Acta Protozool. (2002) 41: 415 - 419

ACTA
PROTOZOOLOGICA

Short Communication

A Free-Living Amoeba with Unusual Pattern of Mitochondrial Structure Isolated from Atlantic Salmon, *Salmo salar* L.

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Summary. A strain of non-encysting free-living amoeba (LOS7N) isolated from the gills of *Salmo salar* L. was characterised morphologically and by the sequence of SSU rRNA gene. High level of sequence similarity (99.58%) with the strain F-13 (ATCC 30942) denominated as *Saccamoeba limax* Page, 1974 was at variance with results of comparative ultrastructural study. Strain LOS7N differed from the previous and thus far the only ultrastructural description of *S. limax* (Page 1985) in having regularly arranged, straight tubular non-branching cristae of mitochondria.

Key words: free-living amoeba, mitochondrial cristae, *Salmo salar*, SSU rRNA gene.

INTRODUCTION

The lack of concordance between morphological similarity of organisms and their relatedness inferred from SSU rDNA sequence-based phylogenetic analyses mentioned for many protistan groups has been recognised also in amoebae (Amaral Zettler *et al*. 2000, Bolivar *et al*. 2001). Variable and changeable morphological features used to describe amoeboid protists gradually loose the character of taxonomic criteria although some of them still are of considerable descriptive value discriminating, e.g., so called limax amoebae from acanthamoebae (Page 1974). Of the major ultrastructural features used for identification, the form of mitochondrial cristae is considered paramount (e.g., for discrimination of the family Vahlkampfiidae or Acrasida). The morphology of cristae has long been recognised to be very conservative and of great phylogenetic value (Cavalier-Smith 1996/97). Recent papers by Sims *et al*. (1999), Amaral Zettler *et al*. (2000) and Bolivar *et al*. (2001) clearly indicate phylogenetic diversity of amoeboid organisms and evidence the need to re-evaluate criteria for their classification. Despite examples of incongruence between morphological and molecular characteristics, the attempts to integrate structural and molecular approaches are most desirable. Since cultures of type species of amoebae are not always available,

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well-described and morphologically documented strains should be included in sample sets of taxa used for molecular analyses.

The aim of this paper is to characterise a free-living amoeba isolated from the gills of Atlantic salmon, *Salmo salar* L., both morphologically and on molecular level and draw attention to inconsistency of our data with literature.

MATERIALS AND METHODS

Methods of isolation of amoebae, their culturing on agar plates and cloning procedures, as well as methods of light and electron microscopic examination, were as reported in previous papers (Dyková *et al*. 1997, 1998, 1999).

DNA isolation, amplification and sequencing

Total genomic DNA was isolated from trophozoites of cloned strain LOS7N/I using standard phenol/chloroform extraction technique with ethanol precipitation (Sambrook *et al*. 1989). Universal eukaryotic primers 5´AYCTGGTTGATYYTGCCAG-3´ and 5´TGATCCATCTGCAGGTTCACCT-3´ reported by Medlin *et al*. (1988) were used for amplification of the SSU rRNA gene. PCR reaction was carried out in a volume of 25 µl using a standard technique with 1 unit of Taq polymerase (TaKaRa, Japan), 250 µM of each dNTP, 10 pmol of each primer and 2.5 µl of 10x Taq polymerase buffer. Conditions for PCR were as follows: initial denaturation temperature 95°C for 5 min followed by 30 amplification cycles at 95°C for 1 min, 52°C for 1.5 min and 72°C for 2 min; final extension 72°C for 10 min. PCR product was purified from an 1% agarose gel and cloned into pCR® 2,1 TOPO Cloning vector using the TOPO-TA Cloning Kit (Invitrogen). Sequencing was carried out on an automatic sequencer CEQ™ (Beckman Coulter) using CEQ Dye Terminator Cycle Sequencing Kit (Beckman Coulter) according to the manufacturer's protocol.

Phylogenetic analyses

The SSU rRNA gene of the strain under study was aligned together with sequences of Gymnamoebia *sensu stricto* according to Bolivar *et al*. (2001) and *Hexamita inflata* as an outgroup. The alignments were performed using Clustal X program (Thompson *et al*. 1997) with various alignment parameters and corrected by eye in the program BioEdit sequence alignment editor (Hall 1999). Ambiguously aligned regions were removed. The phylogenetic relationships between taxa were determined using maximum parsimony (MP) and distance method carried out in the program package PAUP*, Version 4,0b10 (Swofford 2001). MP analysis was done using the heuristic search with random addition of taxa and transversion/transition (Tv/Ts) ratios of 1:1 and 1:2. Gaps were treated as missing data. The distance method was performed using heuristic search and minimum evolution (ME) as an objective setting with Kimura two-parameter substitution model. Genetic distances were calculated using K2P algorithm. Clade support was assessed by bootstraping (1000 replicates).

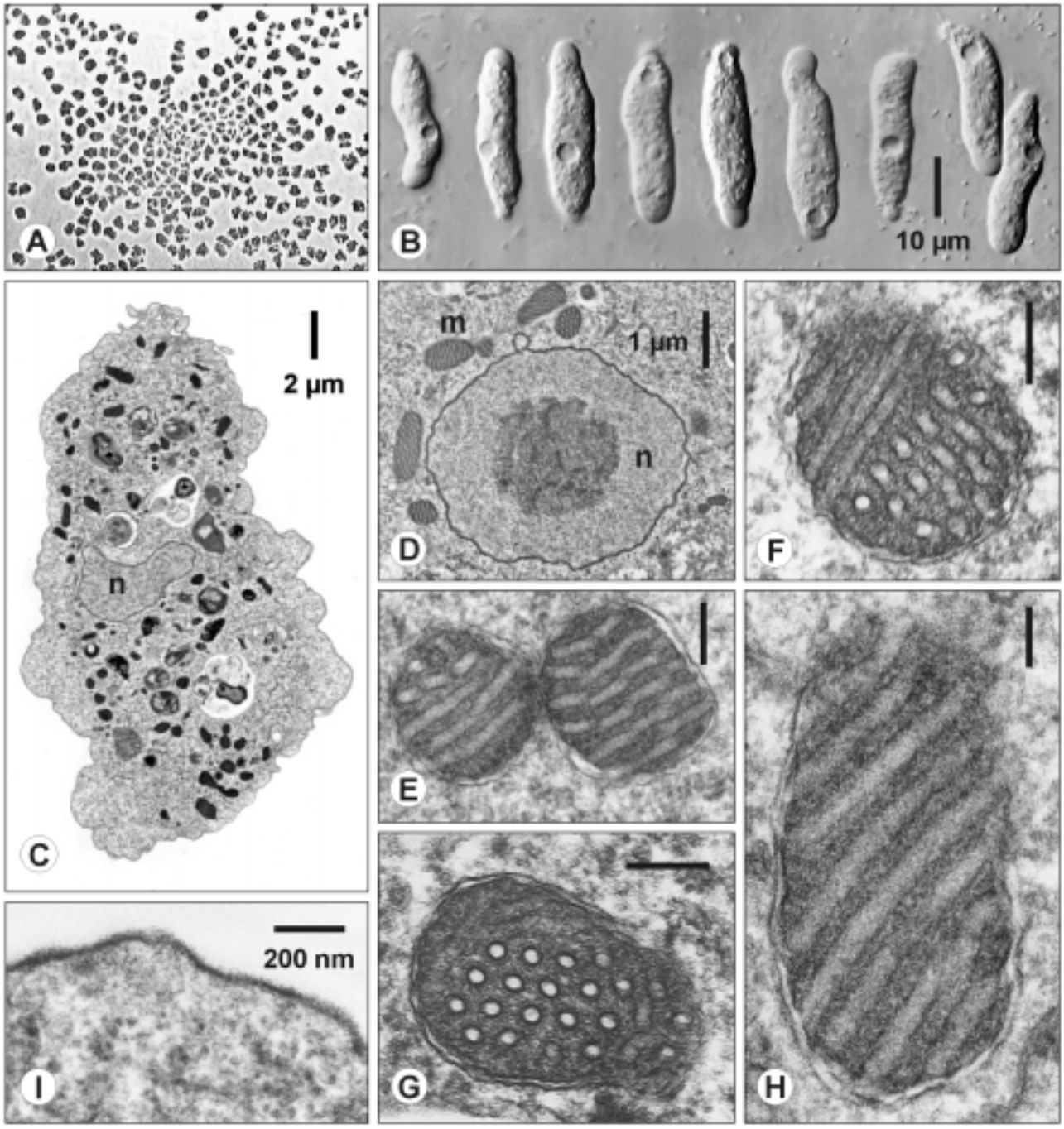
RESULTS

The gills of *Salmo salar* L. fry (size category 2.4-2.8 cm) that was used for salmon restocking of the river Elbe basin in the Czech Republic were found infected with free-living amoebae. An amoeba strain (LOS7N) was isolated in July 2000 and a clone LOS7N/I was derived from this strain in passage No. 9 in October 2000.

Description of the cloned strain LOS7N/I

Trophozoites cultured on agar plates (Fig. 1A) were mostly polymorphic (when attached to agar surface). Locomotive forms observed in hanging drop preparations had monopodial, cylindrical or subcylindrical shape with a short anterior hyaline zone (Fig. 1B). They possessed conspicuous contractile vacuole and small knob-like uroid. The length of limax-like locomotive forms was 34.5 (30-40) µm; the breadth did not exceed 10 µm. Encystment was not observed during the six month period of clonal culturing. Strain morphology as observed in the light microscope resembled, to some extent, that of species of the genus *Saccamoeba* Frenzel, 1892, emend. Bovee, 1972 and *Trichamoeba* Fromentel, 1874. Crystal structures recorded in the cytoplasm of species of both these genera were not observed in trophozoites of our strain.

Transmission electron microscopy (Figs 1C-H) revealed a conspicuous feature of ultrastructure: a highly characteristic organisation of mitochondria. Smooth outer membrane-bounded mitochondria were oval or elliptical in sections. The interiors of mitochondria were crowded with tubular cristae of uniform diameter (40-45 nm). The tubular form of cristae was evident when cut transversely (Fig. 1G). The other planes of sectioning evidenced that cristae were straight, not branched, and packed in close parallel arrays (Figs 1E, F). They extended all the way across the organelle, maintaining the same distances (Fig. 1H). The surface coat of amoeba trophozoites was rather amorphous; glycostyles or other surface structures were not discernible (Fig. 1I). The cisternae of endoplasmic reticulum, encountered rather rarely, had the form of flattened tracks. The Golgi apparatus consisted of flat saccules piled up



Figs 1 A-I. Light micrographs of living amoebae of the cloned strain LOS7N/I and the features of their ultrastructure; **A** - cultured trophozoites attached to agar surface as seen through Petri dish; **B** - trophozoites in hanging drop preparation (Nomarski differential interference contrast); **C** - general structure with part of nucleus in the plane of section, numerous densely stained mitochondria and rests of phagocytized material in the cytoplasm; **D** - nucleus surrounded by mitochondria; **E, F** - straight, non-branching cristae of mitochondria in various planes of sectioning; **G** - tubular form of mitochondrial cristae as seen in transverse section; **H** - parallel arrangement of tubular cristae; **I** - cell surface. Scale bars: E-G, I - 200 nm; H -100 nm

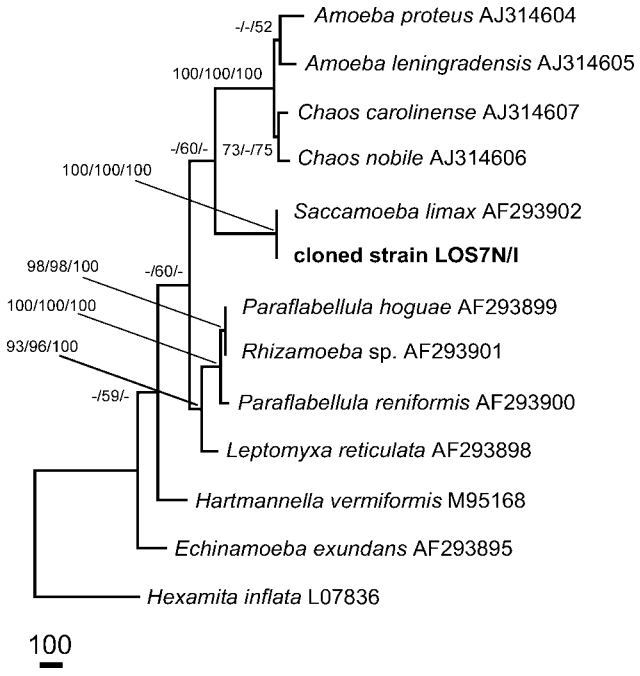


Fig. 2. Maximum parsimony phylogenetic tree of the SSU rRNA sequences rooted at *Hexamita inflata* (one most parsimonious tree, Tv/Ts = 1:1, 1287 steps, CI = 0.74, RI = 0.72). Bootstrap values (MP Tv/Ts = 1:1, MP Tv/Ts = 1:2, distance K2P method) are indicated for the nodes gaining more than 50% support. The scale is given under the tree. The accession numbers are in parentheses

in close parallel arrays. The cytoplasm of trophozoites contained remnants of phagocytized material, mainly of bacterial origin (Fig. 1C).

SSU rRNA data and phylogenetic analysis

The length of SSU rRNA gene of the strain under study (LOS7N/I) was 1902 nucleotides with a G+C content 37.7%. The sequence has been deposited in the EMBL/GenBank database under Accession Number AY145442. The analyses were based on sequences of 13 taxa. The final alignment consisted of 1426 nucleotide sites (776 sites were excluded). The number of parsimony informative characters was 413. LOS7N/I clustered in all analyses performed with strain F-13 (ATCC 30942) denominated in Amaral Zettler *et al.* (2000) as *Saccamoeba limax* Page, 1974. The similarity of sequences of these two strains (computed from the alignment) was 99.58%. The comparison of entire length of sequences revealed eleven nucleotide changes. Both strains branched with *Amoeba-Chaos* clade with low bootstrap support (60%) in maximum parsimony (MP), Tv/Ts = 1:2; under 50% in MP, Tv/Ts = 1:1 and minimum evolution (ME). The topology of the phylogenetic tree is shown in Fig. 2.

DISCUSSION

More than 70 cloned strains of fish-infecting amoebae that we have isolated thus far have been characterised at the cellular and ultrastructural levels. Among them, the strain LOS7N/I is unique in having regularly arranged tubular non-branching mitochondrial cristae. The level of similarity in sequences of LOS7N/I strain and F-13 (ATCC 30942) strain denominated as *Saccamoeba limax* (Amaral Zettler *et al*. 2000) was high enough to suggest species identity. However, the comparison of ultrastructural data available calls in question the assignment of our strain (LOS7N/I) to the genus *Saccamoeba sensu* Page (1985). The strain F-13 (ATCC 30942) denominated as *S. limax* and included in the set of strains sequenced in the study of Amaral Zettler *et al*. (2000) was analysed also by Bolivar *et al*. (2001). Its sequence is deposited in GenBank under accession number AF 293902. To the best of our knowledge, ultrastructural study of this strain has not been published to date. Two strains of *S. limax* (CCAP 1534/6 and CCAP 1572/3) stored in the UK National Culture Collection (UKNCC, formerly CCAP) were described by Page (1985) as having tubular but seldom branching mitochondrial cristae. Their appearance, documented with electron micrographs as irregularly twisted and, most probably, blindly ending crists in the interior of mitochondrion matrix, differs substantially from that of our strain (LOS7N/I). Similarly, another species of the genus *Saccamoeba* (*S. stagnicola*) was characterised by Page (1985) as having tubular mitochondrial cristae of twisting appearance that branch rather rarely.

Our comparisons that focused on the organisation of tubular mitochondrial cristae included also species of non-vahlkampfiid genera forming limax-like monopodial trophozoites (Page 1980). Although in the re-diagnosis of the genus *Trichamoeba* Fromentel, 1874 tubular cristae of mitochondria were characterised as occasionally branched, an electron micrograph of *T. sinuosa* (Siemensma and Page 1986, Fig. 12) shows mitochondria almost identical with those of *Saccamoeba* spp. documented by Page (1985, Fig. 35). The genus *Rhizamoeba*, separated from *Trichamoeba* by Page (1972) on the basis of differences in uroidal structures, as well as *Hydramoeba* Reynolds and Looper, 1928, separated from *Trichamoeba* due to parasitic way of

life of the only species of the former genus, were not sufficiently described to compare their mitochondria with the strain under study.

Our data have shown that neither morphology-based systematics (Page 1988, Page and Siemensma 1991) nor molecular data available can immediately solve the generic assignment of our strain LOS7N/I. Nevertheless, combined with new data on other strains and species, they can contribute in the future to a better understanding of phylogenetic and taxonomic relationships of limax amoebae and amoebae in general. Continued interest in taxonomic studies correlating morphological and molecular characters of amoeboid organisms will undoubtedly be the basis for this.

Acknowledgements. Funding support was provided by the Grant Agency of the Academy of Sciences of the Czech Republic (Project IAA6022202) and by the Ministry of Education, Youth and Sports of the Czech Republic (Project MSMAJ06/98: F1-123100003).

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Received and accepted on August 28th, 2002