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Phylogenetic position and notes on the ultrastructure of *Sappinia diploidea* (Thecamoebidae)

Rolf Michel,[1] Claudia Wylezich,[2] Bärbel Hauröder[1] and Alexey V. Smirnov [3]

[1] *Central Institute of the Federal Armed Forces Medical Services, Koblenz, Germany*

[2] *Department of General Ecology and Limnology, Zoological Institute, University of Cologne, Cologne, Germany*

[3] *Department of Invertebrate Zoology, St. Petersburg State University, St. Petersburg, Russia*

Summary

An unusual amoeba, *Sappinia diploidea*, possessing diplokaryon-like nuclear apparatus and a complex life cycle, was isolated from the bark of trees and a freshwater pond, identified and re-investigated. The phylogenetic analysis based on SSU rRNA gene sequences shows close relatedness of this species to the members of the genus *Thecamoeba*. It suggests the existence of the monophyletic family Thecamoebidae, comprising the genera *Thecamoeba* and *Sappinia*, but not comprising *Dermamoeba algensis*, whose phylogenetic position remains ill-resolved. Until now there was no laboratory culture of this interesting species; as a result of the present finding, a strain of *Sappinia diploidea* is deposited with CCAP (UK) and is available for further studies; a neotype is established for this species.

Key words: amoeba, Thecamoebidae, SSU phylogeny, diplokaryon, ultrastructure, *Sappinia diploidea*

Introduction

Sappinia diploidea is a remarkable naked lobose amoeba, classified in the class Lobosea, order Euamoebida, family Thecamoebidae in the classification scheme by Page (1987). It possesses a pair of closely associated nuclei, resembling the diplokaryon of microsporidia, *Giardia* and some other organisms. It deserves special attention because of the complex behaviour during encystment, resembling the sexual process. Hartmann and Nägler (1908) described encystment of two binucleate amoebae within a common wall with subsequent fusion of cells and their nuclei. According to their observations, a single binucleate amoeba leaves

such a cyst. These observations were confirmed by a number of researchers, but the fusion of nuclei was never documented clearly (Goodfellow et al., 1974). Interestingly, *Sappinia* was isolated more than once. In particular, it was noted from soil (Nägler, 1909) and, remarkably often, from faeces of lizards (Hartmann and Nägler, 1908), elk, bison and perhaps cattle and was therefore characterized as coprozoic (Levine, 1961; Goodfellow et al., 1974). Gelman et al. (2001) suggested that this species was responsible for a case of amoebic encephalitis, but their identification was based solely on the presence of two nuclei in the studied amoebae. However, all laboratory cultures of *Sappinia* were lost and this remarkable amoeba species remains outside the scopes of attention of investigators.

The present paper provides new LM and EM images of the amoeba, possessing all characters of *Sappinia diploidea* (including nuclear events in cysts) but showing some variability of surface structure. We have sequenced the SSU rRNA gene of this strain and have confirmed its close relatedness with *Thecamoeba*, which is in good congruence with the morphological characters.

Material and Methods

Three strains of *Sappinia diploidea* were isolated from the bark of trees: strain "Sd-plat-4/02" and "Pl-247" from sycamore trees in Andernach and Koblenz (Germany), respectively. The strain "Busnog" was isolated from the bark of old fallen beech tree covered with moss at Lake Snogeholm in the south of Sweden. Strain "sd-bio" originated from the pond within a biotope at Hardert (Germany). All strains were isolated at room temperature on NN agar plates according to Page (1988) from mixed cultures with mainly *Thecamoeba* species among others. Clonal selection was avoided with respect to the possible presence of different mating types. Stock cultures were maintained by serial transfer every 14 days up to once a month, the intervals depending on the growth rate. Strains with poor growth were accelerated by addition of tiny non-defined amoebae as food. Since the migration speed of *Sappinia* was higher than that of the food amoebae, they could be separated easily from each other in order to get pure populations for electron microscopy and especially for molecular biological investigation.

For electron microscopy amoebae and cysts were harvested from the plates and centrifugated (500 g for 10 min). The resulting pellets were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 1 h, washed twice in the same buffer, postfixed for 1 h in 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.2), and embedded in Spurr resin. Sections were stained with uranyl acetate and Reynold's lead citrate and examined using a Leo EM 910 transmission electron microscope (Leo, Oberkochen).

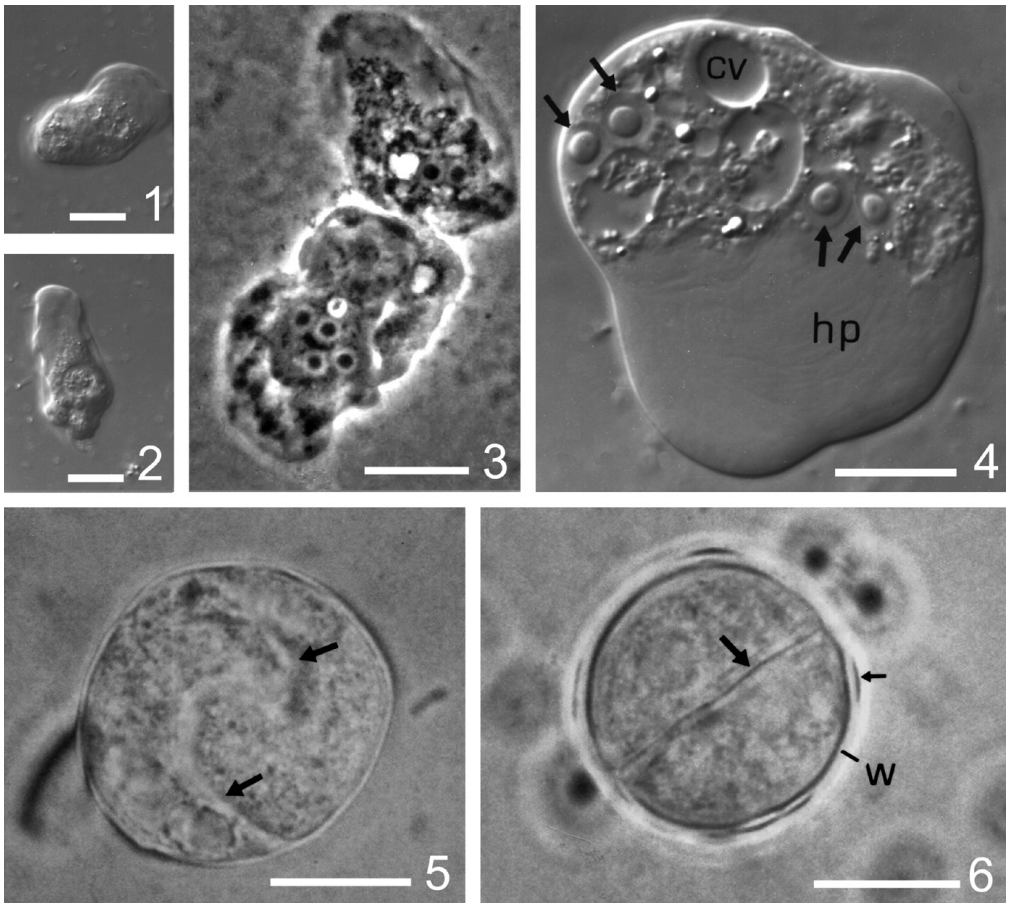
DNA of *Sappinia diploidea* strain "Sd-plat-4/02" was extracted using a modified (Wylezich, 2004) CTAB procedure (Clark, 1992). The SSU rRNA gene was amplified by PCR using eukaryote specific primers complementary to the 5′- and 3′-end of the gene and standard amplification techniques (Medlin et al., 1988). The amplification product of *S. diploidea* was purified with the Rapid PCR Purification System from Marligen Biosciences (Biocat, Heidelberg, Germany) and directly cycle sequenced with the BigDye Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems, Darmstadt, Germany). After cycle sequencing, the reactions were purified with AutoSeq G-50 columns (Amersham, Braunschweig, Germany) and separated on an ABI 3100 Automated Sequencer. All these steps were performed following the manufacturer's protocols. Both strands were completely sequenced in an overlapping mode using PCR primers and different internal sequencing primers: 590F, 600R, 1280F, 1300R (Wylezich et al., 2002), Kin-500for and 1450rev (Scheckenbach et al., 2005).

The sequences obtained were aligned with other sequences retrieved from GenBank/EMBL using ClustalX program (Thompson et al., 1997), and the alignments were then edited manually. Ambiguously aligned regions were excluded. The opisthokont *Diaphanoeca grandis* was selected as an outgroup. Maximum likelihood analyses were done with TREE-PUZZLE, v. 5.2 (Schmidt et al., 2002) using the HKY 85 model of substitution (Hasegawa et al., 1985) and gamma-shaped distribution of rates of substitution among sites, with eight rate categories and 10,000 puzzling steps. Maximum parsimony and neighbor joining analyses (K2P model, Kimura, 1980) were performed with PHYLIP v. 3.63 (Felsenstein, 1989). Bootstrap analyses were repeated 1,000 times to estimate the robustness of the phylogenetic trees (Felsenstein, 1985).

Results and Discussion

Morphology and behaviour

All studied strains show identical LM characters and sizes. The locomotive amoebae were oblong, 55-60 µm in length and had length/breadth ratio about 2.0. During normal locomotion the hyaline cytoplasm occupied the anterior quarter to third of the cell (Figs 1-2). Amoebae had a smooth dorsal surface, sometimes with a few tiny lateral wrinkles. Two rounded nuclei, 3.8-4.6 µm in diameter, were tightly apposed to each

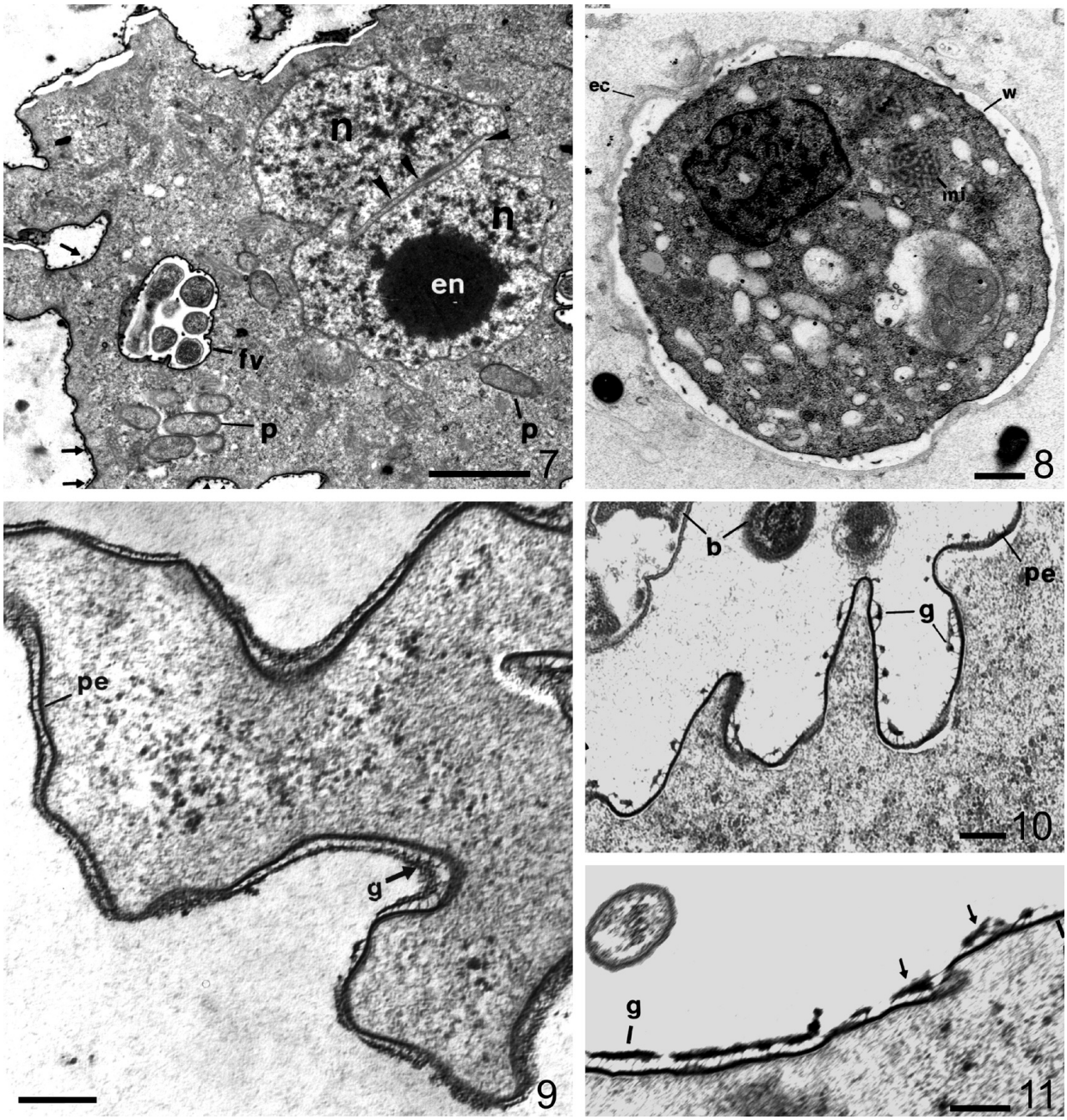


Figs 1-6. Light microscopy of *Sappinia diploidea*. **1-2** - trophozoites of the strain "Sd-plat-4/02" showing normal habitus, with the hyaloplasm occupying the anterior quarter to third of the cell; **3** - two trophozoites of the strain "sd-bio" with two and four nuclei arranged in pairs; **4** - flattened trophozoite ("Sd-plat-4/02") slightly pressed with the coverslip, revealing four nuclei - in two pairs (arrowed) - and the contractile vacuole; **5** - precystic copulation stage of two trophozoites of *Sappinia* ("Busnog"), the two individuals are still separated by a S-like gap or furrow indicated by arrows; **6** - young cyst with two amoebae still separated by a visible border (large arrow), the cyst wall consists of endocyst and very faint ectocyst (small arrow). *Abbreviations*: cv - contractile vacuole, hp - hyaloplasm, w - cyst wall. Scale bars: 20 μm.

other (Figs 3-4) acting as a single unit within the cytoplasmic stream. Each nucleus had vesicular nucleolus about 2.3 μm in diameter. Some cells had two pairs of nuclei (Figs 3-4). The contractile vacuole was highly deformable in moving cells. In full correspondence with the observations by Hartmann and Nägler (1908) and Goodfellow et al. (1974), after a considerable period of multiplication amoebae in culture grouped in pairs; in every pair cells were closely apposed to each other separated by a distinct S-like cleft (Fig. 5). The entire pair of cells was rounded, 30-34 μm in diameter. Subsequently, cysts 30-34 μm across, enclosed by a double wall were formed; cells were still separated in these cysts (Fig. 6). Further events were not traced yet.

Electron microscopy

Our TEM observations are generally congruent with those done by Goodfellow et al. (1974). The interior of the trophozoit was dominated by two characteristic nuclei closely adjacent to each other (Fig. 7). The membranes at the site of contact between the nuclei were parallel to each other and connected by fine fibrils reminding a desmosome-like connection zone. The karyoplasm contained numerous heterochromatin granules. Bacteria located within the cell were either enclosed as prey in membrane-bounded food vacuoles or located directly in the cytoplasm, resembling endocytobionts. Mitochondria had tubular cristae.



Figs 7-11. Electron microscopy of *Sappinia diploidea*. **7** - strain "Busnog", two nuclei closely adjacent to each other, due to the section plane endosome is visible only in one of them, the karyoplasm contains numerous heterochromatin granules, parallel membranes at the attachment site of the nuclei are connected by fine fibrils (arrowheads), bacteria located within the cell are either enclosed as prey in membrane-bounded food vacuoles or directly within the cytoplasm as endocytobionts; **8** - cyst of strain "Sd-plat-4/02" showing one nucleus and mitochondria within the vacuolated cytoplasm; **9** - strain "Sd-plat-4/02" showing deeply stained cell membrane, covered with a conspicuous glycocalyx which apparently consists of perpendicular fibrils arising from the pellicle and branching at the distal side into a horizontal fuzzy coat; **10** - strain "Busnog", the glycocalyx consists of single elements which are widely separated from each other - so that they do not form any continuous layer; **11** - strain "Pl-247", intermediate variant of the cell coat: the elements forming the cell coat (arrowed) sometimes are separated from each other, but in some areas they form nearly continuous layer. *Abbreviations*: b - food bacteria surrounding the cell, en - endosome, fv - food vacuoles, g - glycocalyx, mi - mitochondria, n - nucleus, p - endocytobionts, pe - deeply stained cell membrane, w- inner cyst wall, ec - ectocyst. Scale bars: 7-8 - 10 μm, 9-11 - 200 nm.

Table 1. Sequence lengths and G/C contents of possible members of Thecamoebidae and Vannellida. Organisms sequenced in this study are highlighted in bold.

Species and GenBank accession numbers	Sequence lengths (bp) [1]	Sequence lengths (bp) [2]	G/C contents (%)
***Thecamoeba quadrilineata* DQ122381**	**2210**	**2113**	**54.0**
Thecamoeba similis AY294145	2409	2366	37.7
***Sappinia diploidea* DQ122380**	**2492**	**2446**	**46.0**
Dermamoeba algensis AY294148	2037	1970	43.7
Mayorella sp. AY294143	2131	2112	39.0
Platyamoeba stenopodia AY294144	2143	2110	58.2
Platyamoeba plurinucleus AY121849	1942	1866	36.4
Platyamoeba placida AY294150	1893	1857	39.3
Lingulamoeba leei AY183886	1838	1741	38.1
Vannella anglica AF099101	1962	1866	37.1
Vannella aberdonica AY121853	1913	1863	38.8
Vannella miroides AY183888	1959	1862	38.6

[1] Sequence lengths from Genbank entries
[2] Sequences cut off for beginning and ending at the same sequence position

Despite the most careful search in numerous cells, we did not succeed in finding any cytoplasmic structure resembling dictyosomes.

Trophozoites of different strains show slightly varying structure of the cell surface, which differs from an amorphous one described by Goodfellow et al. (1974). The cell membrane was covered with a thin amorphous layer, followed by a loose layer of irregular perpendicular fibrils branching at the distal side into a horizontal diffuse layer. Its density varied from continuous layer (strain sd-plat, Fig. 9) and up to single elements (strain "Busnog", Fig. 10) decorating the cell surface, and its thickness varied from 29 to 90 nm, depending on the strain (Figs 9-11). The reasons of this variability are unclear; it seems to be a consistent trait of the strains investigated. Series of micrographs from all three strains were thoroughly inspected, but we did not find any hint for the existence of more regular pattern than the one described above.

A double-walled cyst is shown in the Fig. 8. The cytoplasm contained numerous vacuoles of different size (among them there is a large vacuole, resembling an autolytic vacuole, found in early cyst stages) and few mitochondria with tubular cristae. The nucleus contained numerous heterochromatine granules; nuclear pores were well-visible; the nucleolus is out of section in this figure.

Phylogenetic relationships

Sequence lengths and G/C contents of both isolates are shown in the Table 1. In preliminary analyses (not shown) including SSU rRNA sequences from all groups of Amoebozoa the sequences of *Sappinia diploidea* "Sd-plat-4/02" and the *Thecamoeba quadrilineata* show a close relationship to *Thecamoeba similis* and *Platyamoeba stenopodia* from GenBank, published by Fahrni et al. (2003). Hence, an alignment was made with the available SSU rRNA sequences from all possible representatives of the family Thecamoebidae (including *Platyamoeba stenopodia*) and selected species of Vannellida, Acanthopodida and Dactylopodida (sensu Smirnov et al., 2005). With this final alignment (most conserved 1275 characters) we have reconstructed gene trees using maximum likelihood (ML), maximum parsimony (MP), and distance matrix (neighbor joining, NJ) methods. The ML phylogenetic tree with the corresponding NJ and MP bootstrap values is shown in Fig. 12.

Out tree generally is congruent with the latest Amoebozoa trees by Cavalier-Smith et al. (2004), Smirnov et al. (2005) and Kydryavtsev et al. (2005). In all our trees *Sappinia diploidea* groups close to the members of the genus *Thecamoeba* which, in their turn, closely and consistently groups together. *Dermamoeba algensis* forms the sister taxon of the Vannellida/Dactylopodida clade and is relatively distant from other members of Thecamoebidae (probably, the artifact of the analysis because this grouping contradicts almost all morphological characters), while *Platyamoeba stenopodia* appears to be a sister group of *Thecamoebida* (in correspondence with data of other authors). The NJ analysis switches the branching order of the *S. diploidea* and *P. stenopodia*, but the bootstrap value for the sister group relationships of *Thecamoeba* strains and *P. stenopodia* is relatively weak (52 %, not shown).

Conclusions

The results of our LM and TEM studies are identical with those by Goodfellow et al. (1974) and other authors and allow us to conclude that the interesting amoeboid organism that we re-isolated from

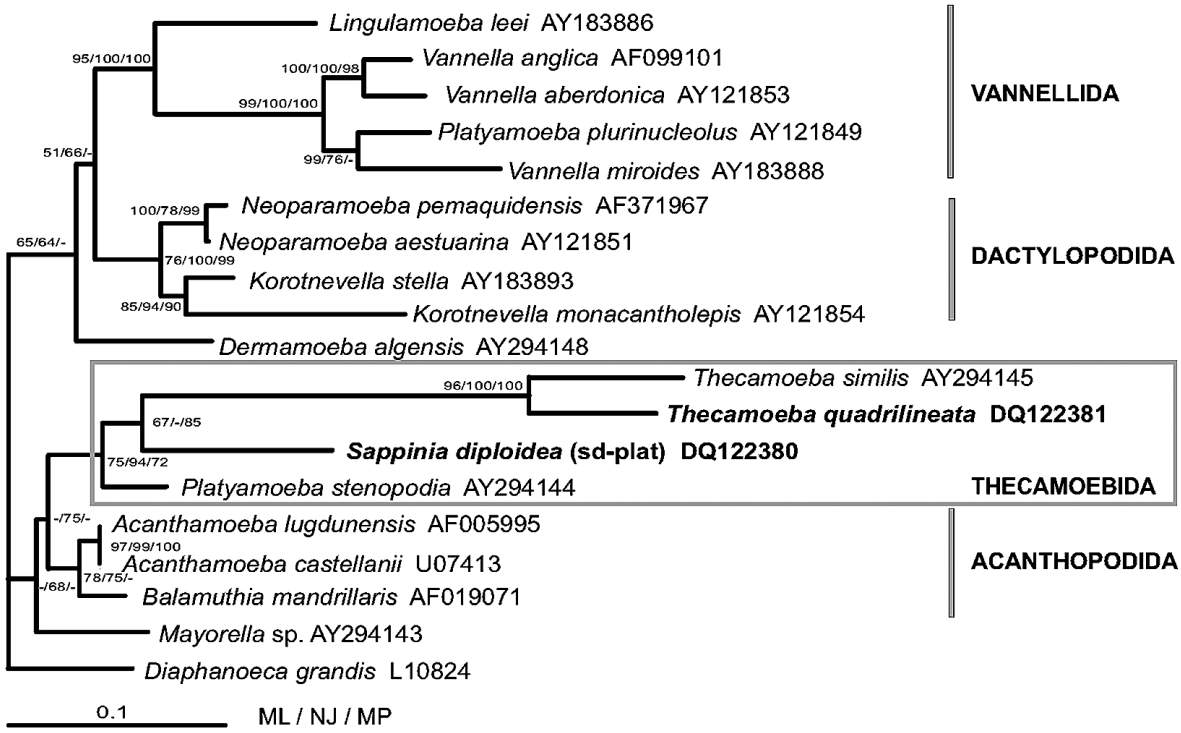


Fig. 12. Maximum likelihood tree (alpha shape parameter: 0.46) for reconstructing of the phylogenetic position of *Sappinia diploidea* ("Sd-plat-4/02") using ML, NJ and MP algorithms. Quartet puzzling support values (ML, 10,000 steps) and bootstrap values (NJ and MP, 1,000 replicates) are shown for branching nodes with more than 50% support (ML/NJ/MP). The phylogram was rooted using *Diaphanoeca grandis*. Organisms sequenced in this study are highlighted in bold. Genbank accession numbers of the species used are indicated. The scale bar represents a distance of 0.1 substitutions per site.

several localities is indeed *Sappinia diploidea*. The cell surface structure is more elaborated and complex in our strains rather than in those studied by Goodfellow et al. (1974). This difference may be fixation- dependent, as noted for many other amoebae species (Smirnov and Goodkov, 1998; Goodkov et al, 1999; Smirnov, 1999), or may reflect different physiological statement of the fixed cells. Given that *Sappinia diploidea* apparently has a relatively complex life cycle, we cannot exclude the possibility that the glycocalyx structure and its density vary at the different stages of this cycle. This suggestion may explain why Goodfellow et al. (1974) noted no elaborated glycocalyx.

The fact that we have isolated our strains from the bark of two different trees increases the known range of habitats of this amoeba species. Our phylogenetic analysis confirmed the close relationship of *Sappinia* with *Thecamoeba*, and the inclusion of the second *Thecamoeba* species in the analysis makes the family Thecamoebidae phylogenetically sound as it was suggested in Adl et al. (2005). Thus, we define the only remaining large morphological family of amoebae that was not yet confirmed with the molecular data. One of strains of *Sappinia* isolated as a result of this study is deposited with the CCAP (UK) under the reference numbers1575/2 (initially designated as Sd-plat-4/02). Since there is no recognised type material on *Sappinia diploidea* (Hartmann and Nägler 1908), we suggest to announce this strain as the type one (neotype).

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Address for correspondence: Rolf Michel. Central Institute of the Federal Armed Forces Medical Services, Koblenz, Germany. E-mail: rolf_michel@hotmail.com