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Protacanthamoeba bohemica sp. n., Isolated from the Liver of Tench, *Tinca tinca* (Linnaeus, 1758)

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Summary. A new species of amphizoic amoeba, *Protacanthamoeba bohemica* sp. n., isolated from the liver of *Tinca tinca* (Cypriniformes: Cyprinidae) is described. Trophozoites typical of the genus *Protacanthamoeba* Page, 1981 differ distinctly from those of the type species of the genus in having constantly more numerous and much longer acanthopodia. No relevant ultrastructural distinctions were observed. SSU rRNA gene sequence acquired for *P. bohemica* (the first within the genus) have been deposited in the GenBank database under accession number AY960120. A phylogenetic analysis based on SSU rRNA gene sequences assigned *P. bohemica* to the *Acanthamoeba* and *Balamuthia* clades.

Key words: amphizoic amoebae, morphology, phylogeny, *Protacanthamoeba bohemica* sp. n.

INTRODUCTION

Series of studies revealed that free-living amoebae infect fish as well as some aquatic invertebrates, playing parts of innocuous epi- or endobionts or infectious agents of diseases (Sawyer 1976, Johnson 1977, Jones 1985, Dyková and Lom 2004). The reasons why many amoebae can convert from free-living to epi- or endobionts of fishes are enigmatic. The study of amoebae capable of colonising fish tissues is undoubtedly the most important step towards resolving this enigma. In general, the morphological diversity and polymorphism of amoebae with poorly documented descriptions of species sometimes make even generic diagnosis of newly isolated strains extremely difficult. Some genera of free-living amoebae seem to be morphologically well defined, but they were often erected to include one species, the description of which was based on a single isolate. When the type strains of such taxa have not been maintained in culture collections, newly isolated strains identifiable with them gain importance. Studies of such strains ensure that after the progress achieved in the establishment of genera of free-living amoebae at light-microscopical and ultrastructural levels, other objective characters resulting from molecular studies might be added to generic and species diagnoses. This objective is the focus of our study.

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Based on an amoeba strain isolated from the liver of a cyprinid fish *Tinca tinca* (Linnaeus, 1758), we describe a new species of the genus *Protacanthamoeba* that have been represented to date by the type species *P. caledonica* Page, 1981 and the species *P. invadens* (Singh *et* Hanumaiah, 1979) described originally as *Acanthamoeba* sp. To the best of our knowledge, only slides of *P. caledonica* stained with Kernechtrot are deposited as a type material in The Natural History Museum of London, England (Holotype 1980: 12:22.1).

MATERIALS AND METHODS

Strain origin, cultures, sample preparation and morphological studies. The clone designated as TT3H/I was derived from amoeba strain isolated from the liver of *Tinca tinca*, which was collected in Spolský pond, South Bohemia, Czech Republic, in January 1997. The amoebae were cultured routinely on non-nutrient agar (NNA) seeded with an autoclaved suspension of *Escherichia coli* (Kalinina and Page 1992). Comparison of growth was done in cultures kept simultaneously at fluctuating RT, 4ºC, and constant (20ºC) temperature of an incubator. The hanging drop preparations were used to observe and take measurements of trophozoites. The thin layer of 1.5% agar was used to observe and document cysts. Fixation for electron microscopy was done either with 2% osmium tetroxide (selected blocks of agar with submerged trophozoites and cysts) or cacodylate buffered 2.5% glutaraldehyde (fixation of cultures *in situ* on agar plates) followed by postfixation with 1% osmium tetroxide (Dykstra 1993). Both materials, either blocks of agar or peletted material, were embedded in Spurr's resin (Spurr 1969). The ultrathin sections were double stained with uranyl acetate and lead citrate and examined under a Jeol JEM 1010 electron microscope at 80 kV accelerating voltage.

DNA isolation, PCR amplification and sequencing. Total DNA was extracted from the clonal culture of TT3H strain using the DNeasy™ Tissue Kit (Qiagen), according to the manufacturer's protocol. The SSU rRNA gene was amplified by PCR, using a set of universal eukaryotic primers (Medlin *et al*. 1988). Thermal cycling was initiated by heating for 5 min at 95ºC, followed by 30 cycles (each comprising 94ºC for 1 min, 55ºC for 1 min and 72ºC for 2 min) and terminated by elongation at 72ºC for 10 min. The amplified product was purified, cloned into the pCR® 2.1 TOPO cloning vector (TOPO-TA Cloning kit, Invitrogen) and sequenced using the automatic sequencer CEQ™ 2000 (Beckman Coulter) with the CEQ DTCS Dye Kit (Beckman Coulter).

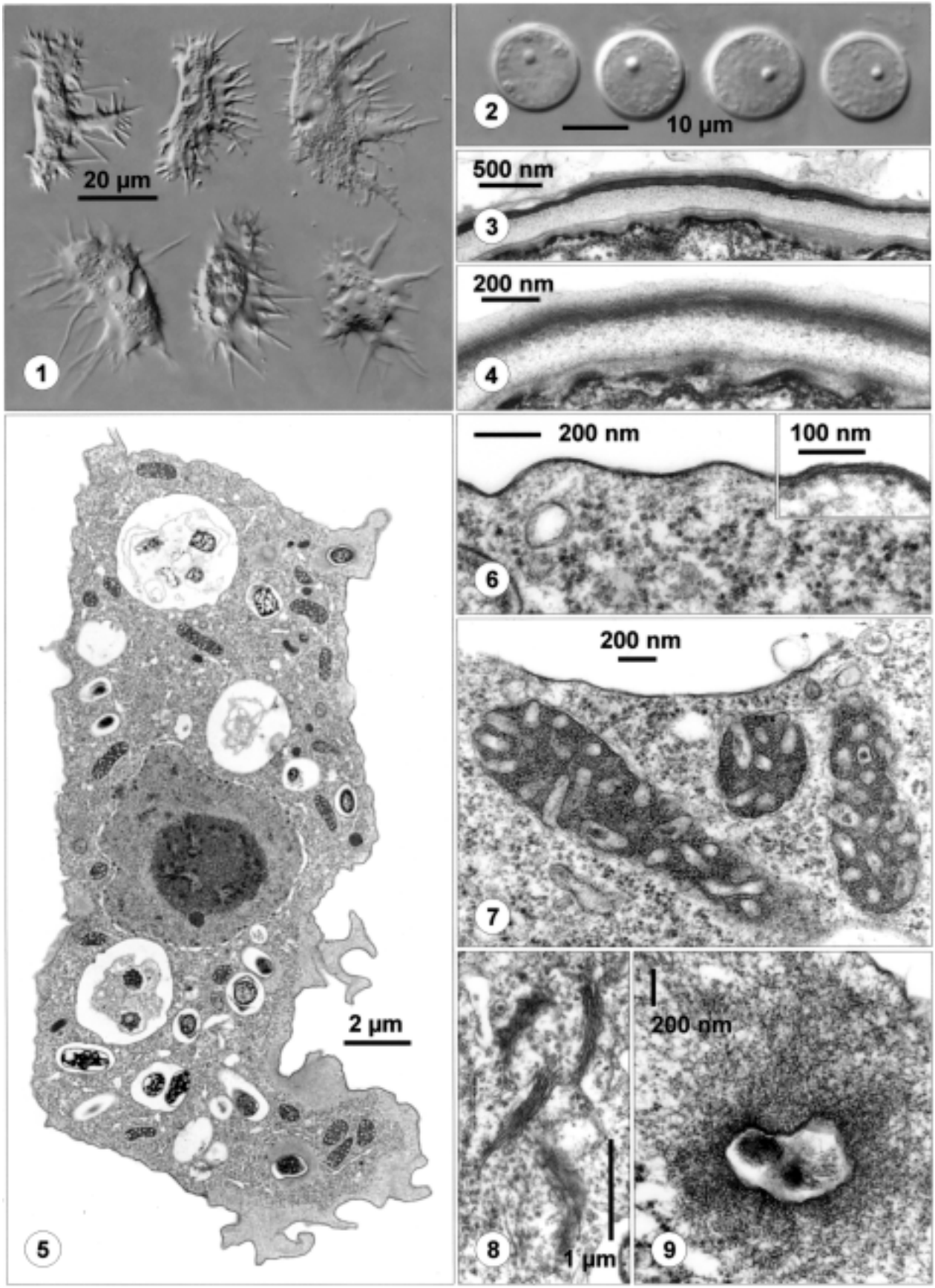
Alignments and phylogenetic analyses. The sequence of the SSU rRNA gene from the studied strain was aligned with two sets of sequences retrieved from GenBank. The first dataset was compiled in order to find out phylogenetic position of the representative of newly sequenced amoeba genus. This dataset included representatives of important lineages of Amoebozoa sensu Bolivar *et al*. (2001) and species that have trophozoites with some morphological similarities. It was completed with representatives of the Metazoa, Fungi, Viridiplantae, Alveolata and Stramenopiles. Of the basal taxons of Eukaryota, *Hexamita inflata* and *Trichomonas vaginalis* were chosen as an outgroup (Arisue *et al*. 2005). In total, 45 sequences were aligned using the Clustal_X program (Thompson *et al*. 1997) with various alignment parameters. Corrections were done by eye using the BioEdit sequence alignment editor (Hall 1999). The alignment consisted of 1518 unambiguous aligned positions with 750 parsimony informative characters. The second dataset reduced to sequences closely related to the sequence of the strain under study consisted of 19 sequences. Alignment comparisons were limited to 1865 sites, from which 477 were parsimony informative.

Phylogenetic analyses of both datasets were performed using the maximum parsimony (MP) and maximum likelihood (ML) methods. They were carried out with the program package PAUP*, version 4.0b8 (Swofford 1999). Search for the MP tree was done heuristically with the random addition of taxa (10 replications). Gaps were treated as missing data. Transition:transversion (Ts:Tv) ratios were set to 1:1, 1:2 and 1:3. Clade support was assessed with bootstraping of 1000 replicates and calculation of Bremer indices (Bremer 1994). For ML analysis, the likelihood ratio test (LRT) implemented in Modeltest v. 3.06 (Posada and Crandall 1998) was used to determine the best model of evolution. Based on the LRT, the ML analysis of the first dataset was performed with the GTR + Ã model of evolution (á shape parameter 0.4036), while for the second dataset, the GTR + Ã + I model of evolution was applied (á shape parameter 0.5241; proportion of invariable sites 0.2691). A bootstrapping of 1000 replicates was employed to estimate clade support.

RESULTS

Light and electron microscopy of trophozoites and cysts. Since their primary isolation, trophozoites cultured on agar plates revealed remarkable features. They formed dense growing zones toward the periphery of the Petri dishes, consisting of distinctly irregular cells that left behind a zone of cyst formation. Trophozoites submerged deeply into NNAs of 1.5, 2.5 and 3% strength. They multiplied slowly and hence subculturing was done usually once in two, to three weeks. To achieve a good harvest of trophozoites, clonal cultures were maintained at 4ºC. At higher temperatures (RT or

Figs 1-9. Light- and electron micrographs of *Protacanthamoeba bohemica* sp. n. **1** - trophozoites as observed in hanging drop preparations with Nomarski DIC; **2** - cysts as seen in Nomarski DIC; **3, 4** - two segments of cyst walls with layers differing in electron density; **5** - overview of a thin section through trophozoite. In addition to three food vacuoles with remnants of phagocytosed material, the cytoplasm also contains phagocytosed bacteria surrounded by a rim of hyaloplasm (see also detail in Fig. 9); **6** - the cell surface of trophozoite; **7** - mitochondria with tubular cristae; **8** - cisternae of Golgi complex in a parallel arrangement; **9** - hyaloplasmic microfilaments accumulated around phagocytosed material.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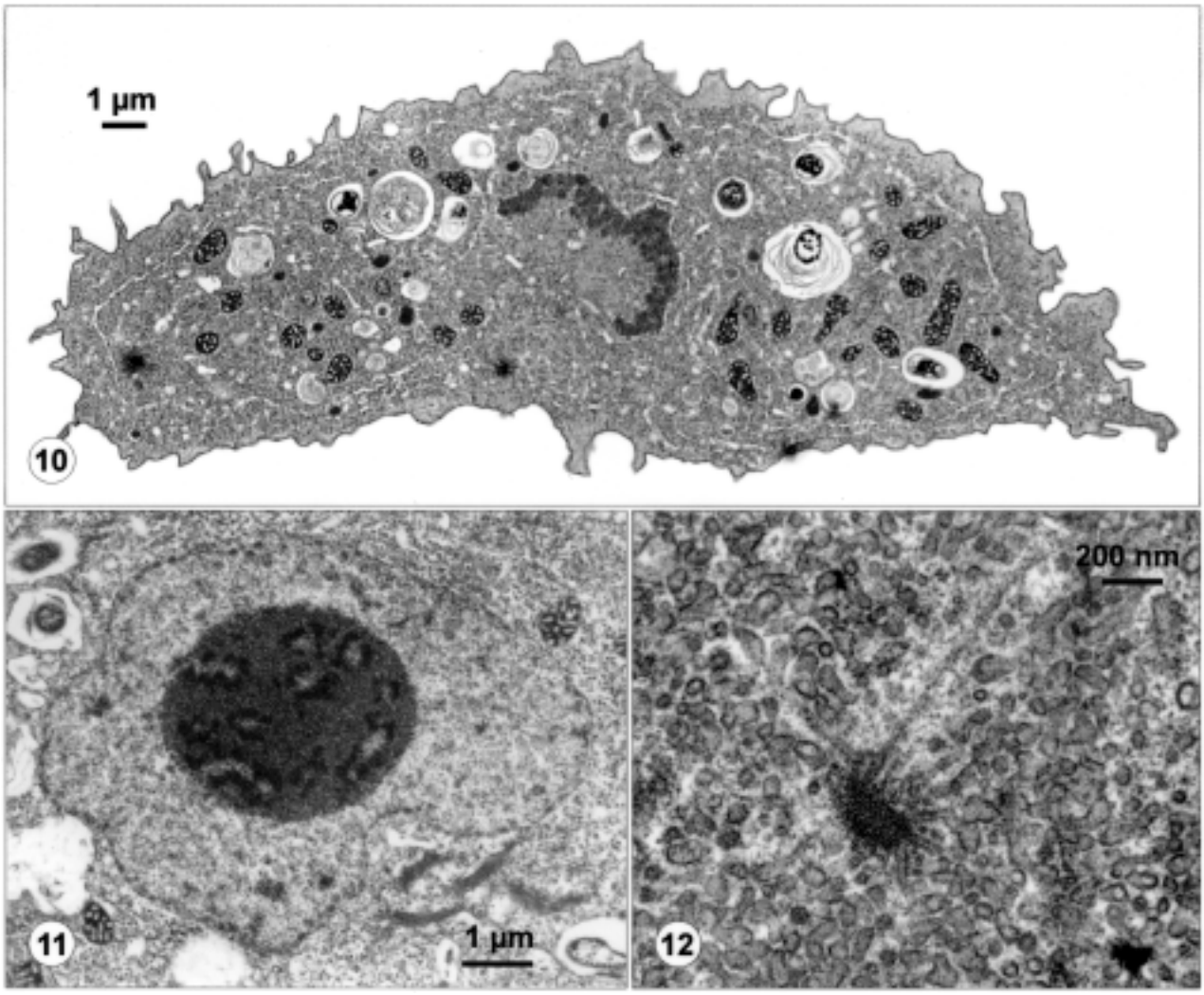

Figs 10-12. Details of ultrastructure of *Protacanthamoeba bohemica* sp. n. **10** - part of the nucleus (lacking nuclear envelope) and remnants of a nucleolar mass; **11** - the heterogeneous appearance of nucleolus; **12** - centriole-like body with radiating microtubules.

20°C), the encystment of trophozoites was extremely fast. Cysts were spherical with a smooth appearance. When observed in hanging drop preparations, polymorphic trophozoites had distinctive acanthopodia extending from the zone of hyaloplasm (Fig. 1). There was no signifficant difference from acanthopodia, but they were more numerous and much longer than observed in *Acanthamoeba* strains previously isolated from fish. In addition, one extremely long acanthopodium frequently predominated. Long furcated acanthopodia characterised our strain irrespective of agar concentrations and temperatures applied, invariably during long-term culturing. Floating forms were rounded and had several pseudopodia usually equal or slightly longer than the diameter of the cell. The plasma membrane of trophozoites was thin (12-14 nm) and the cell coat was not detected in ultrathin sections despite of the two fixation protocols applied (Figs 5, 6). The cytoplasm contained bean-shaped mitochondria with branching tubular cristae (Fig. 7). The stacks of Golgi cisternae were located in the vicinity of nucleus (Fig. 8). In addition to ingested microorganisms, the cytoplasm of trophozoites contained parallel arrays of endoplasmic reticulum and aggregates of microfilaments (Fig. 9). They were observed in hyaloplasmic regions, usually accumulated around partly phagocytosed material (Fig. 10). A somewhat spherical nucleus with numerous nuclear pores (visualised after osmium tetroxide fixation by electron dense material located in

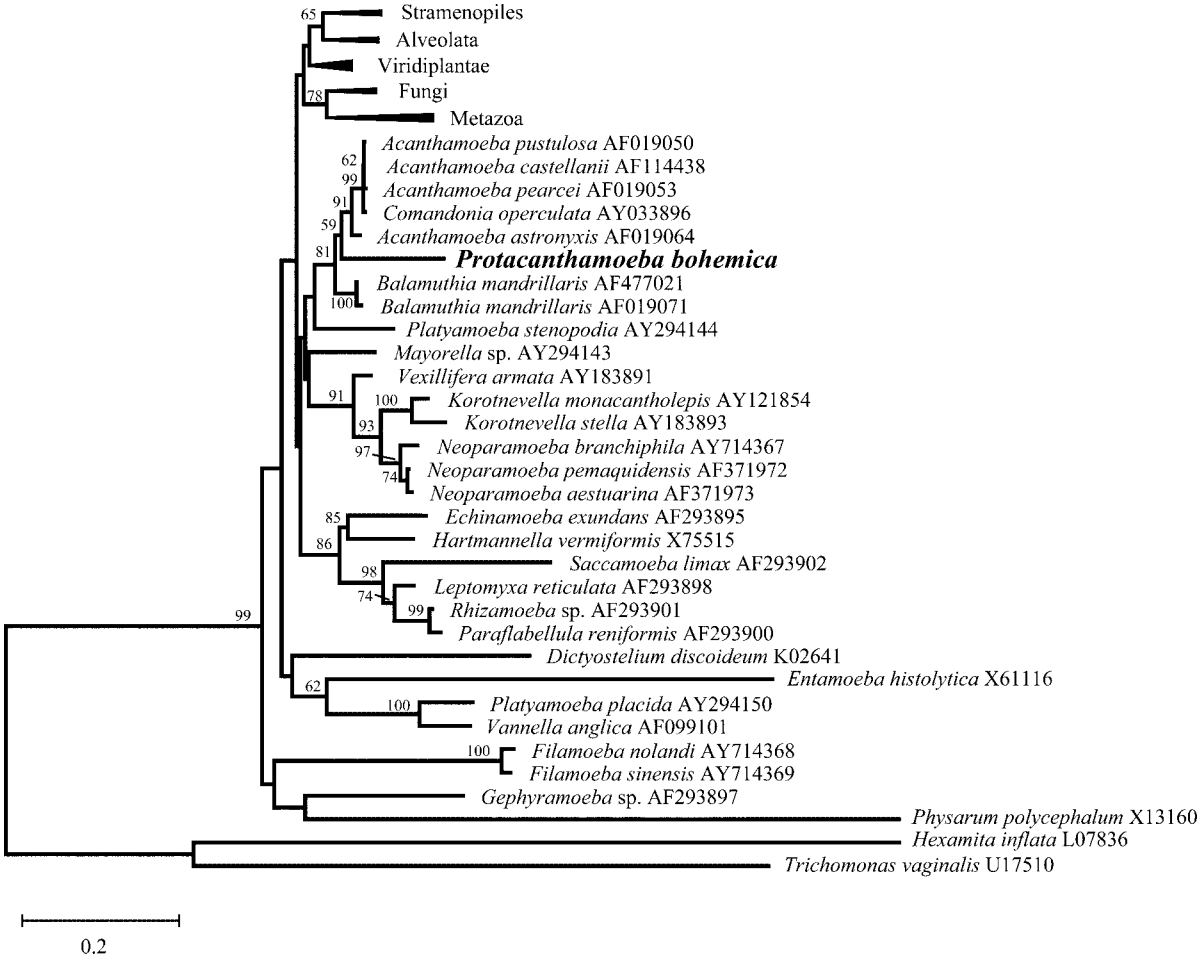

Fig. 13. Maximum likelihood tree (- ln = 19757.4333) of the SSU rRNA sequences. The distance scale is given under the tree. Bootstrap values are given for the nodes gaining more than 50% support. Stramenopiles = *Labyrinthuloides minuta* (L27634), *Fucus distichus* (AB011423); Alveolata = *Oxytricha nova* (X03948), *Gymnodinium fuscum* (AF022194); Viridiplantae = *Zea mays* (AF168884), *Equisetum ferrissii* (AF313576), *Chara polyacantha* (AF032742); Fungi = *Saccharomyces cerevisiae* (J01353), *Chytridium confervae* (M59758), *Mycale fibrexilis* (AF100946); Metazoa = *Xenopus laevis* (K01373), *Hydra circumcincta* (AF358080).

the perinuclear cisterna) contained a nucleolus mostly of heterogeneous appearance (Figs 5, 11). The disappearance of the nuclear envelope and rearrangement of the nucleus (Fig. 10.) were observed together with a centriole-like body and microtubules projecting from it (Fig. 12). Smooth, solid walls of spherical uninucleated cysts already observed in Petri dish cultures were also found in their native preparations (Fig. 2.) and when the silver staining method (Pussard and Pons 1977) was applied. Ectocysts were smooth and no cyst pores, irregularities, arms or separations of endocysts were observed. Three to four layers could be recognised in the walls of mature cysts (Figs 3, 4). In addition to poorly defined amorphous material, a dense region with membranes parallel to the cell surface formed the outermost layers of cysts. Dense amorphous and finely granular translucent layers followed these two layers. When only electron-density was used as a marker, cyst wall structure could be interpreted as subdivided into outer, middle, and inner layers. The cytoplasm of the mature cyst was dense. In addition to the nucleus and mitochondria, spherical lipid bodies and several membrane-bound dense bodies were observed in ultrathin sections.

On the basis of light microscopical and ultrastructural features of trophozoites and cysts, the strain under study was assigned to the genus *Protacanthamoeba* Page, 1981 as a representative of the new species *P. bohemica* sp. n.

Protacanthamoeba bohemica sp. n.

Origin of type material: Liver of tench, *Tinca tinca* (Linnaeus, 1758) (Cypriniformes: Cyprinidae) collected

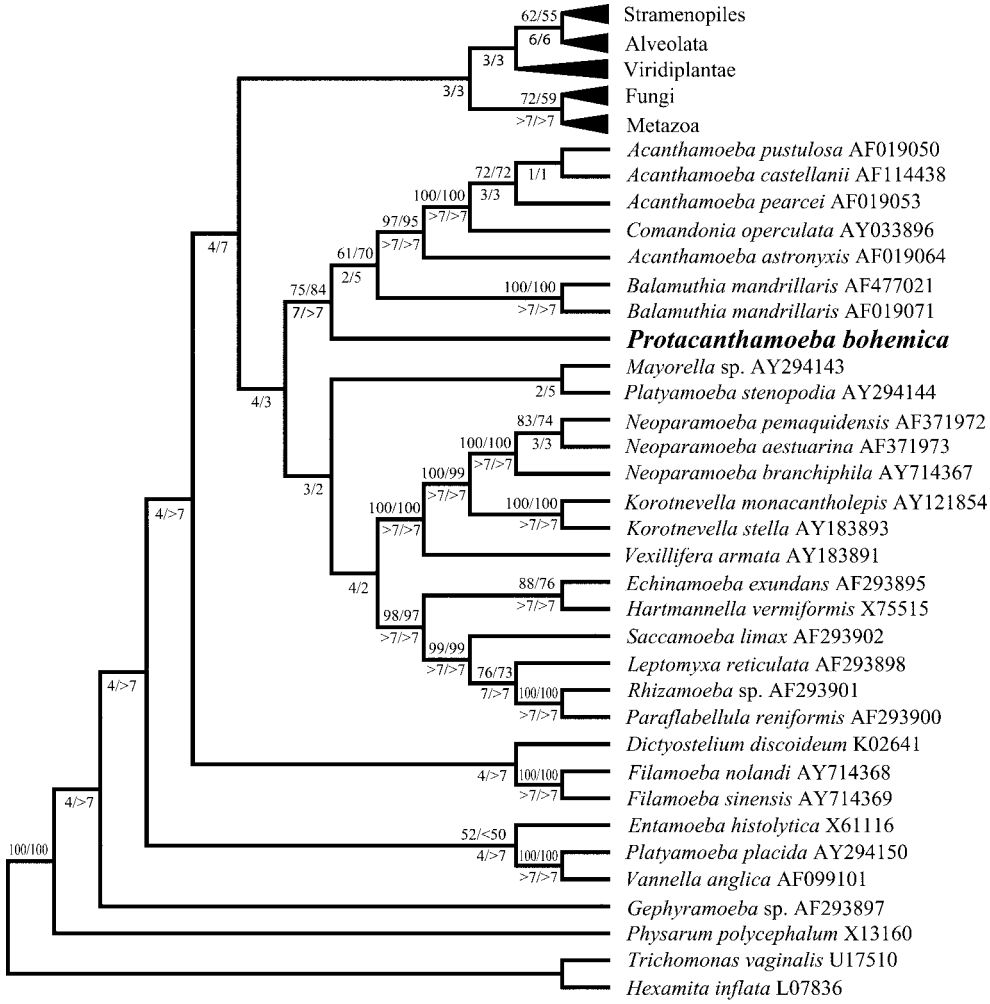

Fig. 14. Maximum parsimony tree of the SSU rRNA sequences (Ts:Tv = 1:2, 5915 steps, one most parsimonious tree). Bootstrap values (for nodes gaining more than 50% support) for MP Ts:Tv = 1:2 and MP Ts:Tv = 1:3 are given above the lines. Bremer indices for MP Ts:Tv = 1:2 and MP Ts:Tv = 1:3 are shown below the lines. See the legend to Fig. 13 for species representing: Stramenopiles, Alveolata, Viridiplantae, Fungi and Metazoa taxons.

from Spolský pond, South Bohemia, Czech Republic, January 1997.

Type material: Cryopreserved clonal culture of the strain denominated TT3H, deposited at the Institute of Parasitology, Academy of Sciences of the Czech Republic, České Budějovice; photosyntypes (light micrographs), nos. 12540-12565 and transmission electron micrographs, nos. 15484-15491; 15637-15640; 15834-15841.

Etymology: The species name is given according to the geographic origin of the fish host (South Bohemia).

Diagnostic summary: Greater dimensions of trophozoites attached to and slowly moving on the under side of coverslips in hanging drop preparations 20-40 µm; acanthopodia (produced from a broad hyaline zone) slender, flexible, often furcate with pointed tips, 5-16 µm in length; nuclear diameter 5-7 µm; diameter of smooth-walled spherical cysts lacking pores 13-17 µm; floating stages with slender pseudopodia. At the light microscopical level, *P. bohemica* differs from the type species of the genus in having trophozoites with more numerous and substantially longer acanthopodia. Smooth walled cysts lacking pores have a smaller range of their diameter. Details of ultrastructure are identical with those described for *P. caledonica* by Page (1981).

Molecular phylogeny based on SSU rRNA gene sequences. The SSU rRNA gene sequence obtained for *P. bohemica* (GenBank accession number AY960120) consists of 2028 bp. The GC content is 55.87%. The

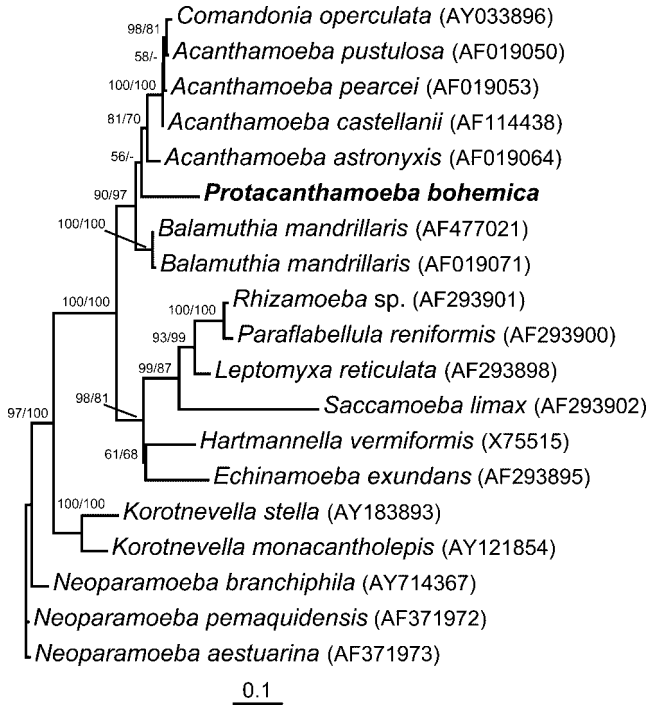

Fig. 15. Maximum likelihood tree (GTR + Γ + I model; -ln = 9278.7826) of the SSU rRNA sequences. Accession numbers of the sequences are in brackets. The distance scale is given under the tree. Bootstrap values (ML and MP Ts:Tv = 1:2) are given for the nodes gaining more than 50% support.

genetic similarities computed from the second alignment (comparing more sites than the first one) revealed homology with the highest identity for *P. bohemica* and *Acanthamoeba* spp. (about 90%) and *Balamuthia mandrillaris* (89 %). For comparison, identities within *Acanthamoeba* sequences were more than 96 % and identities of *B. mandrillaris* and *Acanthamoeba* sequences were about 94%. The sequence of *P. bohemica* clustered together with sequences of *Acanthamoeba* spp. and *B. mandrillaris* in all performed analyses except for MP - Ts:Tv = 1:1. In the alignment of the first dataset, the integrity of the *Acanthamoeba* + *Balamuthia* + *Protacanthamoeba* cluster was supported with a high bootstrap value in ML, MP - Ts:Tv = 1:2 and MP - Ts:Tv = 1:3 (Figs 13, 14). Strong nodal support for this cluster resulted also from the calculation of Bremer indices in both MP - Ts:Tv = 1:2 and MP - Ts:Tv = 1:3 (Fig. 14). *P. bohemica* formed the third lineage associated with *Acanthamoeba* spp. and *B. mandrillaris* within one clade. In comparison with *Acanthamoeba* spp. and *B. mandrillaris* sequences, the branch of *P. bohemica* was relatively long. In both datasets, the ML analysis revealed a closer relation of *P. bohemica* to *Acanthamoeba* spp. than to *B. mandrillaris*, but bootstrap support was low (Fig. 15). MP analyses placed *P. bohemica* in a sister position to *Acanthamoeba* spp. and *B. mandrillaris*, but the clustering of the latter two was not strongly supported as well. Mutual relations among the *Acanthamoeba* spp. + *P. bohemica* + *B. mandrillaris* clade and other amoebozoan lineages differed depending on the type of analysis applied.

DISCUSSION

The comparison of the newly described species *P. bohemica* with *P. caledonica* (the type species of the genus) could be based on relevant, well-documented features. Although a thorough description was not available for *P. invadens* (Singh and Hanumaiah 1979), the second species of the genus, critical comparison could be utilized of *P. caledonica* and *Acanthamoeba invadens* Singh *et* Hanumaiah, 1979, completed by Page (1981) who finally transferred this species to the genus *Protacanthamoeba*. Page (1981) considered the trofozoites of *P. invadens* identical with *P. caledonica* and using the available documentation, he calculated the diameter of cysts of *P. invadens* to be 20-27 µm. The growth at 42ºC and pathogenicity in mice when introduced intracerebrally or intranasally discriminated *P. invadens* from the other two *Protacanthamoeba* spp. Contrary to the thermophily of *P. invadens*, *P. bohemica* grew best at low temperatures around 4ºC.

Although the amoebae SSU rDNA data have been greatly expanded to date, and several evolutionary important lineages were identified within the Gymnamoebia sensu Peglar *et al*. (2003), representatives of numerous morphologically well-defined genera have not thus far been subjected to phylogenetic analyses. This situation is the same for genus *Protacanthamoeba*. Results of our phylogenetic analyses demonstrate a close relationship of *P. bohemica* with *Acanthamoeba* spp. and *Balamuthia mandrillaris*. This result supports to a certain extent close relationships of the genera *Acanthamoeba* and *Protacanthamoeba* proposed by Page (1981) and reflected in the etymology of the name he proposed for the latter genus.

The question as to whether *P. bohemica* is more closely related to *Acanthamoeba* spp. or *Balamuthia mandrillaris* is presently not resolved, but its close relation to both *Acanthamoeba* spp. and *B. mandrillaris* has been well established. MP analyses and comparison of genetic distances supported a closer relation of

Acanthamoeba spp. to *B. mandrillaris*, but ML analysis placed *P. bohemica* and *Acanthamoeba* spp. together.

Phylogeny based on SSU rRNA gene sequences is congruent with the trophozoite morphotype common to *Acanthamoeba* and *Protacanthamoeba* species. Taking into account substantial difference of *Protacanthamoeba* and *Acanthamoeba* cysts, and the diagnostic importance given to cyst morphology within the genus *Acanthamoeba*, the foresight of F. C. Page has to be admired.

The fish-isolated strain of *P. bohemica* is the second representative of the genus described based on both light microscopical and ultrastructural data. Moreover, it is the first that could be included in phylogenetic analyses addressing amoebozoan diversification.

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