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Two New Species of the Genus *Stenamoeba* Smirnov, Nassonova, Chao et Cavalier-Smith, 2007

Iva DYKOVÁ[1,2], Martin KOSTKA[1,3] and Hana PECKOVÁ[1]

[1] Institute of Parasitology, Biology Centre, Academy of Sciences of the Czech Republic, České Budějovice, Czech Republic; [2] Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic; [3] Faculty of Agriculture, University of South Bohemia, České Budějovice, Czech Republic

Summary. As a result of light microscopical, ultrastructural and molecular study of three amoeba strains isolated from organs of three freshwater fish hosts, *Stenamoeba amazonica* sp. n. and *S. limacina* sp. n. are described as new amoeba species. The mutual comparison of isolated strains has extended the knowledge of morphological diversity within the genus *Stenamoeba* Smirnov, Nassonova, Chao et Cavalier-Smith, 2007. Molecular data obtained for these strains have complemented the phylogenetic tree that so far has contained only one nominal species within the single-genus *Stenamoeba* clade.

Key words: *Stenamoeba amazonica*, *Stenamoeba limacina*, new species, taxonomy, phylogeny.

INTRODUCTION

Recently, Smirnov *et al.* (2007) treated taxonomy of vannellid amoebae (Amoebozoa; Vannellidae), made a comprehensive phylogenetic analysis of their SSU rDNA sequences and rectified some of the incongruences between the traditional morphology-based taxonomy and molecular data. They declared that in vannellid amoebae the presence/absence of glycostyles is an invalid character at the generic level, suppressed the genus *Platyamoeba* Page, 1969, transferred species of this genus into *Vannella* Bovee, 1965 and emended diagnosis of the latter genus. In their study, special attention was paid to *Platyamoeba stenopodia* Page, 1969, the generic assignment of which had been debated earlier due to morphological features (Page and Blakey 1979, Smirnov and Goodkov 1999) and later due to the lack of phylogenetic relationship with other vannellids (Fahrni *et al*. 2003, Smirnov *et al.* 2005). Smirnov *et al.* (2007) erected a new genus *Stenamoeba* Smirnov, Nassonova, Chao et Cavalier-Smith, 2007 with *Stenamoeba stenopodia* (Page 1969) as the type and only species.

In this study we present another two *Stenamoeba* species which we isolated from the organs of two freshwater fishes.

Address for correspondence: Iva Dyková, Institute of Parasitology, Biology Centre AS CR, Branišovská 31, 370 05 České Budějovice, Czech Republic; Fax: 00420 385310388; E-mail: iva@paru.cas.cz

MATERIALS AND METHODS

Amoeba strains included in this study were isolated from organs of freshwater fishes: two strains (P119 and P126) from gills of *Psectogaster amazonica* Eigenmann et Eigenmann (Characiformes) and *Calophysus macropterus* (Lichtenstein) (Siluriformes), respectively, both collected in the Amazon River (Peru, Iquitos) and one strain (4692L) from kidney tissue of *Gobio gobio* Linnaeus (Cypriniformes) caught in the Lužnice River (Czech Republic, South Bohemia). Amoeba strains were subcultured weekly on non-nutrient amoeba saline agar (NN recipe in the Catalogue of the UK National Culture Collection, 2001).

Light-microscopical observations were made of live amoebae attached to the under side of cover slips in hanging drops, using Nomarski optics. Material for electron microscopy was collected after fixation of cultured amoebae on the surface of agar plates in cacodylate-buffered 3% glutaraldehyde. Pelleted trophozoites and cysts were postfixed in 1% osmium tetroxide and embedded in Spurr resin. Ultrathin sections were examined with a JEOL JEM 1010 electron microscope operating at 80 kV. Images were collected with Megaview II soft imaging system using analySIS software.

DNA was extracted from the cell pellets using the JETQUICK Tissue DNA Spin Kit (Genomed, Germany) according to the manufacturer's protocol. The SSU rRNA gene was amplified in two overlapping fragments, using primer pairs ERIB1 (5'-ACCTGGTTGATCCTGCCAG-3') – 1350R (5'-CCGTCAATTCCTTTAAGTTTC-3') and 1200F (5'-GATCAGATACCGTCGTAGTC-3') – ERIB10 (5'-CTTCCGCAGGTTCACCTACGG-3'). PCR was carried out in 25 µl reaction volume using a standard technique with 1 µM of each primer, 200 µM of each dNTP, 2.5 µl of 10 × Taq polymerase buffer and 1 unit of TaqDNA polymerase (Top-Bio, Czech Republic). The reactions were run on a Tpersonal Thermocycler (Biometra). The cycling conditions included a 5 min. initial denaturation at 95°C, then the SSU rRNA gene was amplified in 5 cycles (each comprising 94°C for 1 min., 46°C for 1.5 min. and 72°C for 2 min.) followed by 25 cycles (each comprising 94°C for 1 min., 50°C for 1.5 min. and 72°C for 2 min.) and a final 10 min. extension at 72°C. The amplified products were gel-purified using JETQUICK Gel Extraction Spin Kit (Genomed, Germany) and cloned into pDrive Cloning Vector using the QIAGEN PCR Cloning Kit (Qiagen GmbH, Germany). Positive clones were sequenced in both directions on an automatic sequencer ABI 3130x1 using the ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA) with a combination of flanking and internal primers as mentioned elswhere (Dyková *et al.* 2008).

The main dataset used for phylogenetic analyses consisted of two new complete SSU rDNA sequences of strains 4692L and P119 plus another two *Stenamoeba* and 24 other amoebozoan sequences from GenBank with emphasis on lineages that were shown to be the closest relatives of *Stenamoeba* (i.e. *Thecamoeba*, *Sappinia*, Acanthamoebidae). See Fig. 12 for GenBank accession numbers of the sequences used. All datasets were aligned by ClustalX 2.0.6 (Larkin *et al.* 2007). The alignments were carefully checked for ambiguously aligned positions (which were manually deleted) in BioEdit (Hall 1999). The length of the final alignment was 1357 bp. For further analyses, other alignments were also prepared that included partial SSU rDNA sequence of strain P126 and even shorter segment of SSU rDNA of *Stenamoeba* strain A2 NM-2009a from GenBank (Acc. No. GQ996534).

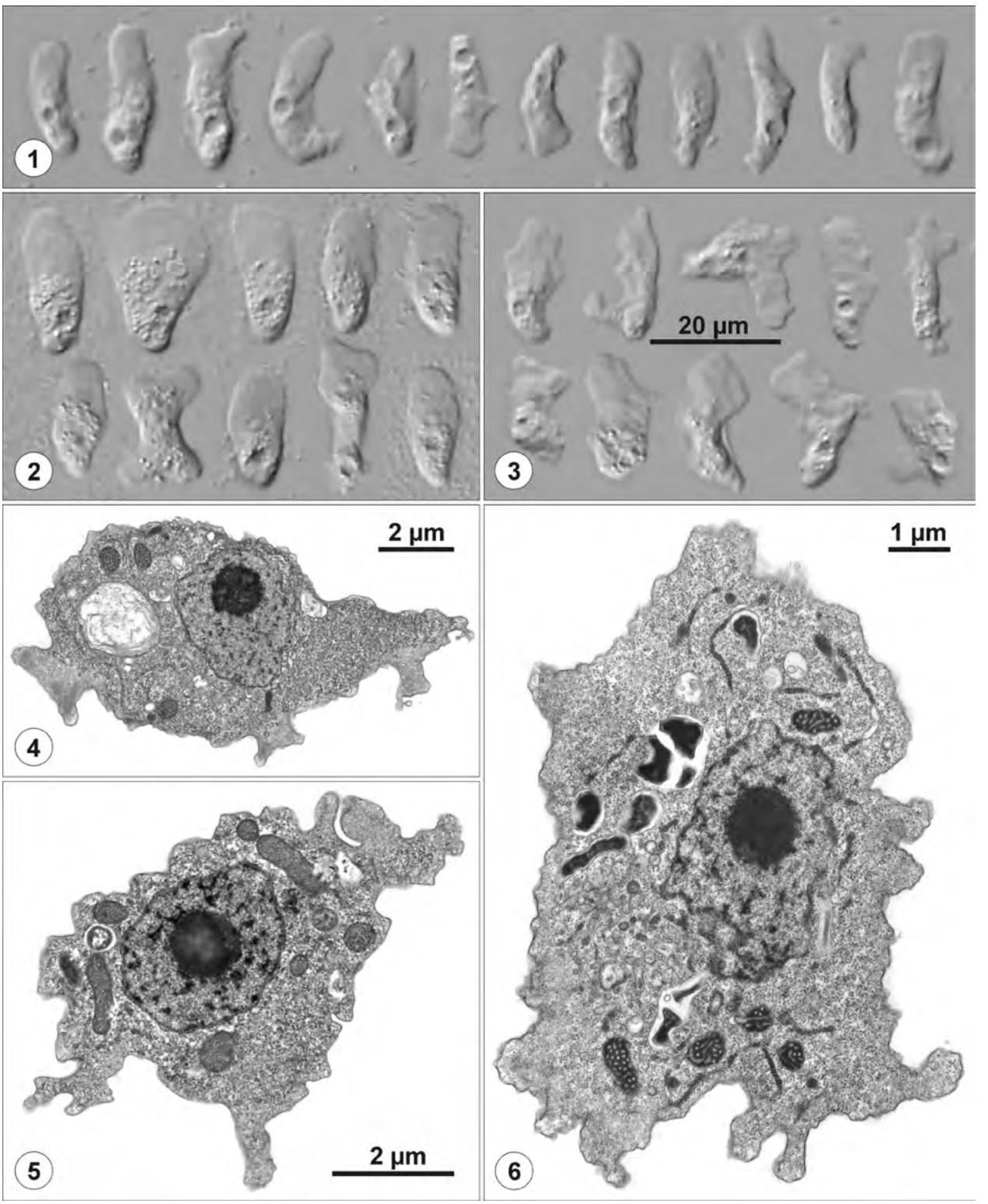
Maximum likelihood (ML) analyses of the alignment were conducted in the program RaxML 7.0.3 (Stamatakis 2006) employing GTR+Γ model and rapid bootstrapping (1,000 replicates). Maximum parsimony analysis (MP) and analysis using Fitch-Margoliash method with LogDet distances (LD) were run in PAUP* 4b10 (Swofford 2003). Heuristic searches were repeated 1,000-times starting from trees constructed by random taxa addition and swapped by TBR algorithm. They were then bootstrapped (1,000 replicates, only 10 starting trees constructed by random taxa addition for each replicate). To determine percent identities of SSU rDNA sequences of stenamoebae, we used an alignment containing all aforementioned *Stenamoeba* sequences except for too short A2 NM-2009a (five sequences in total). The alignment was made in ClustalX and trimmed in accordance with the shortest sequence of strain P126. Percent identity matrix was computed in BioEdit.

RESULTS

Light microscopy and ultrastructure

Since the beginning of subculturing, trophozoites of two amoeba strains from gills of the Amazon River fishes (P119, P126) presented light microscopical features of *Stenamoeba* (Figs 2, 3). The shape of trophozoites of the amoeba strain from kidney of gudgeon (4692L) resembled that of "limax" amoebae for a long period of subculturing due to overload of trophozoites with bacteria. The strains under study were tentatively assigned to the genus *Stenamoeba* based on the following light microscopical features: trophozoites attached to under side of cover slips flat, oblong or linguliform, with a large anterior part of hyaloplasm; nucleus usually located in posterior part of hyaloplasm near its border with granuloplasm; single contractile vacuole mostly in posterior part of granuloplasm; cysts rounded.

Although uniform in principal characters of generic diagnosis, trophozoites of these three strains differed in some light microscopical features. Strain 4692L was the most similar one to the original Page's (1969) description of *Platyamoeba stenopodia* (=*Stenamoeba stenopodia*), including the range of measurements. Its trophozoites maintained "limax" outline, with only slightly narrowed posterior region that sometimes had a knobby appearance (Fig. 1). Surface transitory wrinkles were observed in flattened trophozoites only. Of the biological features, notable was an extremely low ability of trophozoites to attach to coverslips. Among trophozoites that in hanging drop preparations fell down to the bottom of depression slides, both floating forms



Figs 1–3. Trophozoites of *Stenamoeba* species as seen in Nomarski differential interference contrast. **1** – *Stenamoeba limacina* sp. n., type strain (4692L); **2** – *Stenamoeba amazonica* sp. n., type strain (P119); **3** – *Stenamoeba* sp, strain P126. Scale bar applies also for Figs 1, and 2.

Figs 4–6. Ultrathin sections of trophozoites *Stenamoeba* spp. **4** – *S. amazonica*, type strain P119; **5** – Strain P126; **6** – *S. limacina*, type strain 4692L.

with irregular hyaline pseudopods with bluntly rounded tips and linguliform individuals were observed.

Trophozoites of both strains isolated from gills of Amazon River fishes (P119, P126) were larger and dorso-ventrally much thinner (Figs 2, 3) than trophozoites of strain 4692L (Fig. 1). Elongated trophozoites of strain P119 displayed a more regular outline and a smoother cell surface than trophozoites of strain P126 that were of the same size range as P119 but were less uniform in shape and with surface folds and transitory wrinkles.

Essential ultrastructural features shared by all three strains (Figs 4–11) were a thin amorphous glycocalyx covering cell surface, mitochondria with tubular interlaced cristae, vesicular nucleus with rounded, electron-dense nucleolus and ill-defined chromatin patches in nucleoplasm. Average diameter of nuclei (when round), 2.6 μm. Cysts, av. diameter 5.6 μm, were single-walled. No species- or strain-specific ultrastructural details were observed.

SSU rDNA sequences and phylogeny

The obtained SSU rDNA sequences of strains 4692L and P119 were 2249 and 2286 bp long, respectively. Only one segment (930 bp) of SSU rDNA sequence was obtained for strain P126. The sequences are deposited in GenBank data base under Acc. Nos. GU810183–GU810185.

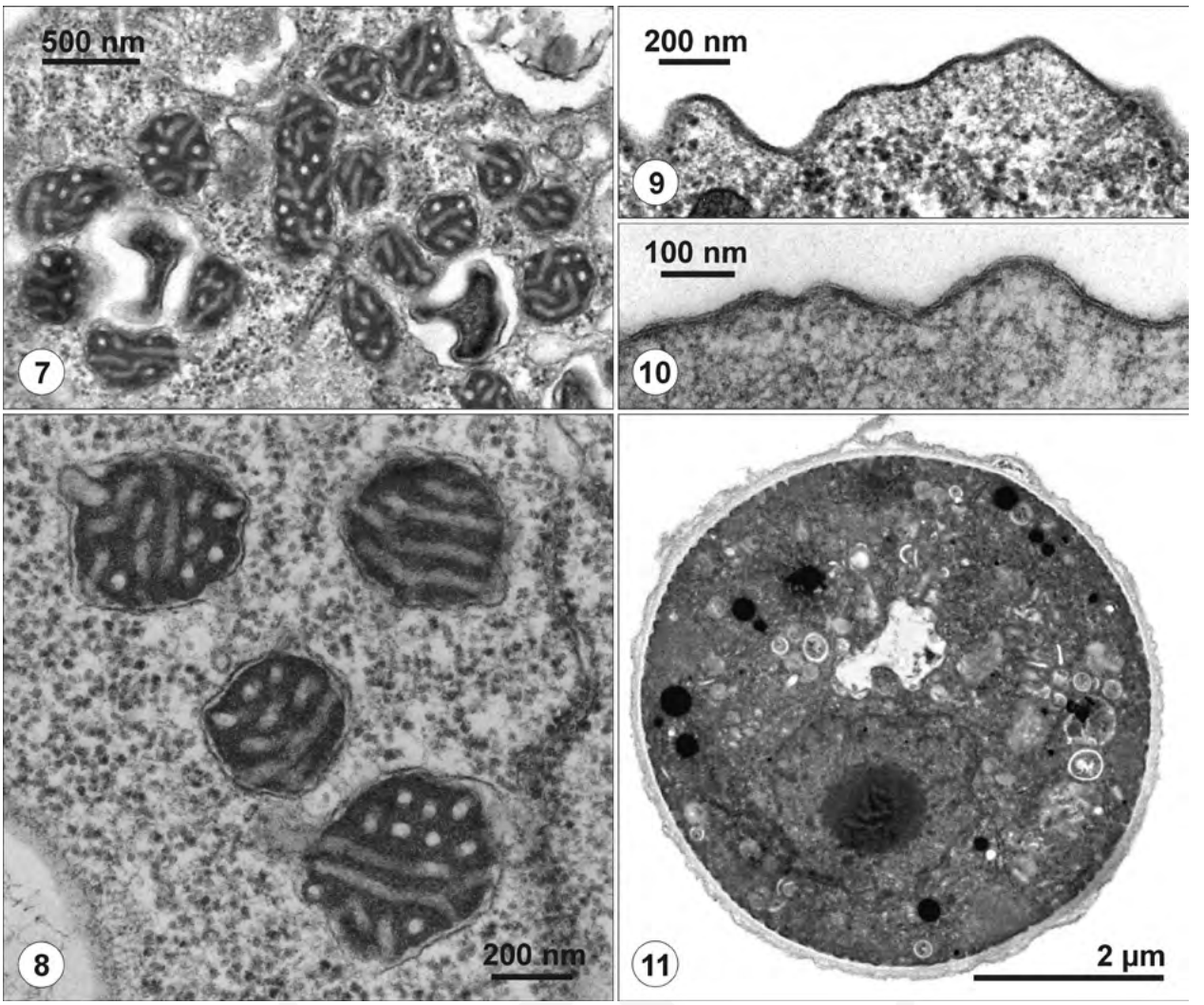
The tree resulting from phylogenetic analyses of the main dataset (including complete SSU rDNA sequences only) shows clearly (with very high bootstrap support) that both sequences are closely related to those of the two earlier sequenced stenamoebae (Fig. 12). Within the genus *Stenamoeba*, two groups are formed. One comprises strain P119 and the type strain of *S. stenopodia* and is well supported by bootstrap values. The other one comprises strains CRIB68 and 4692L; its bootstrap support is good (ML 88%, MP 92%, LD 99%). Amoebae of the genera *Thecamoeba* and *Sappinia* were resolved as a sister group of *Stenamoeba*. However, when the partial sequence of strain P126 is included in phylogenetic analyses, the overall resolution of the resulting tree worsens. This strain still clusters with stenamoebae (with strain 4692L) in ML trees, but with little bootstrap support (< 50% for P126 + 4692L and < 50% for the whole genus *Stenamoeba* itself). In LD tree the strain formed basal branch to other stenamoebae and was not at all grouped with them in MP tree. Inclusion of another short SSU rDNA sequence (A2 NM-2009a strain) did not improve topology resolution – it in fact worsened for LD tree where *Thecamoeba* was reconstructed as an ingroup of *Stenamoeba*, which is clearly an artifact. The exact phylogenetic position of strain P126 thus remains unclear, at least until its complete SSU rDNA sequence is obtained. It should be noted that its branch in the tree was considerably longer than those of other stenamoebae. Direct comparison of sequences shows that the available part of SSU rDNA of P126 strain shares 86% identity with respective parts of SSU rDNA sequences of CRIB68 strain and 4692L strain. On the contrary, the sequence identity of the latter two amoebae is 95%. The sequence identity calculated for strain P119 and the type strain of *S. stenopodia* is 91%. Note that the length of the alignment used to compare the sequences was 956 bp (limited by partial sequence length of P126 strain). When whole SSU rDNA sequences of all stenamoebae except strain P126 were compared, the percent identities were generally lower by 2–8% and were highest for the two pairs, CRIB68 vs. 4692L and P119 vs. *S. stenopodia* (90% in both cases). If SSU rDNA sequence of A2 NM-2009a strain alone is included in the main dataset used for phylogenetic analyses, it clusters with strain CRIB68 with rather low bootstrap support (ML 69%, MP and LD < 50%). Otherwise, the topology remained the same as in the case of the tree in Fig. 12, only the bootstrap support for (4692L + CRIB68 + A2 NM-2009a) group lowered (ML 74%, MP 80%, LD 51%). Note that the segment available for A2 NM-2009a strain (395 bp) is rather conserved: there are only two mismatches (one of them insert) in this sequence when compared to CRIB68. Two mismatches are also between CRIB68 and 4692L in this region, but 4 mismatches between 4692L and A2 NM-2009a. For comparison, there are 3 or 5 mismatches in the region in the case of P119 vs. *S. stenopodia* or CRIB68 vs. *S. stenopodia*, respectively.

Taxonomic conclusions

Based on the unique combination of morphological and molecular data, two species of the genus *Stenamoeba* Smirnov, Nassonova, Chao et Cavalier-Smith, 2007 are described.

***Stenamoeba limacina* sp. n. (Figs 1, 6, 8, 10)**

Flattened amoeba of elongated limax-like shape with anterior part round or truncate; anterior half hyaline, posterior half granular; single contractile vacuole; length in locomotion 15.4–19.2 μm, length/breadth ratio 3.1–4.0; transitory folds or wrinkles on the surface of trophozoites if not overloaded with phagocytosed bacteria; no pseu-



Figs 7–11. Details of ultrastructure characterizing the new *Stenamoeba* spp. **7** – mitochondria of *S. amazonica*; **8** – mitochondria of *S. limacina*; **9** – cell surface of *S. amazonica*; **10** – cell surface of *S. limacina*; **11** – cyst structure of *S. amazonica*.

dopods formed in locomotion; floating form with blunt radiating pseudopodia; cysts spherical, single-walled. Trophozoites not prone to attach to coverslips.

Type strain: 4692L isolated from the kidney tissue of *Gobio gobio* Linnaeus (Cypriniformes), caught in the Lužnice River (Czech Republic, South Bohemia), June 1991.

Material deposition: Cryo-collection of the Institute of Parasitology, Biology Centre of the Academy of Sciences of the Czech Republic, České Budějovice, Czech Republic. GenBank Acc. No.: GU810183.

Etymology: The specific name refers to the limax-like shape of trophozoites.

***Stenamoeba amazonica* sp. n. (Figs 2, 4, 7, 9, 11)**

Flattened amoeba with outline usually elliptical or linguliform; length (18.5–23.2 µm) greater than breadth (length/breadth ratio 1.5–2.6); anterior half hyaline and dorsoventrally thinner than granular posterior half; single contractile vacuole; surface of trophozoites smooth; floating form with blunt radiating pseudopodia; cysts spherical, single-walled.

Type strain: P119 isolated from gills of *Psectogaster amazonica* Eigenmann et Eigenmann (Characiformes), collected in the Amazon River (Peru, Iquitos), November 2004.

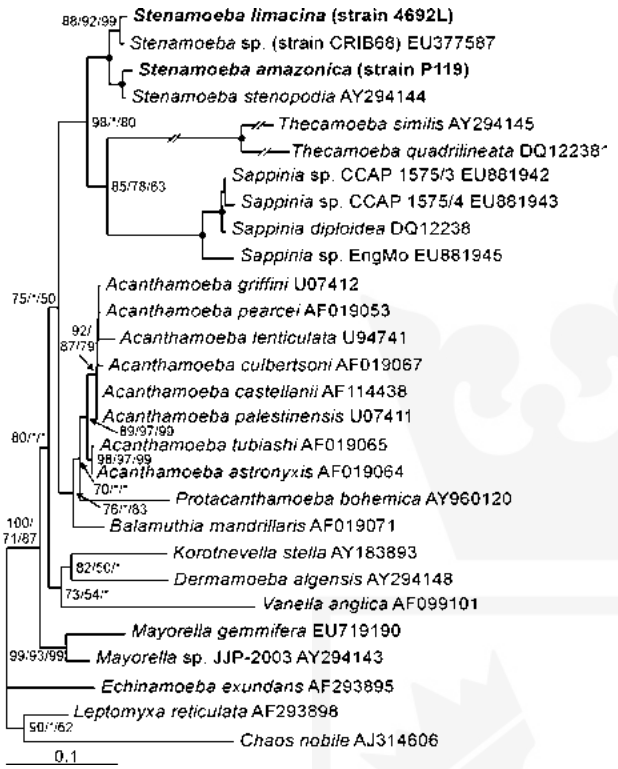


Fig. 12. Maximum likelihood (ML) tree of 28 amoebozoan SSU rDNA sequences. The two new sequences are in bold. Other sequences are supplemented with their GenBank accession numbers. Bootstrap numbers at the nodes are for ML, maximum parsimony and Fitch-Margoliash methods with LogDet distances. Nodes that scored 99 or better bootstrap support from all three methods used are indicated by black dots. Lengths of branches of *Thecamoeba* spp. are halved. All other branches are drawn to the scale.

Material deposition: Cryo-collection of the Institute of Parasitology, Biology Centre of the Academy of Sciences of the Czech Republic, České Budějovice, Czech Republic. GenBank Acc. No.: GU810184.

Etymology: The species name refers to the geographic origin of the fish host.

Strain P126 (Figs 3, 5)

Flattened amoeba of irregular outline; length (18.4–22.0 μm) always greater than breath, (length/breadth ratio 2.2–2.7); anterior part hyaline and dorsoventrally thinner than posterior granular part; indistinct wrinkles on the surface of trophozoites, single contractile vacuole; floating form with blunt radiating pseudopodia; cysts spherical. Its morphology is similar to that of *S. limacina* and *S. amazonica*, but its phylogeny is too inconclusive for a safe generic assignment.

Strain isolated: From gills of *Calophysus macropterus* (Lichtenstein) (Siluriformes), collected in the Amazon River (Peru, Iquitos), November 2004.

Material deposition: Cryo-collection of the Institute of Parasitology, Biology Centre of the Academy of Sciences of the Czech Republic, České Budějovice, Czech Republic. GenBank Acc. No.: GU810185.

DISCUSSION

In our long-term studies of amphizoic amoebae from fishes, isolates of flattened amoebae other than vannellids were not frequent. It is possible that *Stenamoeba* species are more frequent in water or sediment samples than in fish material.

The species described in this study demonstrate a newly revealed morphological diversity within the genus *Stenamoeba*. Each of the two new species shows a peculiarity worth of mentioning. Thus, *S. limacina* in primary isolates (i.e. before the repeated subculturing makes obvious the flattened form of trophozoites) could easily be misidentified as a member of the Tubulinea. Interestingly, this species not only has a great morphological resemblance to *S. stenopodia* but it also shares the low adhesiveness of trophozoites observed in *S. stenopodia* by Page (1969). The lack of wrinkles on the surface of the type strain of *S. amazonica* brings the morphology of this species close to vannellids and suggests that temporary folds, wrinkles or ridges, included in the generic diagnosis of *Stenamoeba*, do not occur in all species.

The overall topology of the tree is in agreement with other recent phylogenetic studies, including the sister-group status of *Stenamoeba* and Thecamoebidae (e.g. Tekle *et al.* 2008).

When only the four complete *Stenamoeba* SSU rDNA sequences are analysed, the support of their monophyly and for internal topology within the genus is very good. However, inclusion of the partial sequence of P126 strain greatly worsens the good resolution of the tree topology. We believe that this is caused partially by its incompleteness and by its relative divergence from SSU rDNA of other *Stenamoeba* sequences used and that the topology within the genus *Stenamoeba* as shown in the Fig. 12 is correct, despite its dramatically lowered bootstrap support upon inclusion of P126 in analyses.

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