

# Morphological and molecular characterization of *Euplotes encysticus* (Protozoa: Ciliophora: Euplotida)

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*The ciliate Euplotes encysticus Yonezawa, 1985 was insufficiently described so far regarding its living morphology, infraciliature and silverline system. Recently, a China population was isolated from brackish water (salinity 6‰) near a coastal sandy beach (114°32'E 22°43'N) of the Daya Bay, South China Sea and subsequent investigations were performed on it in detail. Based on data available, the morphology of this species was summarized and a new diagnosis is given which is characterized by: medium-sized body with consistently 9 frontoventral, 2 caudal and 2 left marginal cirri; 30–40 adoral membranelles; 7 dorsal kineties; dorsal silverline system consisting of complex type with irregular network; capable of encystment. Both the morphology and SSr RNA gene sequence show, with high support, a close relationship of E. encysticus and Euplotes muscorum.*

**Keywords:** Daya Bay, *Euplotes muscorum*, infraciliature, South China Sea, SSr RNA

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## INTRODUCTION

The species-rich genus *Euplotes* has been revealed to be much more divergent than previously believed and is of extremely wide distribution and high adaptive potentialities (Curds, 1975; Lobban *et al.*, 2005; Schwarz *et al.*, 2007; Wilbert & Song, 2008). Species identification and separation is still uneasy mainly because: (1) many of the features used for distinguishing species are known to vary considerably even within clonal cultures; (2) there is a large amount of confusion in the literature concerning the identity of certain common species; and (3) there is the added difficulty of having to search through a considerable body of literature before an identification can be attempted (Curds, 1975; Yonezawa, 1985; Song *et al.*, 1998; Lobban *et al.*, 2005; Schwarz & Stoeck, 2007).

In recent decades, from various habitats, many new *Euplotes* species have been studied, and added to this genus (Song *et al.*, 1998; Lobban *et al.*, 2005; Schwarz & Stoeck, 2007; Wilbert & Song, 2008; Jiang *et al.*, in press). Recent studies have demonstrated that a complete description of a ciliate species might include details of living morphology, infraciliature and/or silverline system as well as molecular data in order to solve taxonomic problems (Shao *et al.*, 2008; Song *et al.*, 2009; Chen *et al.*, in press). As regards SSr RNA sequence information, about 1/5 of *Euplotes*

morphospecies have been involved so far (Yi *et al.*, 2009; Jiang *et al.*, in press).

*Euplotes encysticus* was originally reported by Yonezawa (1985) as a fresh water form. Although Matsusaka *et al.* (1989) described its cortical morphogenesis during encystment, the circumscription of this 'new' species has not been clearly provided in the authors' opinion.

As a new contribution, a new definition of *E. encysticus* is provided based on a population adaptable to both brackish (salinity 6‰) and fresh water. Furthermore, the characters were raised to distinguish *E. encysticus* between the most similar congener *E. muscorum* based on both morphology and phylogenetic analyses.

## MATERIALS AND METHODS

### Sample collection, observation and identification

Samples were collected from a coastal sandy beach (22°43'N 114°32'E) of the Daya Bay, South China Sea in December, 2007, by digging a hole about 10 cm deep in the sand, waiting for seawater to seep into the hole, then taking the seawater with some sand back to the laboratory. Water temperature was about 12°C, and salinity 6.0‰. Uniprotistan cultures can be kept at room temperature (about 20°C) using distilled water, to which rice grains were added to enrich bacterial food (Hu, 2008).

Live observations were carried out using an oil immersion objective with bright field and Nomarski differential

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interference contrast optics (Shao *et al.*, 2008). The infracilia-ture was revealed using the protargol method (Wilbert, 1975). In order to demonstrate aspects of the silverline system, the Chatton–Lwoff method was used (Wilbert & Song, 2008). Measurements and counts were performed at a magnification of 1250. Drawings were made with the aid of a camera lucida. Terminology and systematics are mainly according to Lynn (2008) and Song *et al.* (2009).

### Sequence availability and phylogenetic analyses

The SSU rRNA gene sequences of *E. encysticus* and reference sequences from GenBank databases were aligned using the Clustal W implemented in Bioedit 7.0 (Hall, 1999) and refined by removing ambiguous gaps at both termini of the alignment. The Kimura two-parameter distance method (Kimura, 1980) in program MEGA-4.0 (Tamura *et al.*, 2007) was used for distance analyses. *Protocruzia contra* was used as an out-group.

Phylogenetic trees were constructed according to the methods reported in Yi *et al.* (2008). A maximum parsimony (MP) tree was obtained via random addition and swapped using the tree–bisection–reconnection (TBR) algorithm. Gaps were treated as missing data. MP analysis was performed with the software package PAUP\* 4.0b10 (Swofford, 2002), and the support for the internal branches was estimated using the bootstrap method with 1000 replicates.

The program MrModeltest v.2.0 (Nylander, 2004) selected the GTR + I + G as the best model with Akaike information criterion, which was then used for both BI and ML analyses. BI analysis was performed with MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003) using the Markov chain Monte Carlo algorithm. The program was run for 1,000,000 generations with a sample frequency of 100 and a burn-in of 2500. The ML tree was constructed with the PhyML V2.4.4 program (Guindon & Gascuel, 2003).

## RESULTS

*Euplotes encysticus* Yonezawa, 1985 (Table 1; Figures 1 & 3)

### Morphology of live and impregnated specimens

Cell size about 80–90 × 50–65 μm *in vivo*. Body asymmetrically oval with posterior portion slightly wider than anterior; dorsoventrally flattened about 4:1 (Figure 1A, E). Peristome narrow and extremely long, extending approximately 75% of cell length, with a visible protrude at the anterior (Figure 3C, arrow). Adoral zone of membranelles (AZM) curved in varying degrees in posterior portion (Figures 1F, 3K, M). Cytoplasm colourless, often filled with many light-reflecting granules and few food vacuoles (Figure 3A, B). Single contractile vacuole located to right of transverse cirri (Figure 3D, arrow), about 10 μm in diameter, often pulsating quickly (<1 second) with a ~60 seconds interval. Six obvious dorso-lateral ridges (Figure 1E, arrows; Figure 3B, arrows). Dorsal kineties (DK) inserted in concaves down the ridges (Figure 3F, arrows). DK<sub>1</sub>, on left margin of ventral side, noticeably shortened (Figure 1C, arrow); other DKs extending over entire length of cell with about 24 cilia in each. The dorsal cilia are surrounded by ~6 elongated ellipsoidal granules,

**Table 1.** Morphometric data of *Euplotes encysticus*. All data based on protargol-impregnated specimens and measurements in μm.

Character	Min	Max	Mean	SD	CV	N
Body, length	67	90	77.1	6.6	8.5	25
Body, width	44	67	56.1	5.8	10.4	25
AZM, length	50	80	57.3	7.2	12.6	25
BL: length of AZM, ratio	0.6	0.9	0.74	0.1	7.5	25
FVC, number	9	9	9	0	0	25
Transverse cirri, number	5	5	5	0	0	25
Marginal cirri, number	2	2	2	0	0	25
Caudal cirri, number	2	2	2	0	0	25
Dorsal kineties, number	7	7	7	0	0	25
No. of BBP in mid-DK	21	30	24.0	1.7	7.2	25
AM, number	35	43	39.0	2.1	5.3	25

AM, adoral membranelles; AZM, adoral zone of membranelles; BBP, basal body pairs; BL, length of body; CV, coefficient of variation in %; DK, dorsal kinety; FVC, frontoventral cirri; Max, maximum; Mean, arithmetic mean; Min, minimum; N, number of specimens investigated; SD, standard deviation.

which are generally 1.5 × 0.2 μm in size (Figure 1G, arrows; Figure 3E, arrows).

Cirri usually strong. Frontoventral cirri 25–30 μm long, transverse cirri 30–40 μm, caudal and left marginal cirri about 20 μm (Figure 1A; Figure 3A). Paroral membrane (PM) about 10 μm long.

Macronucleus (Ma) usually C-shaped, with the anterior and posterior ends sometimes curving (Figure 1B). Micronucleus (Mi) small, nearly spherical and located in the upper right half of the macronucleus (Figure 1D).

After long time cultivation (about 1 month), cells can encyst because of food exhaustion. Cysts usually irregular spherical with scraggly border, about 40 μm in diameter; contractile vacuole detectable (Figure 3G, arrow).

Locomotion by crawling on crumb or substrate (Figure 1E).

### Silverline system

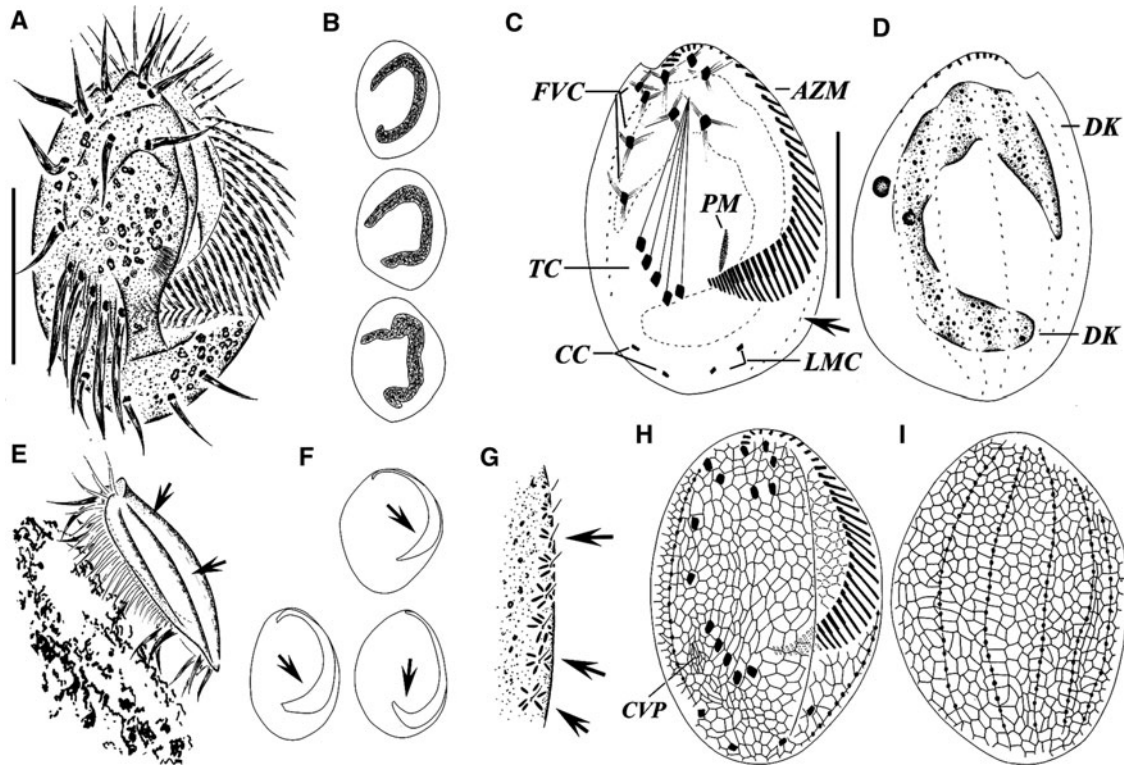
Dorsal silverline system complex type, consisting of 4–6 polygenes between each kinety (Figures 1H, I, 3I, J). Contractile vacuole pore (CVP) located at level of transverse cirri (Figure 1H).

### SSr RNA sequence analyses

The SSr RNA sequence (Accession number: FJ346569) for *E. encysticus* described in this study is 1865 bp long with 43.91% GC content. The alignment of SSr RNA sequences displays 38 bp differences between *E. encysticus* and *E. muscorum* and two populations of *E. encysticus* are almost identical (excluding the termini of the alignment). Treating gaps as missing data, the Kimura two-parameter distance between two *E. encysticus* was estimated to be 0.0006, which ranges from 0.0076 to 0.0082 between *E. encysticus* and *E. muscorum*.

### Phylogenetic position of *Euplotes encysticus* based on SSr RNA sequences (Figure 4)

Trees constructed using different algorithms show similar topology and thus are combined. The analyses provide full



**Fig. 1.** *Euplotes encysticus* from life (A, E, G), after protargol staining (B, C, D, F) and silver nitrate impregnation (H, I). (A) Ventral view of a typical individual; (B) to show different shapes of macronucleus; (C, D) ventral (C) and dorsal views (D), showing the infraciliature; (E) the lateral view, arrows mark the ridges; (F) arrows showing the variability of AZM; (G) dorsal side, showing the granules around the dorsal cilia; (H, I) ventral and dorsal views of the silverline system. AZM, adoral zone of membranelles; CC, contractile vacuole; CVP, contractile vacuole pore; DK, dorsal kineties; FVC, frontoventral cirri; LMC, marginal cirri; Ma, macronucleus; Mi, micronucleus; PM, paroral membrane; TC, transverse cirri. Scale bars = 50  $\mu$ m.

posterior probability and bootstrap support for the monophyly of Euplotidae (100ML/100MP/1.00BI). Two populations of *E. encysticus* cluster together with high support value (93ML/73MP/0.85BI), then form a clear sister group to *E. muscorum*, which is strongly supported (98ML/97MP/1.00BI).

## DISCUSSION

Though the previous form of *E. encysticus* was found in fresh water habitat (Yonezawa, 1985; Matsusaka *et al.*, 1989), it is rather certain that our isolation should be conspecific with the original because of the extreme similarities (Figure 2D–F), i.e. the general pattern of ciliature, the silverline-system, the dorso-lateral ridges, the capability of encystment and the quite adjacent number of all cirri and the adoral membranelles (AZM). The ‘minor difference’ in morphology is that our organism has a bigger body size *in vivo* (80–90  $\times$  50–65  $\mu$ m versus 62–74  $\times$  41–53  $\mu$ m in Yonezawa, 1985). In addition, by 0‰ to 6‰ in salinity, the discrepancy of the original niches of the two populations is rather inconspicuous, and it would even be neglected, because our isolation can reproduce well when it was implanted into fresh water directly.

In comparison with the congener *E. encysticus* shows great similarity with *E. muscorum* Dragesco, 1970 (Figure 2A–J) in morphology. Thus, referring to all the data available we suggest the following points to discern the two species: (1) no cyst has been observed so far

in *E. muscorum* (Dragesco, 1970; Dragesco & Dragesco-Kernéis, 1986; Jo & Shin, 2003); (2) the obvious dorsal-lateral ridges and the granules surrounding the dorsal cirri have never been detected in *E. muscorum*; (3) the AZM of *E. encysticus* outspreads more broadly than that of *E. muscorum* both in living and protargol-impregnated specimens; and (4) the AZM covers 3/4 body length in *E. encysticus*, and 2/3 in *E. muscorum*. The dissimilarity between these two forms is clearly supported by the differences in their SSU rRNA gene sequences, of which *E. encysticus* differs from *E. muscorum* by 2.04% (in comparison to other morphological closely related *Euplotes*: *Euplotes focardii* versus *Euplotes parkei* 1.63% differences; *Euplotes plicatum* versus *Euplotes bisulcatus* 2.47%). These data, along with the phylogenetic position as revealed in molecular trees (Figure 4), support both the validity of *E. encysticus* as a well-outlined and distinctive member of the genus *Euplotes* and the morphological identification of *E. encysticus*.

Yonezawa (1985) raise the freshwater isolation as a new species mainly on: (1) the shape of right side of peristome in *E. encysticus* was straight, but in *E. muscorum* was wing shaped; (2) the position of micronucleus relative to macronucleus: in shallow depression in the centre of macronucleus in *E. encysticus*, while near the anterior corner of macronucleus in *E. muscorum*; and (3) the capability of cysting.

In our opinion, the shape of the lip may vary to a certain extent even in a same population. And as the figures in Jo & Shin (2003) indicated, the micronucleus of *E. muscorum* may be located also in shallow depression in the centre of

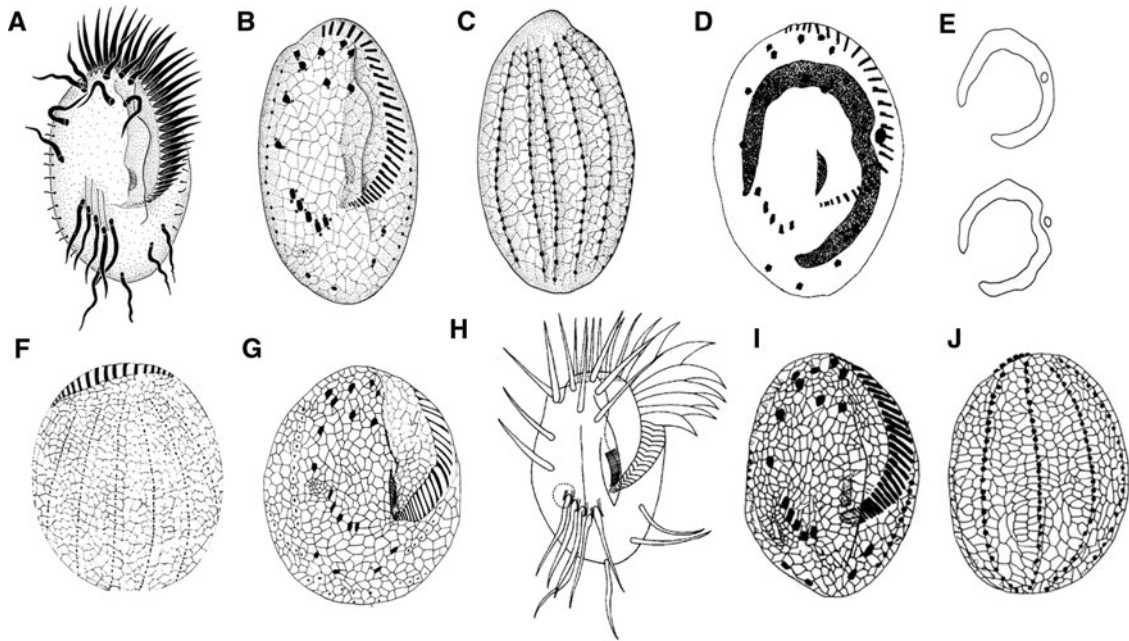


Fig. 2. *Euplotes muscorum* from Dregesco, 1970 (A–C), from Jo & Shin, 2003 (D–G); *Euplotes encysticus* from Yonezawa, 1985 (H) and Matsusaka *et al.* (1989) (I, J).

macronucleus (Figure 2D, E), so these two points are not adopted here as distinguishing characters.

Since no clear diagnosis of *E. encysticus* based on modern methods is available, an improved definition is suggested according to previous and present studies (Yonezawa, 1985; Matsusaka *et al.*, 1989).

SYSTEMATICS

Class: SPIROTRICHEA Bütschli, 1889  
 Subclass: HYPOTRICHIA Stein, 1859  
 Order: EUPLOTIDA Small & Lynn, 1985  
*Euplotes encysticus* Yonezawa, 1985

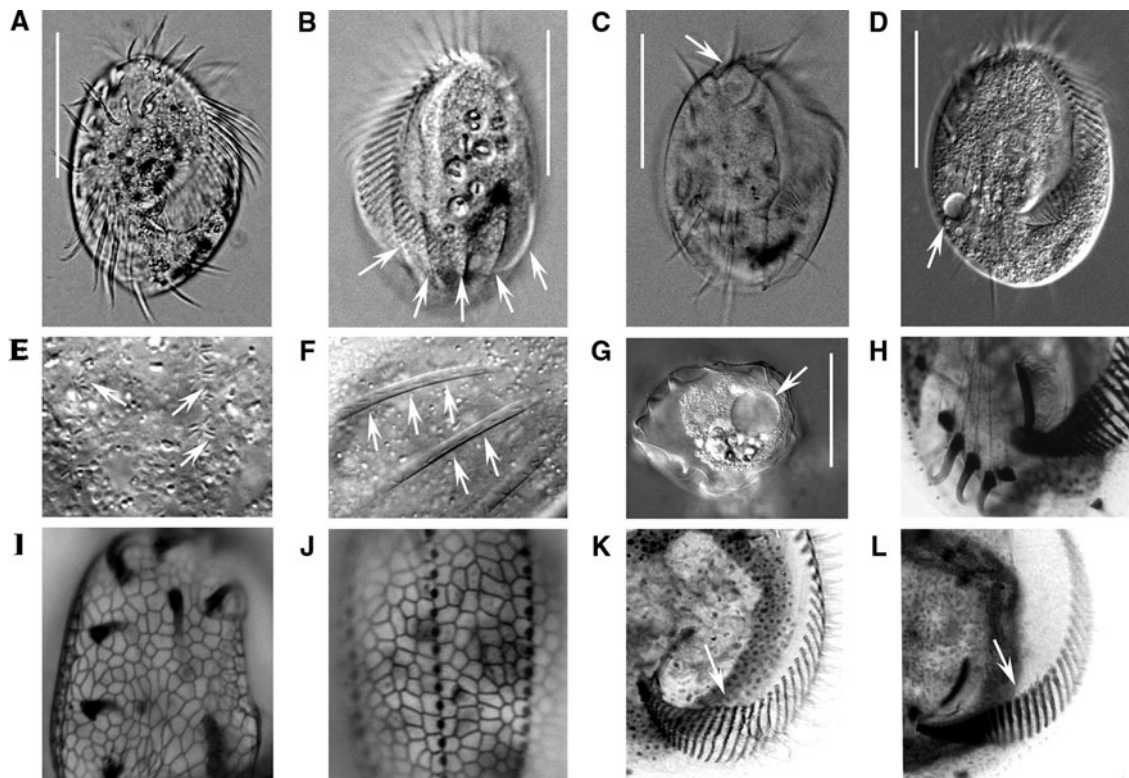


Fig. 3. *Euplotes encysticus* from life (A–G), after protargol (H, K, L) and silver nitrate impregnation (I, J). (A, B) Ventral (A) and dorsal (B) views, arrows mark the ridges; (C) showing the protrusion at anterior portion of cell (arrow); (D) ventral side, arrow refers to the contractile vacuole (CV); (E) arrows indicate the granules around the dorsal cilia; (F) to show the detail of dorsal side, arrows mark the ridges; (G) the cyst, arrow marks the contractile vacuole; (H) to show the transverse cirri; (I, J) ventral (I) and dorsal (J) sides of silverline system; (K, L) to show the AZM curved in different extent (arrow). AZM, adoral zone of membranelles. Scale bars (A–D) = 50 µm; (G) = 20 µm.

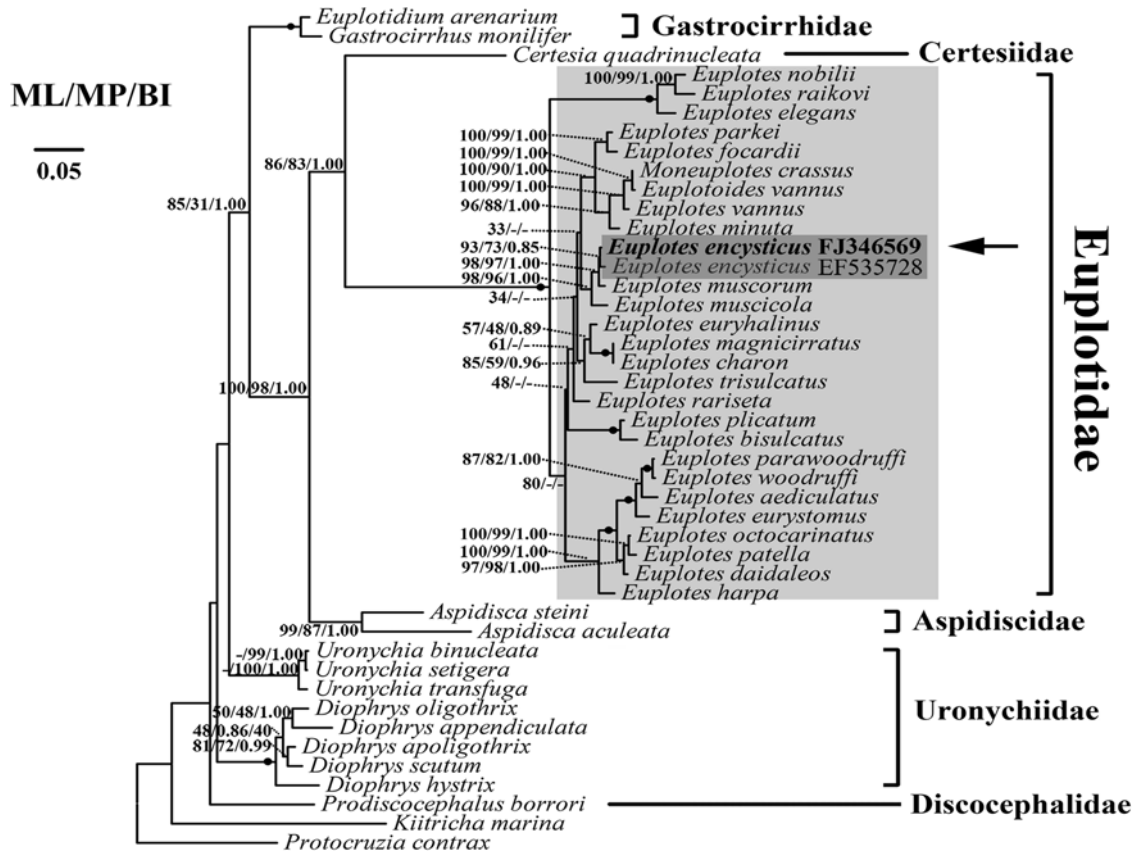


Fig. 4. ML tree inferred from SSU rRNA gene sequences showing the position of *E. encysticus*. Numbers near branches represent the bootstrap values in the following order: ML/MP/BI. “-” reflects minor differences in topology that could not be represented on the consensus tree. The scale bar corresponds to 5 substitutions per 100 nucleotide positions. The highest supported (100ML/100MP/1.00BI) branches are marked with solid circles. Species newly described in this study are shown in bold text.

#### IMPROVED DIAGNOSIS

Medium-sized *Euplotes*, *in vivo* about  $60\text{--}90 \times 40\text{--}65 \mu\text{m}$ ; body oval in outline; buccal field covering  $3/4$  of body, AZM broad; 9 frontoventral, 5 transversal, 2 caudal and 2 left marginal cirri; 30–43 adoral membranelles; 7–8 dorsal kineties within obvious ridges, the central one of which contains about 21–30 pairs of basal bodies, each dorsal cirri surrounded by several granules; dorsal silverline system complex type; capable of encystment.

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