed individually with distilled water. Genomic DNA was extracted from five cells 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 18 s-R (5'-TGA TCC TTC TGC AGG TTC ACC TAC-3') [35]. Bidirectional sequencing was performed by the Shanghai Sunny Biotechnology Company (Shanghai, China). The SSU rRNA gene sequences of T. pyriformis, T. vorax, T. chironomi and T. bergeri were aligned with sequences of other related taxa on the European Bioinformatics Institute web server (URL: http://www.ebi.ac.uk/Tools/msa/muscle/) using the MUSCLE package. Resulting alignments were refined by trimming both ends using BioEdit 7.0.5.2 [36]. Bayesian inference (BI) analysis was carried out with MrBayes on XSEDE v3.2.6 (Ronquist and Huelsenbeck 2003) on CIPRES Science Gateway [37] using the GTR + I + G evolutionary model as the best-fit model selected by MrModeltest v.2 [38] according to the Akaike Information Criterion (AIC). A Maximum Likelihood (ML) tree was constructed using RAxML-HPC2 v. 8.2.10 [39] on the CIPRES Science Gateway with the optimal model GTR + I + G evolutionary model as the best model according to the AIC criterion selected by the program Modeltest v.3.4 [40]. Node support came from 1000 bootstrap replicates. TreeView v.1.6.6 [41] and MEGA v5 were used to visualize tree topologies.

Results

Taxonomy

The term Tetrahymenosis covers diseases affecting a number of fishes, crustaceans, mollus, beetle, dragonfly, salmon, slug, midge larvae and freshwater mussel species that caused by ciliates of the genus *Tetrahymena* which constitutes an abundant group inhabit various aquaculture and natural habitats (Table 1). *Tetrahymena* belongs to the phylum Ciliophora, Class Oligohymenophorea, order Tetrahymenida and family Tetrahymenidae [4]. Currently, the genus *Tetrahymena* Furgason, 1940 (Ciliophora, Hymenostomatida) is comprised of over 40 species [2–5, 13, 19]. *Tetrahymena* is generally pyriform-shaped, uniformly covered with meridional kineties, the buccal cavity is equipped with one curved undulating membrane with paired basal bodies organized in zigzag pattern (i.e. stichodyad), located on right edge of the buccal cavity, and three parallel membranelles. Within species, size variations depend on culture medium, physiological state and life stage (Corliss, 1973). The life cycle of *Tetrahymena* species is typified by the following developmental stages: theront, trophont, tomont and tomite.

Diversity of parasitic *Tetrahymena*

Tetrahymena species commonly exhibits a whole range of existence from the completely free-living state through stages of facultative parasitism to obligate endoparasitism [6]. Corliss (1971a) classified *Tetrahymena* parasitism into three types: facultatively parasitic forms (generally found free-living in nature but for which a parasitic existence is not uncommon), facultatively free-living forms (typically found in association with a host but capable in nature as well as experimentally) and parasitic forms (as endoparasites associated with specific hosts). To date, about ten *Tetrahymena* species (*T. corlissi, T. pyriformis, T. stegomyiae, T. chironomi, T. vorax, T. rostrata, T. limacis, T. rotunda, T. glochidiophila* and *T. papula*; Table 1) have been generally reported as pathogenicity to various invertebrates and vertebrates [16–18, 22].

In the current work, *T. pyriformis, T. vorax, T. chironomi* and *T. bergeri* are the four species isolated from fish farms or markets in Harbin. The cells commonly showed a high densities and vitalities when just isolated from the various fishes (Figs 1, 2). The detailed morphology of the four species are reported before, and the Harbin populations of the four species are similar with those previous descriptions (Figure 2) [1, 3, 7, 42]. The topologies of BI trees are similar to that of ML trees, so only the ML trees are shown in Figure 4 with branch support values for both analyses. In the consensus topology, all the parasitic *Tetrahymena* spp. (Table 1) having sequence data are selected to constructed trees. As is shown, all the parasitic forms cluster in the 'borealis' group. The clustering pattern indicates that 'borealis" *Tetrahymena* spp. might have a greater probability to become parasitism [13, 43]. In addition, Harbin population of *T. pyriformis, T. vorax,* and *T. bergeri* cluster with their previous populations, respectively, which also supports the morphological identifications.

Diagnostic features:

Pathogenicity/Histopathology.

As pathogenic parasites, histopathology of parasitic *Tetrahymena* spp. have been studied in detail and reported many times [16, 18, 22, 44–48]: possessing typical whitish lesions on the body surface, and affected organs include the skin, musculature, viscera, eye socket and spinal cord; masses of ciliates can be detected in copious amount of mucus and between spaces in the damaged tissues. In fishes, extensive studies reporting the organ-specific pathological changes when infected with Tetrahymenosis are available, and typical gross lesions of *Tetrahymena* infection are characterized by protrusion of scales, swelling, ulcerative wounds on the skin, blindness, and protrusion of eyes [47, 48, 22, 18]. In our recent study, four *Tetrahymena* species were isolated *Malapterurus electricus*, *Tetraodon palembangensis*, *Poecilia latipinna*, *Channa aurantimaculata*, *Carassius auratus*, zebrafish, *Phoxinus lagowskii,*

Pelteobagrus fulvidrac from farms in Harbin. At arrival, fish did not have any apparent gross signs of *Tetrahymena* infection. Nonetheless, the parasite was observed in fresh-mount preparations on the skin, in the gills and in internal organs (Figure 2). Noticeable, many cells of *Tetrahymena bergeri* were observed in the skin mucus of *Phoxinus lagowskii*, individuals of *P. lagowskii* exhibited symptoms that no feeding, staying upside down, gasping attack edges and secreting a lot of mucus (Figure 3G, H, arrowhead). *Tetrahymena pyriformis* were observed from guts of diseased zebrafish (Figure 3F, arrowhead). *Pelteobagrus fulvidraco* had many large, round deep skin lesions all over the body with hyperaemic margins exposing the skeletal muscle, and scrapings revealed the presence of *T. vorax* (Figure 2G, arrowheads). Gills of all diseased fish were shriveling, dark and anaemic, gills of several fish had dark-red ulceration (Figure 2I; arrowhead). Some cells of *T. vorax* were also detected in gaps among the gill filaments.

Skin lesions or mucus of fishes were directly stained in vivo by Hoechst33342 staining method, and Figure 3A–C show the process of facultatively parasitic *Tetrahymena* sp. eating bacteria around the cell (Figure 3B), and *Tetrahymena* sp. eating tissues (skin or gill) as well as bacteria of host (Figure 3C). Cytoplasm of *Tetrahymena* sp. always contain many (approximately 2–5 μm in diameter) bacteria-or fish tissue- filled food vacuoles and variable-sized granules, distributed randomly (Figure 3C, arrowhead). The result indicates that facultatively parasitic *Tetrahymena* sp. can physically devour tissue, not only by producing enzyme [49].

Detection and identification using FISH

The probe Tetr2020 evaluated with the probe match tool of the ARB software package and the GenBank BLAST tool showed that they are specific to *Tetrahymena* spp. The probe Tetr2020 (5′- TGTAGTAGCCGTTTCTCAG-3′) had at least two mismatches to other closely related species like *Uronema* spp., *Pseudocohnilembus* spp. and *Cyclidium* spp. FISH with the probes Tetr2020 resulted in the presence of a red fluorescence signal each for *T. pyriformis, T. vorax, T. chironomi* and *T. bergeri* (Figure 3D, E), clearly distinguishable from the faint autofluorescence signal achieved with negative-control hybridizations using the Tetr2020 probe to hybridize the untargeted ciliates *Pseudocohnilembus persalinus*, *Uronema marinum* and isolated from the same hosts (Figure 3F). Thus, the probes Tetr2020 is also a general probe suitable for widely and rapidly detecting Tetrahymenosis.

Discussion

Corliss (1971a) classified *Tetrahymena* parasitism into facultatively parasitic forms (*T. pyriformis* and *T. rostrata*), facultatively free-living forms (*T. chironomi* and *T. corlissi*) and parasitic forms (*T. stegomyiae* and *T. limacis*). However, based on later and the present works (Tables 1, 2), supplementary classification is as follows: facultatively parasitic forms (*T. pyriformis*, *T. rostrate*, *T. bergeri* and *T. vorax*), facultatively free-living forms (*T. chironomi*, *T. corlissi, T. rotunda, T. glochidiophila* and *T. papula*) and parasitic forms (*T. stegomyiae* and *T. limacis*). and the morphology of pathogens and the hosts and data sources are compared in Tables 1 and 2. *Tetrahymena bergeri* is reported in the present work as facultatively parasitic forms in *Channa aurantimaculata* for the first time (free-living reported before), the reason possibly is that high latitude cold areas may have multiple life-styles when cold weather, due to the slow degradations of microorganisms and insufficiency of food [50]. Except for the ten species showed above, many unidentified *Tetrahymena* sp. conducted infections were also recorded, e. g. Stolk (1960) isolated *Tetrahymena* sp. from the central nervous system of larvae of *Cyprinous carpio*, *Abramis brama* and *Ameiurus* sp., Kim et al., (2002) examined the fish imported from Southeast Asian countries into Korea and reported of infection from the consignment of ornamental fish, Laoprasert et al., (2001) reported of tetrahymenosis in guppies from local fish farms, appearing as a white ulcerative disease. Thilakaratne et al., (2003) reported of *Tetrahymena* sp. infection in guppies from commercial ornamental fish farms in Sri Lanka, along with other parasites in different ornamental fish species. Pimenta-Leibowitz et al., (2005) reported that poor environmental and adverse physiological conditions pre-dispose to infections with *Tetrahymena* sp.

As a histophagous parasite, *Tetrahymena* disintegrates host tissue and feeds on cell debris. Infection is showed by whitish patches due to the masses of ciliates in copious amount of mucus, patches usually located on the dorso-lateral skin around the abdomen (Johnson, 1978). Typical gross lesions of *Tetrahymena* infection are characterized by protrusion of scales, swelling, ulcerative wounds on the skin, blindness, and protrusion of eyes (Johnson, 1978). Numerous *Tetrahymena* spp. may form a rim around the eye orbit (spectacled eye). The parasites route of entry into the body has not been reported. In histopathological analyses, the ciliates were found to be distributed between spaces in the damaged tissues, and extensive necrotic changes occur in the muscle and subdermal tissue. Necrosis of the epithelial cells, extending to the musculature, and disfigured dermal and subcutaneous tissue with edema and hemorrhage was also reported [24, 51].

Compared with Scuticociliatosis, Tetrahymenosis can be found in a more wide-ranging hosts (e. g. slugs, hick embryos, dragonfly, helgramite, roach, cockroach and caterpillar), and Tetrahymenosis is easier to discovered in freshwater fish (vs. mostly in marine habitat fishes for Scuticociliatosis) (24, 46, 48, 52). However, their major clinico-pathological manifestations are similar: anemia, weight loss, dark coloration, enteritis, excessive body mucus, yellowish intestinal mucus, loss of scales, hemorrhagic and/or bleached spots on the skin, and dermal necrotic lesions that finally destroy tissues lead to high mortalities [8, 16, 50, 52].

As a test case using probes for the identification of pathogenic ciliates in aquaculture, the present study utilized the probe Tetr2020 to unambiguously detect Tetrahymenosis (Figure 3, D). The general identification by strong fluorescence signals from the oligonucleotide probe can be used for quick and early detection of Tetrahymenosis infections on both invertebrates and vertebrates, especially fish. The FISH probe designed here has the potential to be used for confirming the geographical distribution of parasitic *Tetrahymena* spp. and detecting the possible dispersal of this facultative pathogen [27–30]. The FISH approach also provided some morphological information such as body shape, macro and micronuclei shape and number of micronuclei of parasitic *Tetrahymena* spp.

Conclusions

The manifestations of histopathology in fish typically include lesions on the body surface, and affected organs include the skin, musculature, viscera, eye socket and spinal cord; masses of ciliates can be detected in copious amount of mucus and between spaces in the damaged tissues. Improved classification of parasitic Tetrahymena is provided: facultatively parasitic forms (T. pyriformis, T. rostrate, T. bergeri and T. vorax), facultatively free-living forms (T. chironomi, T. corlissi, T. rotunda, T. glochidiophila and T. papula) and parasitic forms (T. stegomyiae and T. limacis).The phylogenic results indicate that Tetrahymena spp. belonging to 'borealis' group have a greater probability to become parasitism. the method of which can be used for quick and early detection of Tetrahymenosis.

List Of Abbreviations

Fluorescence in situ hybridization
FISH

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication Availability of data and materials

The datasets supporting the conclusions of this article are included within the article. Representative sequences are submitted to the GenBank database (accession numbers are provided in Figure 4).

Competing interests

PXM and CY work at Harbin Normal University, Harbin, China. The study was conducted as part of a research program to investigate Tetrahymenosis in aquaculture in Northeastern China.

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Authors' contributions

The study design, protocol and were prepared by ZSX, PMM, LWY, SM, WCN, LJL, WX and ZL, and report of the study and reviewed by PXM and CY. PXM and his team at Harbin Normal University were responsible for the animal phase and data collection. PXM were responsible for compiling the first draft of the manuscript. All authors revised and approved the final version.

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Tables

Table 1. Morphological identification characteristics of parasitic *Tetrahymena* spp.

Species	Size in vivo	Body shape	Caudal cilia	SK No.	Mi No.	CVP No. & position	References
T. corlissi	an average 52 × 32 µm	elongate, pointed anteriorly and rounded posteriorly	present	25-31	1	2 or 3; SK6-10	16
T. pyriformis	ca. 50 × 30 µm	pear-shaped	absent	15-25	N/A	2; SK5, 6	5
T. stegomyiae	60-100 µm	pear-shaped	absent	25-30	1	-	1
T. chironomi	ca. 40 × 23 µm	elongate	absent	23-28	1	3; SK6-9	1
T. vorax	< 150 µm	posterior end is drawn out into a tail	absent	18-23	0	2-6; SK5-10	7
T. rostrata	50-60 × 30-40 µm	Vary	present	36-58	1	3 or 4; SK6-12	5,7
T. limacis	40-55 × 30-40 µm	Cucumber-shaped with strongly apiculate anterior end	absent	26-46	1	2 or 3; 5-8	58
T. rotunda	40-90 µm (after staining)	bluntly ovoid to globose	present	50-66;	--	not observed	64
T. papula	80-100 µm	piriform, bag-shaped	absent	33-45	1	2-6; SK9-16	78

Table 2. Reports of parasitic *Tetrahymena* species and their hosts.

Parasite	Host	Data source
T. bergeri	*Channa aurantimaculata*	From the present work
T. chironomi	Fourth-instar larva (*Chironomus plumosus*)	1
T. chironomi	*Phoxinus lagowskii*	From the present work
T. corlissi	Beetle, Dragonfly, Guppies, Helgramite, Tadpoles, Tent caterpillar	16
T. corlissi	Guppies (*Poecilia reticulatus*)	54
T. corlissi	Freshwater triclads	73
T. corlissi	Atlantic Salmon *Salmon salar*	23
T. corlissi	Fishes	46
T. corlissi	Fresh fish	17
T. corlissi	Guppies (*Poecilia reticulata*)	24
T. corlissi	Guppies	25
T. corlissi	Guppies (*Poecilia reticulata*)	22
T. corlissi	Dwarf Gourami (*Colisa lalia*)	69
T. corlissi	Freshwater ornamental fish	48
T. corlissi	Guppies (*Poecilia reticulata*)	18
T. corlissi	Golden perch (*Macquaria ambiqua*)	50
T. glochidiophila	Freshwater mussel	21
T. limacis	European slug (*Deroceras retioulatum)*	58
T. limacis	European slug (*Deroceras reticulatum*), native slug (*Prophysaon andersoni*), native snail (*Monadenia fidelis*)	59, 60
T. limacis	Fresh-water clams	53
T. limacis	Land snail (*Trichia lubomirskii*)	56
T. limacis	European slug (*Deroceras reticulatum*)	76
T. limacis	Slug (*Deroceras reticulatum*)	65
T. papula	Tent caterpillar (*Malacosoma americana* larva)	16
T. pyriformis	Insects	75
T. pyriformis	Insects	77
T. pyriformis	Clams	53
T. pyriformis	Beetle, Chick embryos, Dragonfly, Helgramite, Roach, cockroach, Tent caterpillar	16
T. pyriformis	Oligochaete, *Allolobophora caliginosa* var. *trapezoides*	17
T. pyriformis	Mosquitoes	62
T. pyriformis	Adult female cockroaches (*Periplaneta americana*)	67
T. pyriformis	Channel catfish (*Ictalurus punctatus*)	44
T. pyriformis	Freshwater triclads	73
T. pyriformis	Fishes	45
T. pyriformis	Freshwater crayfish (*Cherax quadricarinatus*)	47
T. pyriformis	Ornamental fishes	51
T. pyriformis	Freshwater ornamental fish	48
T. pyriformis	*Phenacogrammus interruptus*	From the present work
T. rostrata	European slug (*Deroceras reticulatum*)	61
T. rostrata	Land snail (*Zonitoides nitidua*)	56
T. rostrata	European slug (*Deroceras reticulatum*)	16
T. rostrata	Field slugs (*Deroceras reticulatum*)	72
T. rotunda	Simulium	9
T. stegomyiae	Mosquito	1
T. vorax	Chick embryos, Cockroach, Dragonfly, Helgramite, Tent caterpillar	16
T. vorax	*Pelteobagrus fulvidraco*	From the present work

Figures

Figure 1

Diseased fishes isolated from farms or aquaculture markets of Harbin, northeastern China infected by Tetrahymena spp. A. Malapterurus electricus; B. Tetraodon palembangensis; C, Poecilia latipinna; D, Channa aurantimaculata; E, Carassius auratus; F, zebrafish; G, H, Phoxinus lagowskii; I. J. Pelteobagrus fulvidraco. Scale bars: A. 10 cm (A, D); 4 cm (B, E, G, H, J).

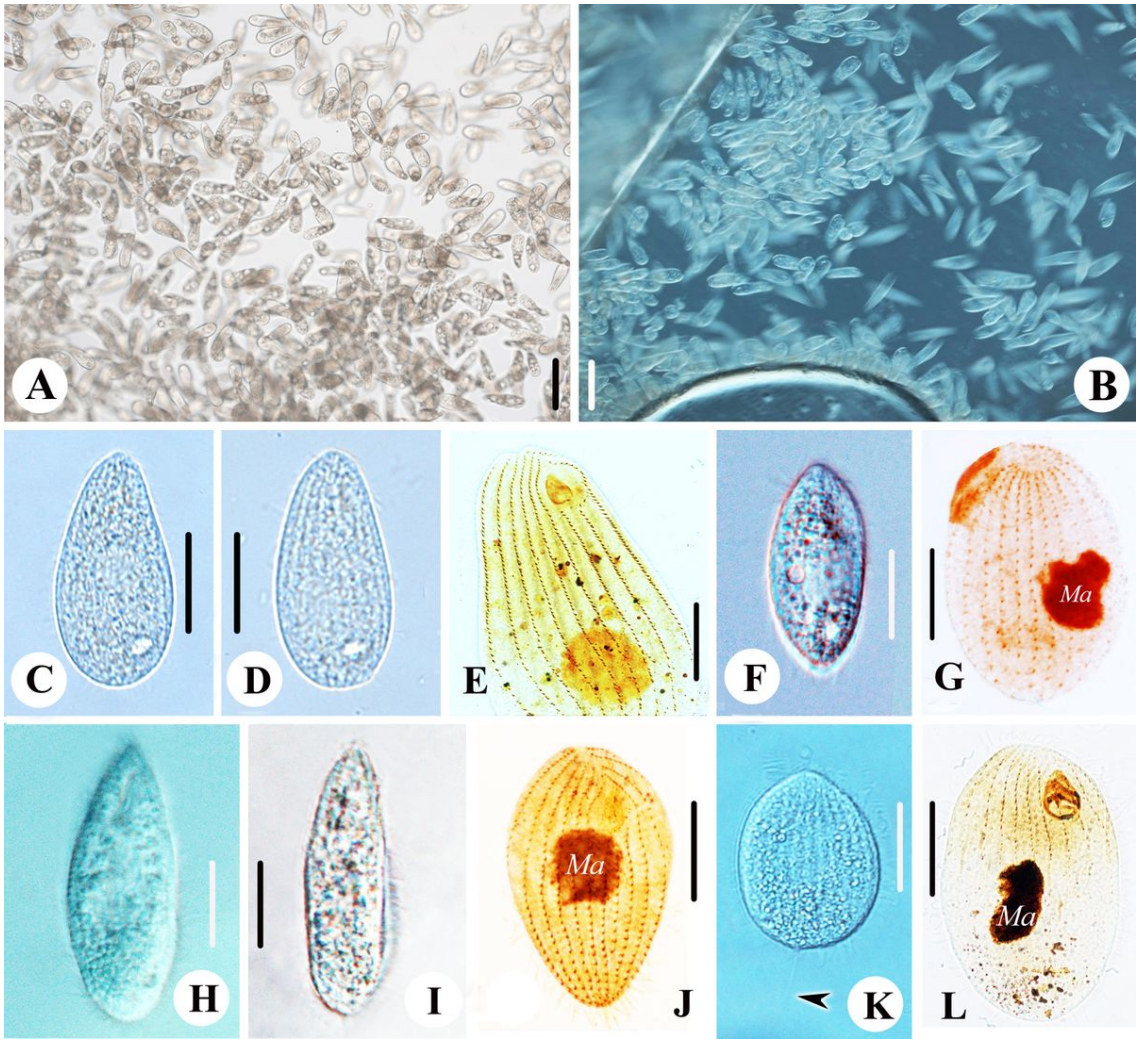

Figure 2

Photomicrographs of parasitic Tetrahymena species isolated from Harbin in vivo (A–D, F, H, K) and after silver carbonate staining (E, G, J, L). (A, B) Views of numerous parasitic Tetrahymena sp. in low magnification. (C–E) Tetrahymena pyriformis. (F, G) Tetrahymena vorax. (H–J) Tetrahymena chironomi. (K, L) Tetrahymena bergeri, arrowhead shows caudal cilium. Scale bars: 100 μm (A, B), 25 μm (C, D, F–L), 10 μm (E).

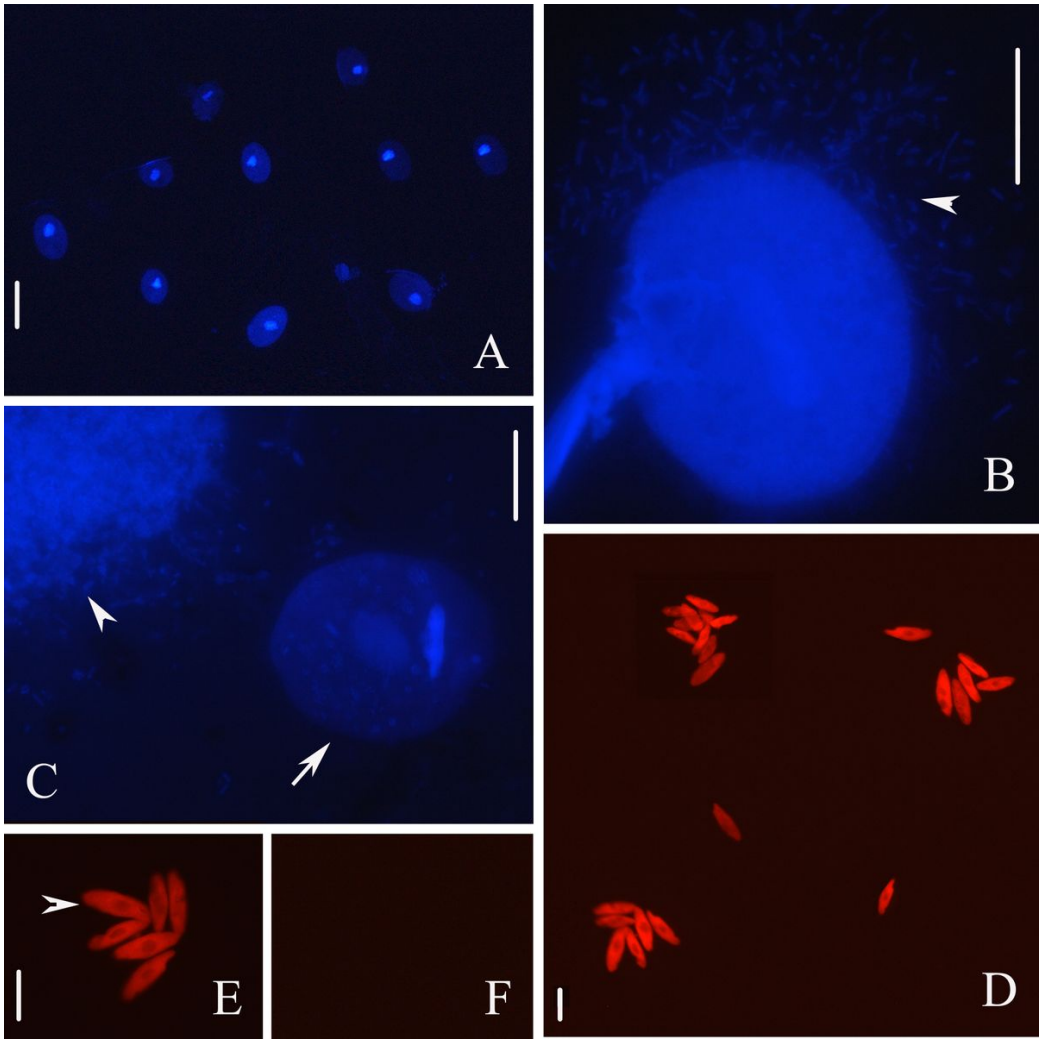

Figure 3

Simultaneous fluorescence in situ hybridization (FISH; D–F) and Hoechst33342 (A–C) staining of parasitic Tetrahymena species (FISH; A–D) and other test ciliates (F). (A) Views of numerous cells of parasitic Tetrahymena sp. in low magnification. (B) To show bacteria (arrowhead) around Tetrahymena sp. (C) To show the process that Tetrahymena sp. (arrow) eating tissue (arrowhead) as well as bacteria of host. (D, E) Presence of red fluorescence signals for T. pyriformis, T. vorax, T. chironomi and T. bergeri (arrowhead, signals of four species were similar, so only that of T. pyriformis are showed). (F) Other test ciliates (not assigned to Tetrahymena) after simultaneous fluorescence in situ hybridization. Scale bars: 50 μm (A, D, E), 20 μm (B, C).

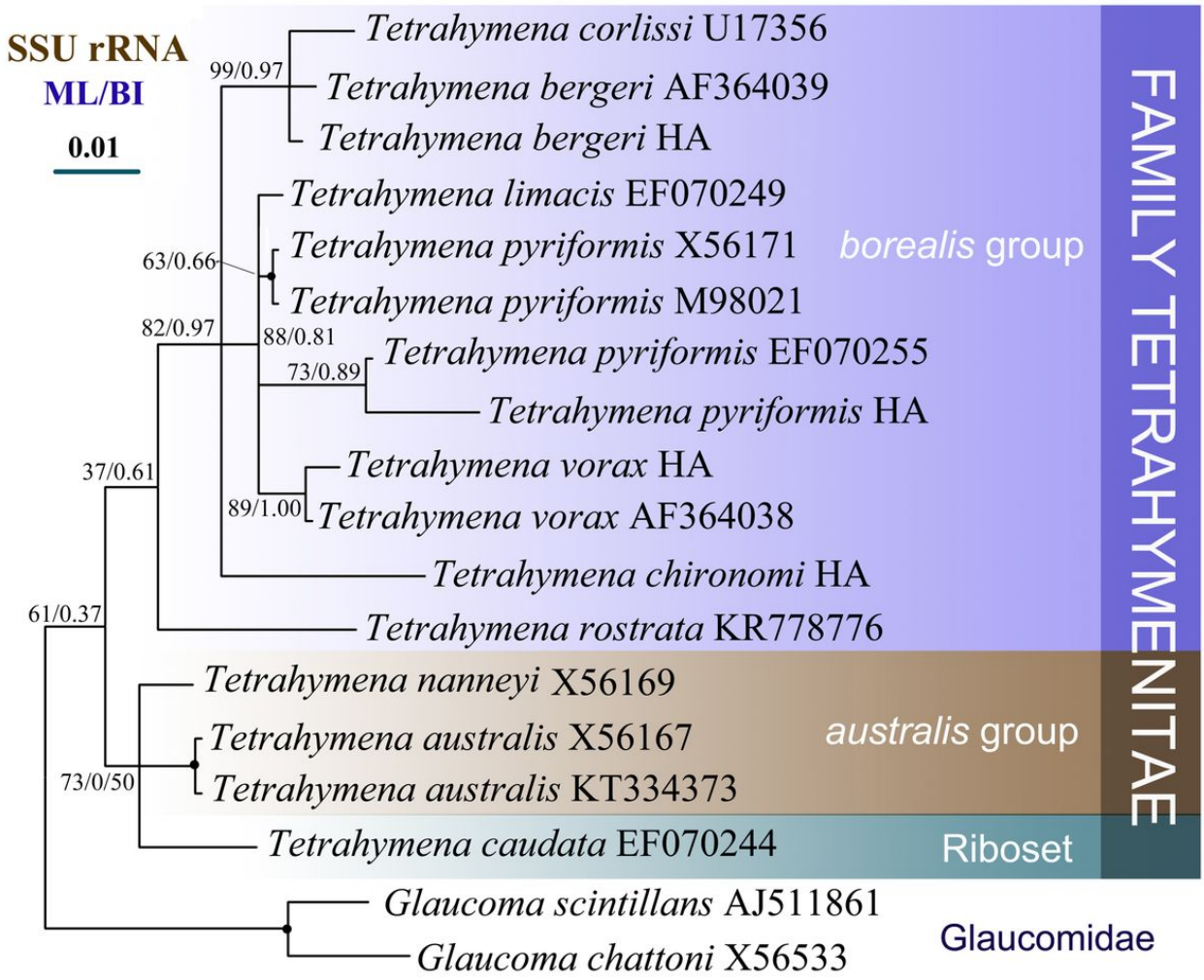

Figure 4

Maximum likelihood tree inferred from SSU rDNA sequences, showing the position of phylogentic relationship of parasitic Tetrahymena species. Numbers at nodes represent the bootstrap values of Maximum likelihood out of 1,000 replicates and the posterior probability of Bayesian Inference. Fully supported (100%/1.00) branches are marked with solid circles. The scale bar corresponds to one substitutions per 100 nucleotide positions. HA refers 'Harbin population'.

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