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Nuclearia pattersoni sp. n. (Filosea), a new species of amphizoic amoeba isolated from gills of roach (*Rutilus rutilus*), and its rickettsial endosymbiont

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Abstract. A new species of amphizoic amoeba, *Nuclearia pattersoni* sp. n., isolated from gills of *Rutilus rutilus* L. is described. It is characterised by elongate flattened trophozoites of irregular shape. The longer dimension of their bodies is 13.2 (11.0–15.7) µm. Filopodia radiating mostly from the poles are 2 to 2.5 times longer than the body. The diameter of less frequently observed spherical trophozoites is 8.2–10.8 µm; their filopodia radiate to all directions. Cyst-like stages have shorter pseudopodia that arise from one pole only. The surface of locomotive forms from agar plate cultures has a thin amorphous glycocalyx, while most cells are covered by two layers of extracellular matrix. Mitochondria have flattened cristae, dictyosomes are located in the perinuclear zone. A conspicuous ultrastructural feature of the morphologically similar *N. simplex*, perinuclear striated band, is not present. Light microscopic and ultrastructural data are completed with the sequence of SSU rRNA gene and phylogenetic analysis including sequences of related taxa. The bacterial endosymbiont found in *N. pattersoni* type strain RR2G2 is assigned to the genus *Rickettsia*.

Similarly as in many other amoeboid organisms, the features of filose nucleariid amoebae discernible by the light microscope are insufficient for safe diagnosis. The nine species distinguishable within the genus *Nuclearia* Cienkowski, 1865 were characterised by Patterson (1984). The comparison of ultrastructural organisation of three named species of the genus, *Nuclearia simplex* Cienkowski, 1865, *N. moebiusi* Frenzel, 1897, and *N. delicatula* Cienkowski, 1865, with an account of the fine structure of *Vampyrellidium perforans* Surek et Melkonian, 1980 (Mignot and Savoie 1979, Patterson 1983, Cann 1986, Patterson et al. 1987), revealed morphological similarities of both genera, justifying their appurtenance to the same family Nucleariidae Cann et Page, 1979, but also a lack of ultrastructural features consistent within these genera (Patterson et al. 1987).

The phylogenetic analyses of the small-subunit rRNA (SSU rRNA) coding regions from four amoebae assigned to the genus *Nuclearia* (strains of the named species mentioned above plus one unnamed) published by Amaral Zettler et al. (2001) placed a monophyletic group formed by the named *Nuclearia* species near the animal-fungal divergence and separated their lineage from testate filose amoebae.

The lack of information available to date on nucleariids and a wide range of suspected relatives inspired us to characterise a nucleariid strain isolated from the gills of roach both on morphological and molecular level.

MATERIALS AND METHODS

The nucleariid filose amoeba was isolated from the gills of roach, *Rutilus rutilus* (Linnaeus, 1758), collected in the pond Novohaklovský in South Bohemia, Czech Republic, in November 1999. The amoeba host was kept in an animal house facility container more than two months prior to isolation attempts. The methods of isolation and culturing of amoebae on agar plates followed those described in previous papers (Dyková et al. 1997, 1998, 1999). Ultrastructure of amoebae was studied using cultures fixed *in situ* (on agar plates) with 3% cacodylate buffered glutaraldehyde and 1% osmium tetroxide and embedded in Spurr resin. A JEOL JEM 1010 electron microscope operating at 80 kV was used for examination of thin sections.

The amoeba strain denominated RR2G2 has been cryopreserved and stored in the collection of the Institute of Parasitology, Academy of Sciences of the Czech Republic, České Budějovice.

DNA isolation, amplification and sequencing

The standard phenol/chlorophorm extraction technique with ethanol precipitation (Sambrook et al. 1989) was applied to isolate genomic DNA from trophozoites of RR2G2 amoeba strain harvested from agar plates. Universal eukaryotic primers 5'-AYCTGGTTGATYYTGCCAG-3' and 5'-TGATCCA-TCTGCAGGTTCACCT-3' reported by Medlin et al. (1988) were used for amplification of the SSU rRNA gene. PCR 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 10 × Taq

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polymerase buffer. The PCR cycling conditions were as follows: initial denaturation temperature 95°C for 5 min followed by 5 amplification cycles (each comprising 94°C for 1 min, 46°C for 1.5 min and 72°C for 2 min), and another 25 cycles (each comprising 94°C for 1 min, 49°C for 1.5 min and 72°C for 2 min). The final 10-min incubation ran at 72°C. The 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.

Alignments and phylogenetic analyses

The alignments performed in this study were done using Clustal X program (Thompson et al. 1997) with various alignment parameters. Ambiguously aligned regions were excluded. The SSU rRNA gene sequence from *Nuclearia* species under study was aligned against all sequences of *Nuclearia* species available through GenBank. *Saccharomyces cerevisiae* and *Candida albicans* were selected as an outgroup. The final alignment consisted of 2430 characters (294 characters were parsimony informative). The dataset used for analysis of *Nuclearia* spp. within related taxa included sequences of representatives of Ichthyophonida and Dermocystida (Mesomycetozoea), Metazoa, Choanoflagellida, Fungi, Cercozoa and Stramenopiles. *Prorocentrum micans* was used as an outgroup. The alignment consisted of 2031 characters from which 686 were parsimony informative.

The sequence of 16S rDNA of a bacterial endosymbiont from *Nuclearia* trophozoites was aligned with sequences of *Rickettsia* spp. from the typhus and spotted fever group, rickettsial endosymbionts from *Acanthamoeba* spp. and those from freshwater leeches. The alignment contained 1062 characters from which 198 were parsimony informative. *Legionella pneumophila* was used as an outgroup to root the 16S rDNA tree.

The phylogenetic relationships between taxa were determined using maximum parsimony (MP), distance, and maximum likelihood (ML) methods carried out in the program package PAUP*, version 4,0b10 (Swofford 2001). The MP analysis was performed using a heuristic search with random addition of taxa. Gaps were treated as missing data. Transversion/transition (Tv:Ts) ratios were 1:2–1:5. 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. All ML analyses were done with the GTR+G+I model of evolution. The distance method was performed using heuristic search with the minimum evolution (ME) as the objective setting. The Kimura two-parameter (K2P) substitution model was used. Clade support was assessed by bootstrapping (MP and ME methods, 1000 replicates; ML method, 300 replicates). The Kishino-Hasegawa (KH) and Shimodaira-Hasegawa (SH) tests (Kishino and Hasegawa 1989) were performed in PAUP* using RELL bootstrap (1000 replicates) in order to assess the significance of differences in likelihood scores of the tree topologies.

RESULTS

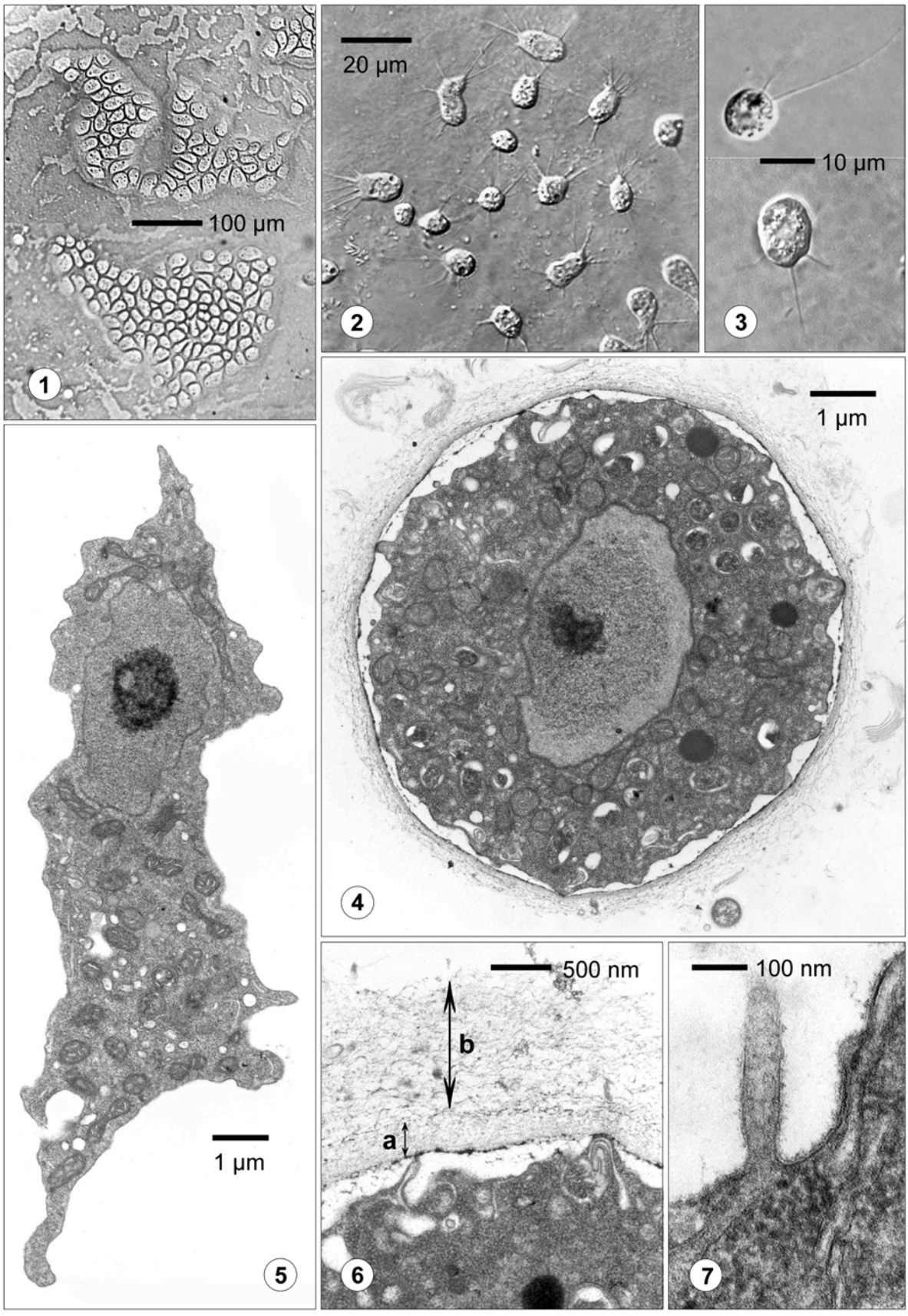
The finding of a uninucleate nucleariid filose amoeba among more than 200 strains of amphizoic amoebae that we had isolated from tissues of freshwater fishes was unique. The type of growth and the formation of walled-off dense aggregates of amoebae on the agar surface (Fig. 1) drew our attention soon after the primary isolate was obtained. The firm adhesion of amoebae to the agar surface made their harvest as well as subculturing quite difficult. In the beginning of culturing the soil extract (SE2) added to Page amoeba saline (Tompkins et al. 1995) improved the results of subculturing. The multiplication, i.e., the growth of cultures was relatively slow, with the generation time 10 to 15 days.

Description of *Nuclearia* strain RR2G2

The strain under study was characterised by an irregular shape of trophozoites that was discernible under low magnification on the surface of agar plates (Fig. 1) and easy to observe in hanging drop preparations (Fig. 2). Elongate flattened trophozoites of irregular shape sometimes with angular outlines were observed more frequently than spherical ones (Fig. 3). The longer dimensions of elongate trophozoites were 13.2 (11.0–15.7) μm (mean with the range in parentheses). The filose pseudopodia radiated mostly from the poles, the longest being up to 2 to 2.5 times longer than the longer dimension of amoebae. Branching of pseudopodia was not observed. Less frequent spherical trophozoites with filopodia radiating from all regions of the cell resembled heliozoan-like stages. Their diameter varied between 8.2 and 10.8 μm. In cyst-like stages, shorter filopodia arose from one pole only.

At the ultrastructural level, most of the characteristics of the genus *Nuclearia* summarised by Patterson (1984) were detected. In a well-growing culture, most cells were covered by a sheath of filamentous extracellular matrix arranged parallel to the plasma membrane and divided sometimes into two layers (Figs. 4, 6). The surface of locomotive forms of amoebae (Fig. 5) was covered with a thin amorphous glycocalyx (Fig. 7). The fine structure of long pseudopodia was homogeneous or filamentous; microtubular arrays were not detected (Fig. 7). The cytoplasm contained food vacuoles with residues of phagocytosed material as well as rod-shaped bacteria of the maximum length 1.5 μm that obviously survived inside the host cells directly in the cytoplasm (Figs. 8–10). Most of the bacteria contained vacuoles or small vesicles about 100 nm in diameter (Figs. 9, 10). The mitochondria had flattened cristae (Figs. 10, 13) and were numerous in most cells as were the dictyosomes located mostly in the perinuclear zone (Figs. 11, 12). The structure resembling vesicular spongiome was observed in the cytoplasm rather exceptionally. Depending on the phase of the division cycle, the fine structure

Figs. 1–7. *Nuclearia pattersoni* sp. n. **Fig. 1.** The agar plate culture. Note the delimitation of growing amoeba aggregates. **Figs. 2**, **3.** Trophozoites in hanging drop preparations. **Fig. 4.** The trophozoite surrounded by an extracellular matrix. **Fig. 5.** The locomotive form. **Fig. 6.** The sheath of extracellular matrix composed of two layers (**a** and **b**) of fine filamentous material. **Fig. 7.** The short segment of long filose pseudopodium.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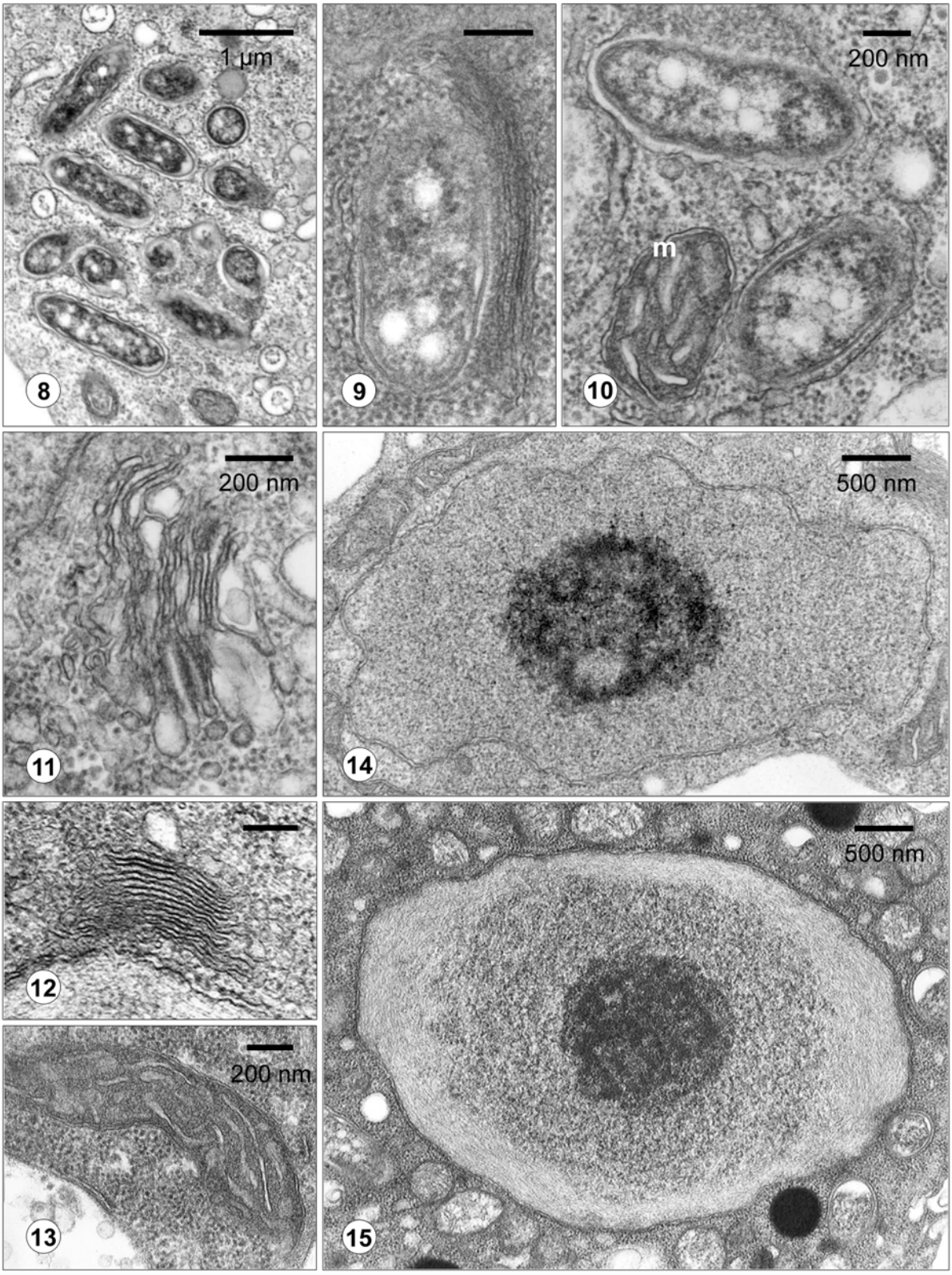
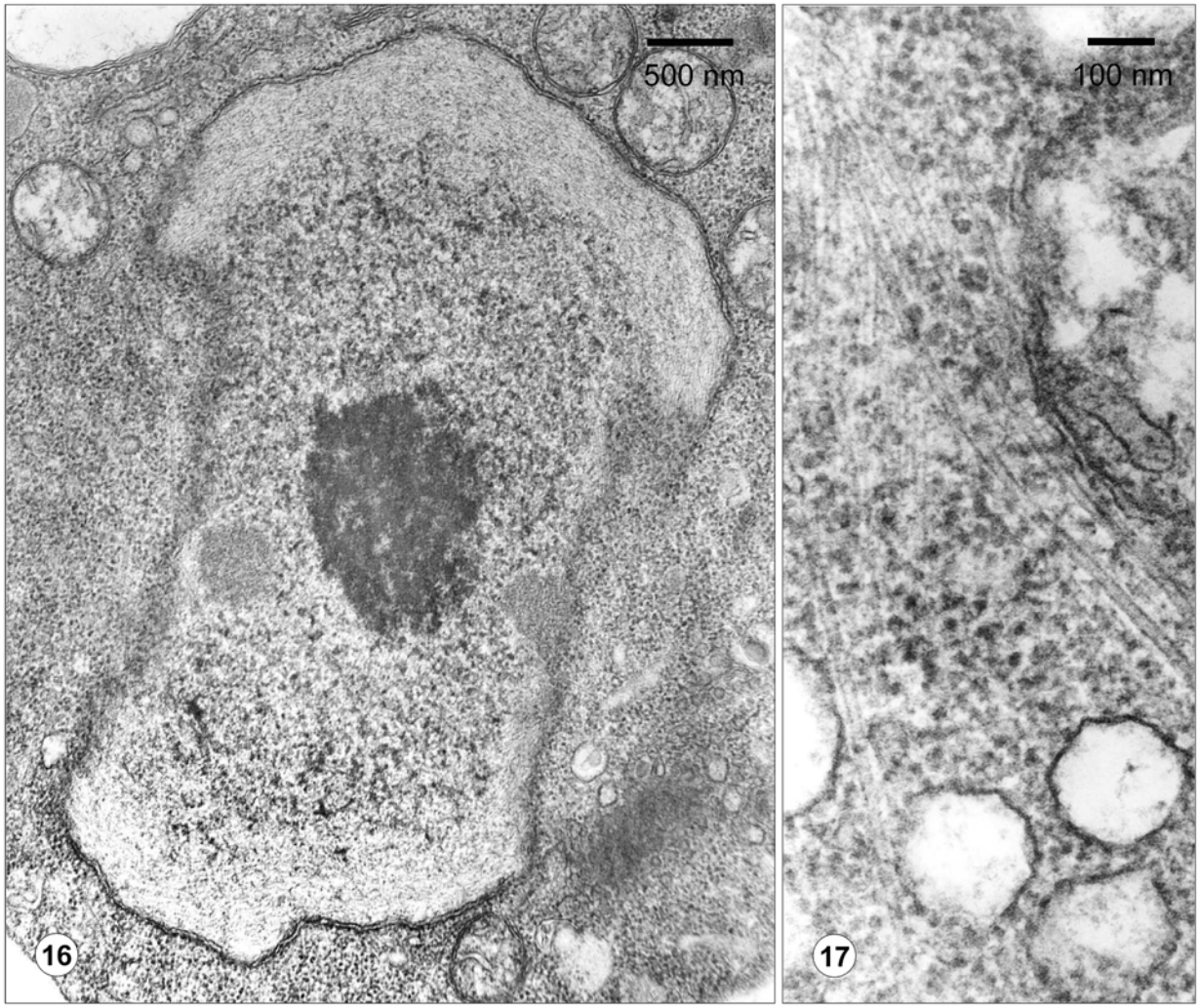


Figs. 8–15. *Nuclearia pattersoni* sp. n. **Figs. 8–10.** The rod-shaped bacteria in the cytoplasm, next to mitochondrion (m). **Figs. 11**, **12.** The dictyosomes located in the vicinity of amoeba nucleus. **Fig. 13.** The mitochondria with flattened cristae. **Fig. 14.** The nucleus in the interphase. **Fig. 15.** The nucleus with a layer of microfilaments arranged parallel beneath the nuclear envelope. Scale bars: Fig. 9, 12 = 200 nm.



Figs. 16, **17.** *Nuclearia pattersoni* sp. n. **Fig. 16.** Dividing nucleus with extranuclear microtubules. **Fig. 17.** Convergent microtubules in the cytoplasm.

of nuclei differed. They consisted of a finely granular material and a densely granular or compact nucleolus (Fig. 14), or contained a prominent peripheral layer formed by concentrically arranged microfilaments (Fig. 15). This type of rearrangement of nuclear ultrastructure was found regularly in cells surrounded by extracellular filamentous matrix. The features of nuclear division were observed as an exception. Since serial thin sections were not available even the documented phase of nuclear division (Fig. 16) was difficult to interpret. In addition to microtubules connected with nucleus, there were also microtubules clustering convergently in the cytoplasm (Fig. 17) but neither centrioles nor MTOCs were safely detected.

SSU rRNA data and phylogenetic analyses of *Nuclearia* strains

The universal eukaryotic primers used for amplification of small subunit ribosomal RNA genes of our *Nuclearia* strain RR2G2 amplified two products of different length, approximately 2000 bp and 1200 bp long, respectively. The sequence of the longer product matched with sequences of *Nuclearia* strains available in GenBank but could not be identified with any of them. The sequence of SSU rRNA gene of *Nuclearia* sp. was 1973 bp in length and contained 43.28% of G+C. It has been deposited in the GenBank database under the accession number AY364635.

Phylogenetic conclusions are shown in Figs. 18–20 and in Table 1. In the phylogenetic tree inferred from the ML analysis (Fig. 18), the amoeba strain under study clustered with *Nuclearia* strain, the sequence of which (AF484687) was deposited in GenBank by Hertel et al. (2002) as belonging to *N. simplex*. All other analysed *Nuclearia* strains formed a clearly distinguished clade with maximum bootstrap support. The ML tree based on analyses of SSU rRNA gene sequences of

Table 1. Kishino-Hasegawa (KH) and Shimodaira-Hasegawa (SH) tests of constrained trees.

Tree	–ln L	Diff –ln L	KH-test P	SH-test P
A=(((F),(((CH),(M)),(((N),(C((D),(I))))))))	19404.25904	(best)		
B=(((F),(((CH),(M)),(((N),((C,(D)),(I)))))))	19405.24897	0.98992	0.770	0.902
C=(((F),(((CH),(M)),((((I),((D),((C),(N))))))))	19406.44874	2.18969	0.744	0.847
D=(((F),(((((C),(D)),(N)),((I),((CH),(M))))))	19413.69069	9.43164	0.290	0.473
E=(((F),(((CH),(M)),((((I),((N),((C),(D)))))))))	19407.27608	3.01703	0.633	0.778
F=(((F),((N),C),(((CH),(M)),((D),(I)))))	19507.06606	102.80702	0.000*	0.000*
G=(((F),(((N),C),(((CH),(M)),((D),(I)))))	19411.32066	7.06161	0.296	0.612
H=((F),((N),(((M),(CH)),((C),((D),(I)))))	19409.59258	5.33353	0.177	0.648
I=((F),((N),(((C),(((M),(CH)),((D),(I)))))	19413.81084	9.55180	0.158	0.480

*P < 0.05

KH and SH tests performed in Paup*, KH test using RELL bootstrap (two-tailed test); SH test using RELL bootstrap (one-tailed test). Number of bootstrap replicates = 1000, *P<0.05 suggests that the constrains are significantly different. Trees A–I correspond to the following topologies: tree A = ML, tree B = MP Tv:Ts 1:2 and MP Tv:Ts 1:3 (1), tree C = MP Tv:Ts 1:3 (2) and MP Tv:Ts 1:4 (2), tree D = MP Tv:Ts 1:4 (1) and MP Tv:Ts 1:5, tree E = ME, tree F = hypothetical – *Capsaspora owczarzaki* within nucleariids in animal/fungal boundary, tree G = hypothetical – nucleariids with *C. owczarzaki* as a sister branch in animal/fungal boundary, tree H = branching of nucleariids according to Hertel et al. (2002), tree I = branching of nucleariids according to Amaral Zettler et al. (2001). Abbreviations: F – Fungi, CH – Choanoflagellida, M – Metazoa, N – *Nuclearia* spp., I – Ichthyophonida, D – Dermocystida, C – *C. owczarzaki*.

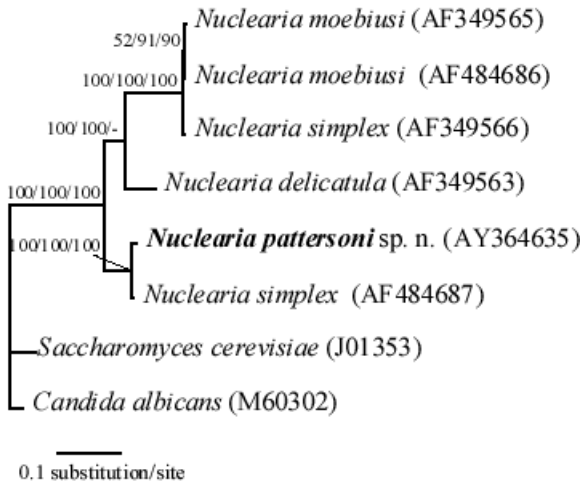


Fig. 18. Maximum likelihood tree of the SSU rRNA gene sequences of *Nuclearia* spp. rooted at *Saccharomyces cerevisiae* and *Candida albicans* (–ln = 6081.51977, α shape parameter = 0.721896, PINVAR = 0.385740). Bootstrap values (ML; MP Tv:Ts = 1:2; distance method K2P) are indicated for the nodes gaining more than 50% support. The distance scale is given under the tree. GenBank accession numbers are in parentheses.

Nuclearia strains and taxa of the animal-fungal boundary (Fig. 19) showed monophyly of the *Nuclearia* clade corroborated with maximum bootstrap values. The relationship of *Nuclearia* strains and representatives of Mesomycetozoea was proved in all analyses performed (ML, MP and ME) (see Fig. 20 for tree topologies). Branching of main taxa (the clade of *Nuclearia* spp., Dermocystida, Ichthyophonida, Choanoflagellida, Metazoa and Fungi) is weekly supported by bootstrap values and the corresponding branches are short. Kishino-Hasegawa (KH) and Shimodaira-Hasegawa (SH) tests (Table. 1) did not reject any of the resulted topologies except a hypothetical one with *Capsaspora owczarzaki* within *Nuclearia* clade.

Based on the morphological description and sequence-based phylogenetic analyses, the *Nuclearia* strain RR2G2 is classified as a new species, *Nuclearia pattersoni* sp. n.

Nuclearia pattersoni sp. n. – type data

Origin of type material: Gills of the roach, *Rutilus rutilus* (Linnaeus, 1758) (Cypriniformes: Cyprinidae).

Type locality: The pond Novohaklovský, near České Budějovice, South Bohemia, Czech Republic (November 1999).

Type material: Photosyntypes (light micrographs), nos. 10 446–10448 and 10469–10473, transmission electron micrographs, nos. 14780–15313, and cryopreserved culture (amoeba strain RR2G2), deposited in the Institute of Parasitology, Academy of Sciences of the Czech Republic, České Budějovice.

Etymology: The species name is given in honour of Professor David J. Patterson in recognition of his great contribution to the knowledge of the genus *Nuclearia*.

Identification of bacterial endosymbiont

In addition to the SSU rRNA gene sequence analysed above and attributed to *N. pattersoni*, another incomplete sequence was obtained when universal eukaryotic primers were used on total DNA from amoeba strain. It was 1065 bp in length with G+C content 51.46% and fell into alpha subdivision of proteobacteria, forming a monophyletic group with rickettsial endosymbionts of leeches (*Torix tagoi* and *Hemiclepsis marginata*) (Fig. 21). The basal position of the endosymbiont of *N. pattersoni* within the clade of endosymbionts of leeches

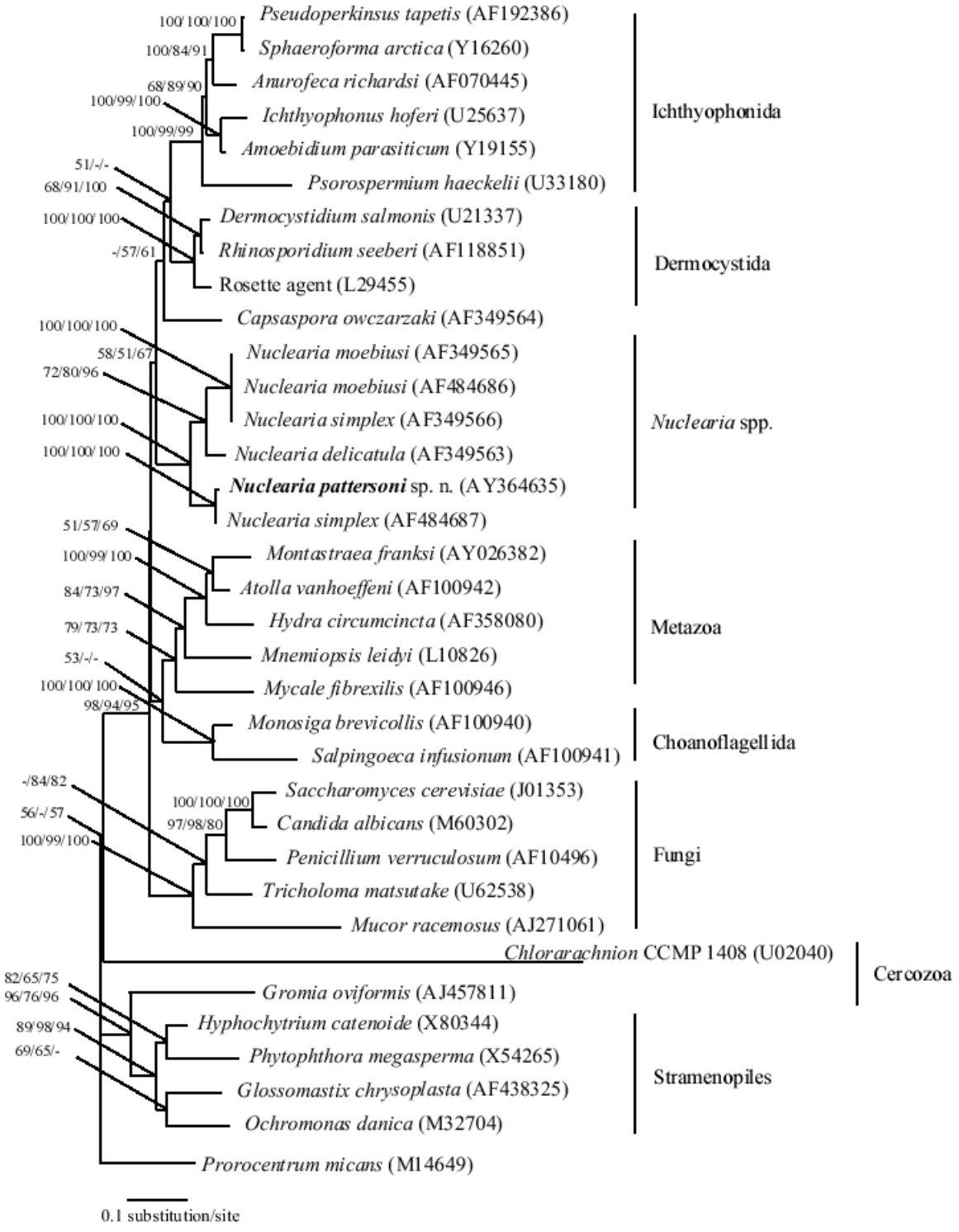


Fig. 19. Maximum likelihood tree of the SSU rRNA gene sequences rooted at *Prorocentrum micans* (–ln = 18386.78468, α shape parameter = 0.46087, PINVAR = 0.161526). Bootstrap values (ML; MP Tv:Ts = 1:2; distance method K2P) are indicated for the nodes gaining more than 50% support. The distance scale is given under the tree. GenBank accession numbers are in parentheses.

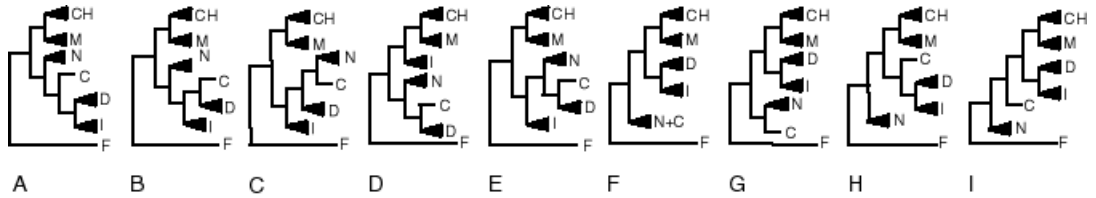


Fig. 20. Schematic representation of the topologies of the trees reconstructed for KH and SH tests described in Table 1.

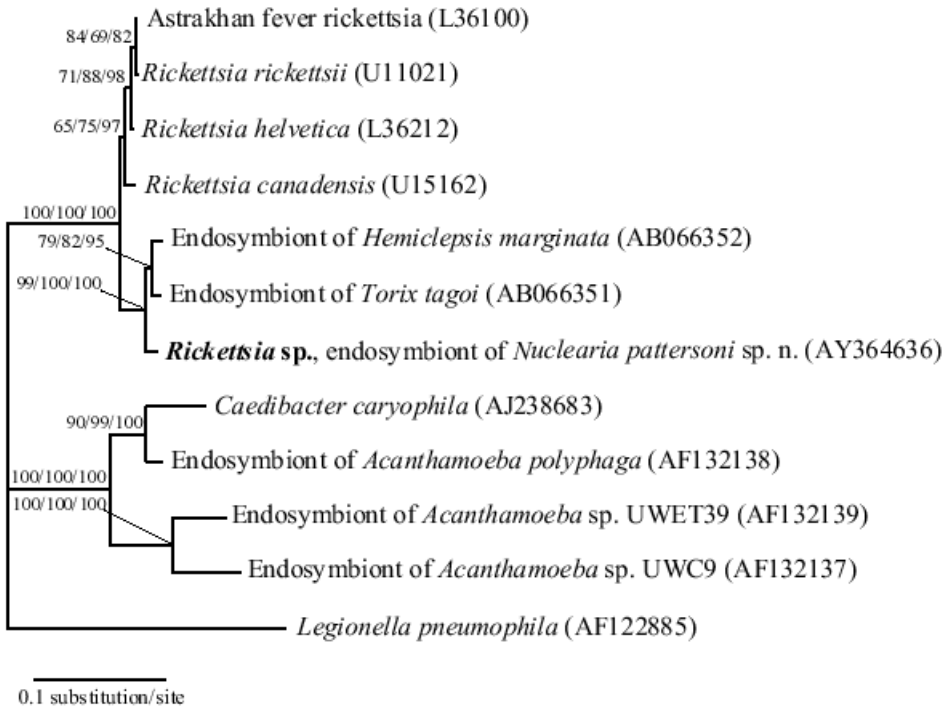


Fig. 21. Maximum likelihood tree of the 16S rRNA gene sequences of rickettsia species rooted at *Legionella pneumophila* (–ln = 3778.91036, α shape parameter = 0.722979, PINVAR = 0.310815). Bootstrap values (ML; MP Tv:Ts = 1:2; distance method K2P) are indicated for the nodes gaining more than 50% support. The distance scale is given under the tree. GenBank accession numbers are in parentheses.

was stable in all performed analyses and corroborated with high bootstrap values. On the basis of molecular characterisation supported by ultrastructural features, the bacterial endosymbiont from *N. pattersoni* was classified as *Rickettsia* sp.

DISCUSSION

The morphological data collected on *Nuclearia pattersoni* support the conclusions by Patterson (1984) and Patterson et al. (1987), who stressed inconsistencies in the characterisation of the genus *Nuclearia.* Our strain as well as two *Nuclearia* species (*N. simplex* and *N. delicatula*) studied by Patterson (1984) are characterised by the presence of extracellular matrix, while *N. moebiusi* is not. The filopodia of *N. delicatula* ramify, while in other species including ours do not. The texture of filopodia, described as fibrilar in *N. simplex*, was homogeneous or filamentous in our material and hyaline or homogeneous in other species. There is also a difference in the number of nuclei. Contrary to the uninucleate *N. simplex*, *N. moebiusi* and *N. pattersoni*, more than two (up to 12) nuclei were described in *N. delicatula.* The striking ultrastructural feature, perinuclear striated band, was described only in *N. simplex* and was not present in *N. pattersoni*.

Several similarities in the fine structure were recognised between *Nuclearia* species and *Vampyrellidium perforans* Surek et Melkonian, 1980 as well as between *N. pattersoni* and *V. perforans*. Both organisms have filose pseudopodia, mitochondria with flattened cristae, and microtubules within the nucleus and within the cytoplasm. The incomplete perinuclear striated band was described in *V. perforans* only.

The spectrum of features of nuclear division observed in our cells fixed for transmission electron microscopy was very poor and hence useless for safe classification of the type of mitosis. In view of the fact that six types of mitoses have been distinguished among protists (Raikov 1994) and two different types were observed in the vampyrellid amoeba *Leptophrys vorax* (Röpstorf et al. 1994), the nuclear division of *N. pattersoni* deserves a thorough study. The improvement of culturing conditions resulting in intense multiplication of amoebae is an essential prerequisite.

Phylogenetic relationships of *N. pattersoni* and previously named *Nuclearia* species were easy to survey in the ML tree (not shown) based on similar dataset as used in the analysis by Amaral Zettler et al. (2001): *N. simplex* (AF349566), *N. moebiusi* (AF349565) and *N. delicatula* (AF349563) formed sister group to *N. pattersoni*, while the strain no. 30864 (AF349564), deposited in ATCC as *Nuclearia* sp. until recently, branched separately. The latter strain was transferred by Hertel et al. (2002) to the genus *Capsaspora*, newly established for amoebae known as symbionts of *Biomphalaria glabrata* and capable of killing sporocysts of *Schistosoma mansoni* (Stibbs et al. 1979, Owczarzak et al. 1980). The results of phylogenetic analyses that we performed with additional sequences from *Nuclearia* strains (Fig. 19) differed from those published by Amaral Zettler et al. (2001). Two strains denominated as *N. simplex* fell into two different clades, one almost identical with *N. moebiusi*, the other one similar to *N. pattersoni*. To the best of our knowledge, morphological descriptions of more recently sequenced strains [*N. moebiusi* (AF484686) and *N. simplex* (AF484687)] are not available for comparison with those used in the study by Amaral Zettler et al. (2001). Thus differing positions of strains assigned to the same species (*N. simplex*) (Fig. 19) should probably be explained by tentative/incorrect morphological determination.

Although more sequences from *Nuclearia* strains were included in our phylogenetic analyses (while the former *Nuclearia* strain ATCC No. 30864 is now considered a species of a different genus as *Capsaspora owczarzaki*) and a modified set of related taxa was analysed, the phylogenetic position of *Nuclearia* spp. clade remains unclear. Our ML analysis indicates close relation to Dermocystida, Ichthyophonida and *C. owczarzaki* but bootstrap support for branching pattern is very low, same as for the other topologies resulted from MP or ME analyses (Fig. 19). The same applies to previously published phylogenetic analyses (Amaral Zettler et al. 2001, Hertel et al. 2002). The KH and SH tests performed in our study did not reject any of these topologies on a significant level. An ancestral position of *C. owczarzaki* in relation to Mesomycetozoea agrees with results of Hertel et al. (2002) but bootstrap support is marginal. This is why we call in question the assignment of *C. owczarzaki* to Mesomycetozoea.

Bacteria have been observed as endosymbionts in a number of freshwater amoeba species (Lee et al. 1985). The development of symbiotic associations was obviously influenced by the fact that amoebae are constantly exposed to microorganisms and that phagocytosis is their principal mode of food uptake. Hertel et al. (2002) quoting Amaral Zettler et al. (2001) stress that members of the genus *Nuclearia* have not been reported to have symbiotic relationships with any other organisms. The fact that trophozoites of *N. pattersoni* harboured rickettsial cells over a long period of subculturing (total DNA was extracted from passage no. 44 two years after primary isolation) gives evidence on a well-established association. The morphology of rickettsial cells found in the cytoplasm of *N. pattersoni* resembled the vacuolated pattern described by Avakyan and Popov (1984) as a result of adaptation to intracellular parasitism.

The fact that phylogenetic analysis brought together the *N. pattersoni* endosymbiont and leech-associated *Rickettsia* spp. suggests that the primary host of an ancestor of this endosymbiont might have been a leech from which the ancestral rickettsia was transmitted by bloodsucking to fish and then through the process of phagocytosis became endosymbiont of amoeba. This indirectly supports the hypothesis that the role of leeches in the aquatic ecosystems is analogous to the bloodsucking arthropods in the terrestrial ecosystems (Kikuchi et al. 2002).

The amplification of the bacterial 16S rRNA gene using the eukaryotic universal primers can be explained by low annealing temperature which allows nonspecific binding of primers and also by a high proportion of the bacterial DNA in samples of total DNA. This could also increase the probability that the primers anneal with the prokaryotic gene rather than the eukaryotic one.

Although the comparison of the morphology of *N. pattersoni* with related data on *N. simplex*, *N. moebiusi* and *N. delicatula* as well as the results of phylogenetic analyses allowed us to establish the new species, the analysis of information available to date evidenced that more data are needed on nucleariid amoebae to determine their relationships within the genus *Nuclearia* and the relationship of these amoebae with other, thus far insufficiently characterised species of similar genera.

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