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Filamoeba sinensis sp. n., a second species of the genus *Filamoeba* Page, 1967, isolated from gills of *Carassius gibelio* (Bloch, 1782)

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Summary. *Filamoeba sinensis*, a new species of amphizoic amoeba isolated from the gills of *Carassius gibelio* (Bloch, 1782) sampled in Hubei Province, China, is described. Both morphology and SSU rRNA gene sequences were employed to compare this species with *Filamoeba nolandi* Page, 1967, the type and only species of the genus. Trophozoites differ from *F. nolandi* in having robust multipolarly branched pseudopodia. A less pronounced hyaloplasmic zone produces a lower number of filiform subpseudopodia. The greatest dimensions of extended trophic forms reach 40-50 µm. Cysts, with a relatively thick and homogeneous wall, are smooth and free of pores. The diameter of rounded cysts ranges from 14 to 20 µm. Cristae of mitochondria are tubular. They are arranged in parallel or irregular mutual positions, their anastomoses or branches were not observed. Phylogenetic analysis revealed a close relationship to *F. nolandi*.

Key words: amphizoic amoebae, *Filamoeba sinensis* sp. n., morphology, SSU rDNA, phylogeny.

INTRODUCTION

Despite the widely recognised diversity of organisms currently assigned to Amoebozoa Lühe, 1913 *sensu* Cavalier-Smith (2003), diagnostic criteria are not well defined within all groups of amoebae. Morphological features that could possibly differentiate the taxa have not been adequately described in "neglected" groups of amoebae. It is most probably due to the exceptional variability of these protists, methods of observation available in amoeba research as time progressed and, possibly, also due to the rare frequency of many findings. As a consequence of diagnostic difficulties, many species have not been reported again since their descriptions and some were never accommodated to suprageneric taxons. Molecular characterisation of amoebae based mostly on SSU rRNA gene sequences has become gradually an integral part of the description of new species. For synthesis of both morphological and molecular approaches in amoeba studies, molecular characterisations of type strains are of a great value, since they facilitate comparisons and serve as a basis for studies of intra-amoebozoan phylogeny.

A freshwater amoeba described as *Filamoeba nolandi* Page, 1967 was isolated from shallow water on

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the sandy shore of Little Deer Lake in Itasca County, Minnesota. Prior to its description in 1964, Page deposited the strain in the former Culture Collection of Algae and Protozoa (CCAP), presently the UK National Culture Collection (UKNCC). Page (1967) did not settle the systematic position of this organism in the original description. In his publications "An Illustrated Key to Freshwater and Soil Amoebae" and "A New Key to Freshwater and Soil Amoebae" Page (1976, 1988) placed the genus *Filamoeba* in the family Echinamoebidae Page, 1975. Phylogenetic analyses published thus far (Amaral Zettler *et al*. 2000, Bolivar *et al*. 2001, Peglar *et al*. 2003) included the SSU rRNA gene sequence of American Type Culture Collection (ATCC 50430) strain of *F. nolandi* (NC-AS-23-1) and not of that deposited in UKNCC as a type strain of the species. Contrary to the type strain, the ATCC strain denoted as *F. nolandi* (NC-AS-23-1) is of marine origin. It was isolated from a sediment core 23 nautical miles off coast of Wilmington, NC, in 1993.

This paper presents a new amoeba species, the second one of the genus *Filamoeba* Page, 1967, which was isolated from a freshwater fish host.

MATERIALS AND METHODS

The strain denoted as CH26, was isolated from gill tissue of *Carassius gibelio* (Bloch, 1782) sampled in Hubei Province, China, in April 2002. It was recovered from decomposing tissue stored for 14 days in eppendorf tubes and washed repeatedly with sterile tap water in order to separate spores of *Myxobolus* sp. detected in gill tissue of the opposite gill arch. A homogeneous population of trophozoites (passage No. 8), cultured on non-nutrient agar was cryopreserved in September 2002. A clone was derived in November of the same year from passage No. 13.

Amoeba trophozoites were observed in hanging drop preparations using an Olympus BX51 microscope equipped with the Nomarski DIC system. For ultrastructural studies, trophozoites and cysts were fixed on agar plates with 2.5% cacodylate buffered glutaraldehyde, then pelleted *via* centrifugation, postfixed with cacodylate buffered 1% osmium tetroxide, dehydrated with series of acetones and embedded in Spurr's resin. The same methods of culturing, observation and harvesting were applied to the strain of *Filamoeba nolandi*. It was ordered for purposes of comparison from UKNCC where it is listed in the type culture collection as CCAP 1526/1 strain.

Our original clone used in this study was cryopreserved and is stored in the culture collection of the Institute of Parasitology, Academy of Sciences of the Czech Republic, České Budějovice.

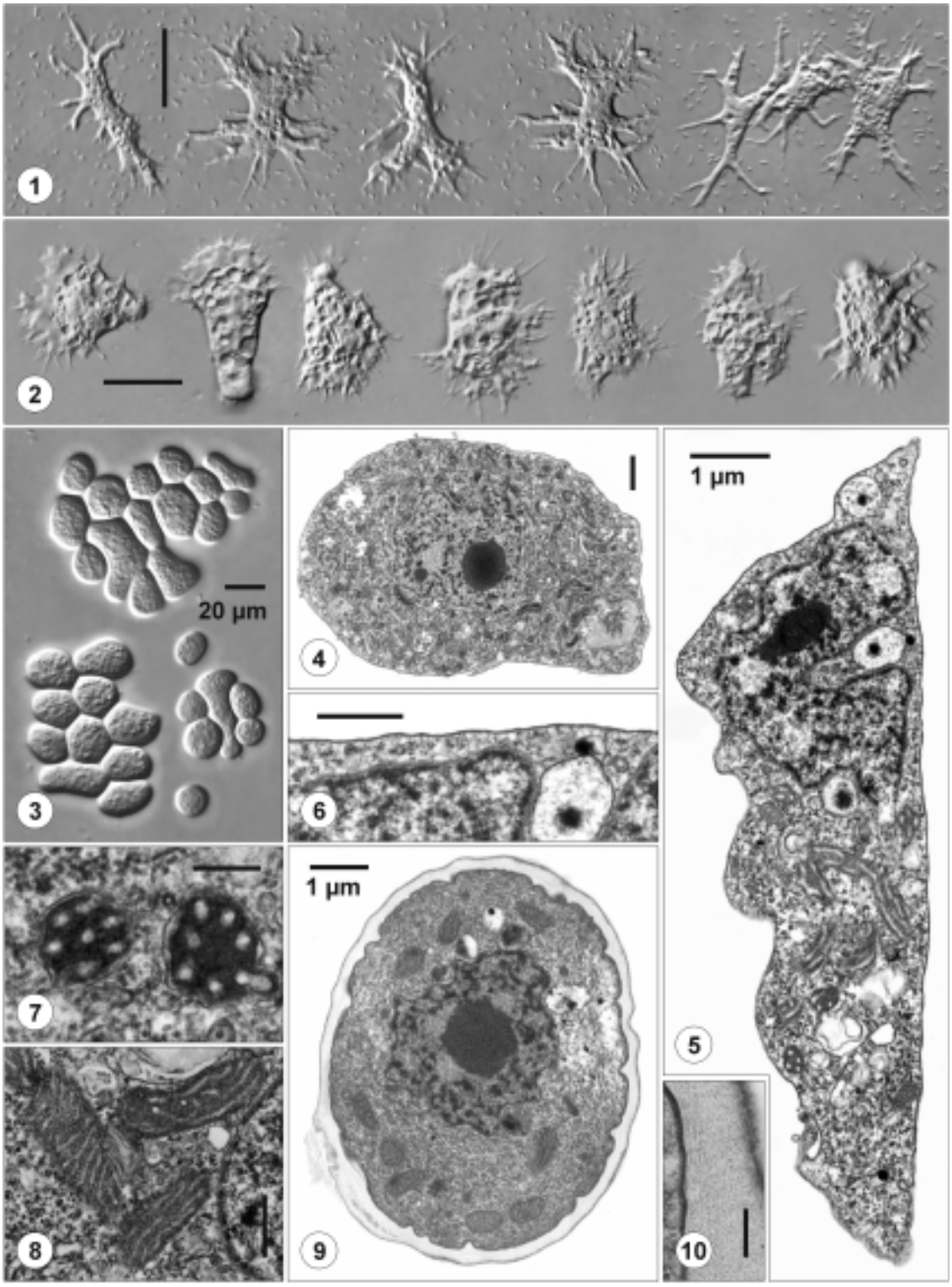
DNA isolation, amplification and sequencing. Morphological characterisation of clones derived from both strains (CH26/I and CCAP 1526/1) was completed with phylogenetic analysis of small-subunit ribosomal RNA gene sequences. DNA was extracted from the cell pellets using the DNeasy™ Tissue kit, according to the protocol of the manufacturer (Qiagen). The SSU rRNA genes of both strains were amplified by polymerase chain reaction (PCR) using universal eukaryotic primers ERIB 1 (5´- ACCTGGTTGAT CCTGCCAG - 3´) and ERIB 10 (5´ - CTTCCGCAGGTTCACCTACGG - 3´) (Barta *et al*. 1997). Each 25 µl reaction included 50 ng of genomic DNA, 10 pmol of each oligonucleotide, 250 ìM of each dNTP, 2.5 µl 10 × PCR Buffer (Top-Bio, Czech Republic), and 1 unit of Taq-Purple polymerase (Top-Bio, Czech Republic). The mixture was preheated 5 min at 95°C. Thermocycling conditions were as follows: 5 cycles consisting of 94°C for 1 min, 44°C for 1.5 min and 72°C for 2 min, 25 cycles consisting of 94°C for 1 min, 48°C for 1.5 min and 72°C for 2 min, then a 10 min incubation at 72°C. The PCR products were isolated from agarose and cloned into the pCR® 2.1 TOPO cloning vector (Invitrogen). Vectors with inserts were sequenced in both directions using CEQ DTCS Dye Kit (Beckman Coulter) on the CEQ TM 2000 automatic sequencer (Beckman Coulter).

Alignments and phylogenetic analyses. The alignments were done using the Clustal_X program (Thompson *et al*. 1997) with various alignment parameters. Ambiguously aligned regions were excluded. The SSU rRNA gene sequences from the newly sequenced *Filamoeba* strains were aligned against sequences of the ATCC strain of *Filamoeba nolandi* and 37 eukaryotic taxa available through GenBank, including representatives of Amoebozoa sensu Bolivar *et al*. (2001). *Hexamita inflata* was selected as an outgroup. The final alignment consisted of 1615 characters (1022 characters were parsimony informative).

The phylogenetic relationships between taxa were determined using maximum parsimony (MP) and maximum likelihood (ML) methods carried out in the program package PAUP*, version 4.0b8 (Swofford 2001). The MP analysis was performed using a heuristic search with random addition of taxa. Gaps were treated as missing data. Transition/transversion/ (Ts:Tv) ratio was 1:2. For ML, the likelihood ratio test (LRT) implemented in the Modeltest v. 3.06 (Posada and Crandall 1998) was used to determine the best model of evolution. ML analyses were done with the TrN+G model of evolution selected by LRT. Clade support was assessed by bootstraping (MP, 1000 replicates; ML, 500 replicates).

RESULTS

Description of *Filamoeba* strain CH26. The strain under study was characterised by an irregular, multipolarly branched, uninucleate trophozoites (Fig. 1). Robust pseudopodia were pointed, non-anastomosing subpseudopodia were filiform and up to 12 µm long. The greatest dimensions of locomotive and extended forms were 40-50 µm. A rounded or lobe-shaped nucleus was mostly difficult to distinguish in Nomarski DIC. The cytoplasm usually contained about 10 contractile vacuoles. Floating forms were rounded. Cysts (Fig. 3) were smooth, rounded, ovoid, irregularly ovoid or reniform with homogeneous and rather thick wall that lacked pores and closely applied to the amoeba plasmalemma.



Figs 1-10. Morphology of strains of *Filamoeba* spp. **1** - trophozoites, representatives of *Filamoeba sinensis* sp. n., strain CH26. **2** - trophozoites, representatives of *Filamoeba nolandi* Page, 1967, type strain from UKNCC (CCAP1526/1). **3** - cysts of *F. sinensis* formed from trophozoites in groups, note their irregular shape. **4, 5** - ultrastructure of *F. sinensis* trophozoites in two different levels of sectioning show a lobular nucleus, rounded nucleolus, several vacuoles in the cytoplasm and mitochondria sectioned in various planes. **6** - the surface of trophozoite of *F. sinensis*. **7** - mitochondria of *F. sinensis* in cross section. **8** - roughly longitudinal sections through mitochondria showing the shape and arrangement of their cristae. **9** - electron micrograph of mature cyst of *F. sinensis*. **10** - detail of cyst wall structure of *F. sinensis*. Scale bars: 20 µm (1-3); 1 µm (4, 5, 9); 500 nm (6, 8); 200 nm (7, 10).

The diameter of rounded cysts was 14-20 µm, the greatest dimension of oval cysts was 37 µm. The outline of cysts was evidently modified by close contact with neighbouring ones, i.e. by the space available for each cysts, which was observed also in old cultures.

At the ultrastructural level (Figs 4-6), trophozoites were truly "naked". The organic layer coating the surface membrane was very thin, almost invisible (Fig. 6). The rounded as well as lobe-shaped nuclei contained rounded nucleoli (Figs 4, 5). Numerous mitochondria, mostly of elongated shape were randomly distributed within the cytoplasm of trophozoites. They had tubular cristae (Fig. 7) situated irregularly or parallel to each other (Fig. 8). Neither cross-, nor longitudinal sections through mitochondria revealed anastomoses or branches of cristae. The cyst wall was homogeneous (Figs 9, 10). The nucleus of cysts sectioned at various levels was mostly rounded, with the diameter about 3 µm.

Based on comparison with the true type strain of *F. nolandi* (CCAP 1526/1) (Fig. 2), which we had cultured under identical conditions, the strain CH26 differed morphologically having robust multipolarly branched pseudopodia, less pronounced hyaloplasmic zone and a lower number of contractile vacuoles. Details of ultrastructure were not given in the original description of *F. nolandi*. The arrangement of tubular cristae in mitochondria of *F. sinensis* showed no signs of ramification.

SSU rRNA data and phylogenetic analysis of *Filamoeba* strains. The complete SSU rRNA gene sequences obtained from strains CH26 and CCAP 1526/1 have been deposited in the EMBL/GenBank database under accession numbers AY714369 and AY714368 respectively. They matched with the sequence of *F. nolandi* (AF293896) available in GenBank, but still could not be identified with it. The sequence of SSU rRNA gene of our strain CH26 is 1839 bp. The GC content is 50.73%. A sequence of the same length but differing in the GC content (49.59%) was obtained for the *F. nolandi* strain CCAP 1526/1. Similarities based on comparisons of complete sequences of SSU rRNA gene were as follows: 96.3% for strain CH26 and *F. nolandi* type strain CCAP 1526/1; 97.1% when CH26 and *F. nolandi* ATCC strain NS-AS-23-1was compared, 97.7% for *F. nolandi* ATCC strain NS-AS-23-1 and *F. nolandi* CCAP strain 1526/1.

As revealed by phylogenetic (ML) analysis (Fig. 11), two *Filamoeba* strains sequenced in this study plus ATCC strains of *F. nolandi* form monophyletic group (supported by 100% bootstrap) that is independent of Gymnamoebia *sensu stricto* according Bolivar *et al*. (2001) and of LH, V and PV lineages sensu Peglar *et al*. (2003). *Gephyramoeba* sp. clusters with *Filamoeba* strains with a bootstrap support lower than 50%. Within the group of *Filamoeba* strains, the true type strain of *F. nolandi* (CCAP 1526/1) is closely related to the ATCC strain of *F. nolandi* of marine origin, while our CH26 strain branches out of these two strains. The MP analysis resulted in three trees with the same length (8701 steps). The phylogenetic position of *Filamoeba* strains as well as relation to *Gephyramoeba* sp. was the same as obtained in ML.

Tentative assignment of strain CH26 to the genus *Filamoeba* Page, 1967, araising from comparison of morphological data available on freshwater naked amoebae, was supported by results of phylogenetic analyses. The SSU rRNA gene sequence we have obtained for the type strain of *F. nolandi* Page, 1976 complemented molecular data on *Filamoeba* spp. represented to date by the one sequence of the ATCC strain of *F. nolandi*, which contrary to the type species is of marine origin.

Details distinguishing the strain CH26 from the type strain of *F. nolandi* Page, 1967 together with results of phylogenetic analyses based on SSU rRNA gene sequences allowed to establish the new species, *F. sinensis*, the second one within the genus.

***Filamoeba sinensis* sp. n.**

Origin of type material: Gills of the Prussian carp, *Carassius gibelio* (Bloch, 1782) (Cypriniformes: Cyprinidae).

Type locality: Fish farm in Hubei Province, China.

Type material: Photosyntypes (light micrographs), nos. 13870-13891, transmission electron micrographs, nos. 17151-17165 and 17251-17275, and cryopreserved culture (clone CH26/I), deposited in the Institute of Parasitology, Academy of Sciences of the Czech Republic, České Budějovice.

Etymology: The species name is given according to the geographic origin of fish host. It was derived from Latin adjective "Chinese".

Diagnostic summary. Trophozoites uninucleate with narrow peripheral hyaloplasmic zone, robust multipolarly branched pseudopodia and numerous contractile vacuoles; greatest dimensions not exceeding 50 µm; mitochondria with tubular cristae arranged in parallel or irregular mutual positions. Cysts rounded (14-20 µm in

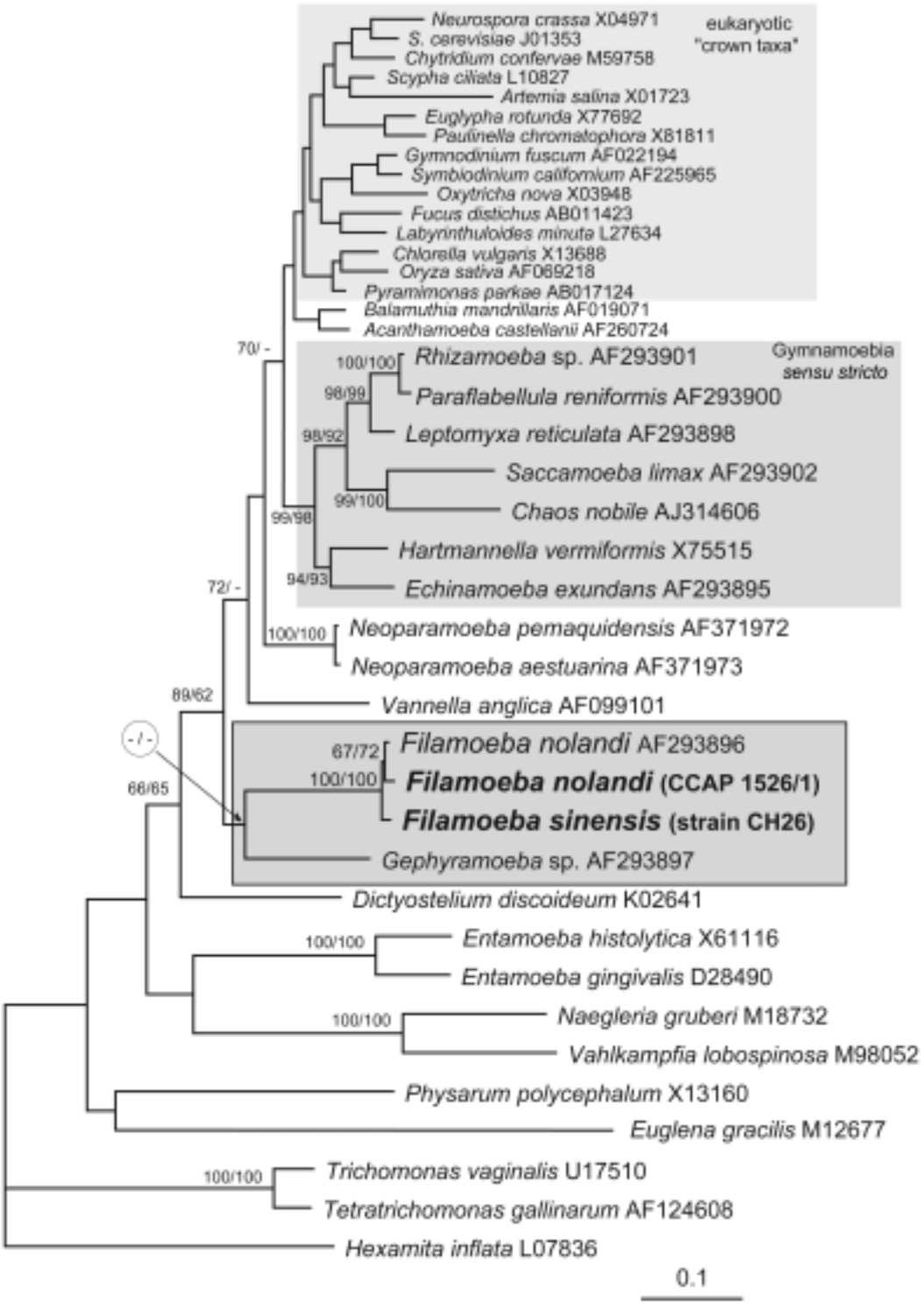


Fig. 11. Phylogenetic position of *Filamoeba* strains inferred from ML analysis (-ln = 25926.0376, α shape parameter = 0.4927). Bootstrap values (ML and MP Ts/Tv = 1:2) are shown for the nodes gaining more than 50% support (except for eukaryotic "crown" taxa). The distance scale (substitution/site) is given under the tree. GenBank accession numbers are indicated.

diameter) or ovoid with homogeneous wall free of pores. *F. sinensis* differs from type species of the genus in morphology of trophozoites and SSU rRNA gene sequence.

DISCUSSION

Tentative generic diagnosis of amoeba under study, morphology of which was unique among amoebae we have isolated from fish, was possible thanks to thorough original description of *F. nolandi* (Page 1967), supplemented with several line drawings of good quality and documentation in monographs by Page (1988, 1991). Description of the type species of the genus *Filamoeba* Page, 1967 was based on light microscopy and some unpublished observations in TEM (Page 1967). For almost 40 years, *F. nolandi* was the unique species representing the genus. The number of reported isolates was surprisingly low and most of them were not documented (Page 1976, Coutinho Salazar 1982, Ariza Astolfi *et al*. 1988). This seems to be the reason why in phylogenetic studies dealing with relationships among amoebae, one only SSU rDNA sequence of *Filamoeba* sp. (ATTC strain) was used.

Amaral Zettler *et al*. (2000), who discovered that distance and likelihood methods united *Filamoeba nolandi* and *Gephyramoeba* sp., also discovered consistent sister relationship of *Acanthamoeba - Balamuthia* with *Gephyramoeba - Filamoeba*. The bootstrap support for common ancestry of both couples was marginal in their analyses. On the basis of an "unexpected relationship" between *Gephyramoeba* and *Filamoeba*, and on the basis of morphological data, Amaral Zettler *et al*. (2000) suggested reconsideration of the current systematic placement of both genera. The strong union of *Gephyramoeba* and *Filamoeba* characterised by Amaral Zettler *et al*. (2000), based on distance and likelihood methods, weakened in studies by Bolivar *et al*. (2001) and Peglar *et al*. (2003). Also in our study, this union is supported by low bootstrap only. In accordance with Peglar *et al*. (2003), representatives of four lineages of gymnamoebae included in our analysis are not related to the cluster of *Filamoeba* strains.

In the overview of morphological features important in amoeboid taxa subjected to phylogenetic analysis, tubular and branching cristae of mitochondria were mentioned by Amaral Zettler *et al*. (2000) in *F. nolandi* and *Gephyramoeba* sp. To the best of our knowledge, this feature was not documented in any ultrastructural study. The branching pattern of mitochondrial cristae of *F. sinensis*, if any, can hardly be identified with typical branching pattern as known, e.g. in *Acanthamoeba* spp.

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