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Electron Microscopical Investigations of a New Species of the Genus *Sappinia* (Thecamoebidae, Amoebozoa), *Sappinia platani* sp. nov., Reveal a Dictyosome in this Genus

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Abstract. The genus *Sappinia* belongs to the family Thecamoebidae within the Discosea (Amoebozoa). For long time the genus comprised only two species, *S. pedata* and *S. diploidea*, based on morphological investigations. However, recent molecular studies on gene sequences of the small subunit ribosomal RNA (SSU rRNA) gene revealed a high genetic diversity within the genus *Sappinia*. This indicated a larger species richness than previously assumed and the establishment of new species was predicted. Here, *Sappinia platani* sp. nov. (strain PL-247) is described and ultrastructurally investigated. This strain was isolated from the bark of a sycamore tree (Koblenz, Germany) like the re-described neotype of *S. diploidea*. The new species shows the typical characteristics of the genus such as flattened and binucleate trophozoites with a differentiation of anterior hyaloplasm and without discrete pseudopodia as well as bicellular cysts. Additionally, the new species possesses numerous endocytobionts and dictyosomes. The latter could not be found in previous EM studies of the genus *Sappinia*. Standing forms, a character of the species *S. pedata*, could be formed on older cultures of the new species but appeared extremely seldom. A loose layer of irregular, bent hair-like structures cover the plasma membrane dissimilar to the glycocalyx types as formerly detected in other *Sappinia* strains.

Key words: *Sappinia platani* sp. nov., Amoebozoa, Thecamoebidae, SSU rRNA gene, diversity, dictyosome, glycocalyx, endocytobionts.

INTRODUCTION

The genus *Sappinia* comprises large, binucleate amoebae with a dense glycocalyx. They are free-living and show a worldwide distribution. *Sappinia* strains have been isolated from faeces (lizard, bison, elk and buffalo; Olive 1902; Hartmann and Nägler 1908; Raper 1940, 1960; Noble 1958; Levine 1961; Goodfellow *et al.* 1974) or from the environment (Michel *et al*. 2006, Brown *et al.* 2007, Wylezich *et al*. 2009). In the past, most isolated strains of this genus have been assumedly identified as *S. diploidea* by light microscopy only (Goodfellow *et al.* 1974, Page and Siemensma 1991,

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Michel *et al.* 2006). One of such *Sappinia "diploidea"* samples has been considered as a potential causative agent of encephalitis in humans (Gelman *et al*. 2001, 2003). Therefore, that species was consistently outlined in the literature on free-living amoeboid pathogens (Schuster 2002, Schuster and Visvesvara 2004, Visvesvara *et al*. 2007, Trebalsi *et al*. 2012). This pathogenic sample, however, has been shown to be more closely related to the other species, *S. pedata*, than to *S. diploidea* based on a real-time polymerase chain reaction assay using the partial SSU rRNA gene (Qvarnstrom *et al*. 2009).

History, phylogeny and medical relevance of this amoeboid group were comprehensively reviewed by Walochnik *et al*. (2010). In short, the genus *Sappinia* Dangeard, 1896 with the single species *S. pedata* had been initially affiliated to acrasid amoebae (Dangeard 1896) whereas the other species *S. diploidea*, firstly described as *Amoeba diploidea* (Hartmann and Nägler 1908), had been classified with the family Thecamoebidae (Goodfellow *et al.* 1974). The latter assumption was confirmed by gene sequence comparisons using the SSU rRNA gene (Michel *et al.* 2006) and later on, by sequence data of the actin genes (Brown *et al*. 2007). Furthermore, both *Sappinia* species branched with each other based on SSU rRNA gene sequences and supported the monophyly of the genus (Brown *et al*. 2007). The inclusion of further strains of *Sappinia*-like amoebae showed a robust monophyletic cluster of all *Sappinia* strains based on SSU rRNA gene sequences (Wylezich *et al*. 2009). On the one hand, this new data confirmed the sister group relationship to the members of the genus *Thecamoeba* and the family Thecamoebidae with inclusion of the genus *Stenamoeba* (Michel *et al*. 2006; Smirnov *et al*. 2007, 2011). Otherwise, a high genetic diversity was revealed within the genus *Sappinia*. In addition to one clade with four strains of "true" *S. diploidea* and a second clade of *S. pedata* strains including the neotypes each, there were three strains (Noaf, Busnog and PL-247) clustering in between of both valid species. Two of these strains (Busnog and PL-247) were formerly identified as *S. diploidea* in Michel *et al*. (2006) based on morphological determination without molecular data. But they formed a sister group to the *S. diploidea* cluster (plus strain Noaf) exhibiting even higher genetic distances using molecular data. It has been assumed that these strains (Busnog, PL-247, and Noaf) represent new species within the genus *Sappinia* as summarized in Figure 1. To affirm this, a detailed description after additional investigations was necessary.

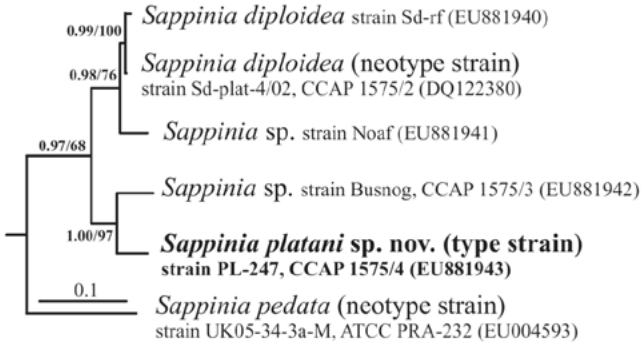


Fig. 1. Phylogenetic tree of the genus *Sappinia* splitted into five species based on SSU rRNA gene sequence data. *Sappinia platani* sp. nov. described in this study is highlighted in bold. The tree is inferred from Bayesian analysis using *Thecamoeba quadrilineata* (DQ122381) as outgroup taxon (not shown). Posterior probabilities are indicated at the nodes. Corresponding bootstrap values for a maximum likelihood analysis are shown (Bayes/ML). GenBank accession numbers for SSU rRNA gene sequences are indicated in parentheses.

The current study reports now on electron microscopical investigations of *Sappinia* strain PL-247 resulting in the description of the species *Sappinia platani* sp. nov.

MATERIALS AND METHODS

The *Sappinia* strain PL-247 (CCAP 1575/4) was isolated from bark of a sycamore tree (Michel *et al*. 2006) and cultured on NN agar according to Page (1988) seeded with *Enterobacter cloacae* as nutrient bacteria.

The SSU rRNA gene of this strain was sequenced previously (Wylezich *et al*. 2009). In order to show the phylogenetic relationship of tentative *Sappinia* species (Fig. 1) sequences were aligned using the Clustal_X program (Thompson *et al*. 1997), and the alignments were then edited manually with BioEdit (Hall 1999). Phylogenetic analyses of the genus *Sappinia* were performed using *Thecamoeba quadrilineata* as outgroup taxon. MrBayes (Huelsenbeck *et al*. 2001) and phyML (http://atgc.lirmm.fr/phyml/, Guindon *et al*. 2005) were used for phylogenetic analyses in the same manner as already described by Wylezich *et al*. (2009).

Light microscopical studies were carried out at brightfield and phase contrast using an Orthoplan microscope (Leitz, Wetzlar, Germany) and an Eclipse E800 phase contrast and fluorescence microsope (Nikon Instruments, Amsterdam, The Netherlands).

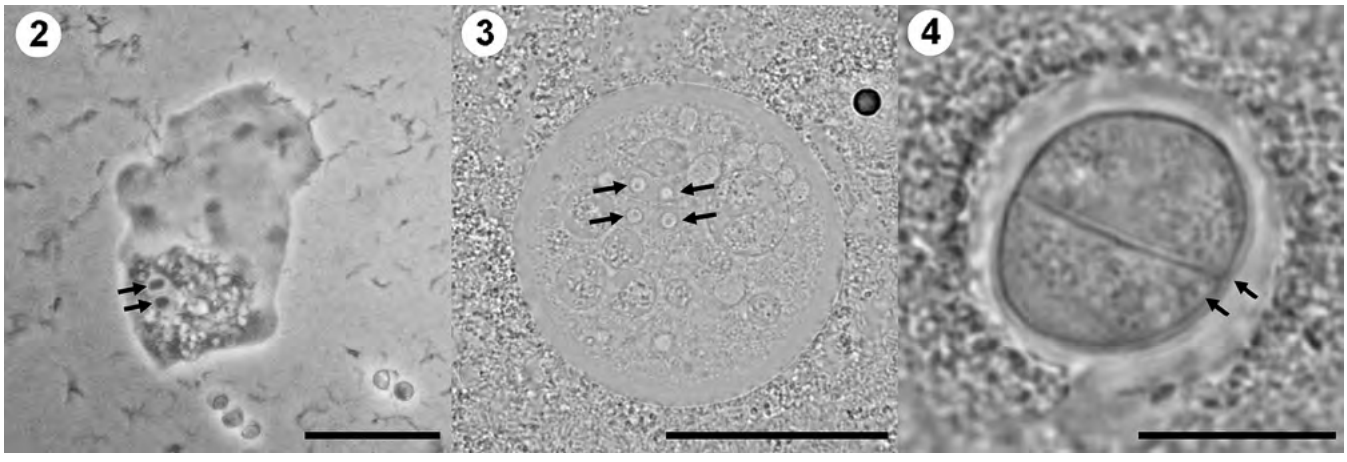
For transmission electron microscopy amoebae were fixed on ice with 2.5% glutaraldehyde followed by 1% osmium tetroxide (both for 60–75 minutes). Both fixatives were prepared in 0.05 M sodium cacodylate buffer (pH 7.4). Cells were washed with the same buffer (3 x 5 minutes) between fixation steps. In the beginning of a fixation amoebae were scraped away from the agar surface using a plastic cell scraper into a drop of liquid medium, and an equal

volume of glutaraldehyde in a 2-fold concentration was added to yield the final concentration of the fixative. During further steps, cells were gently pelleted in glass tubes using a manual centrifuge. In the end of fixation, cells were washed twice with buffer followed by a brief rinse in a series of buffer with decreasing concentration down to distilled water. Amoebae were then embedded in 2% agar prepared with distilled water; after that pieces of agar (~ 1 mm^3) containing cells were cut out, dehydrated in a graded ethanol series (30% – 50% – 70% – 90% – 100%) followed by propylene oxide, infiltration and embedding in Araldite M epoxy resin (Serva). Sections were cut using a Reichert Ultracut E ultramicrotome with a diamond knife, double-stained with 2% uranylacetate in 70% ethanol and Reynolds' lead citrate, and observed using Philips EM 208 or Philips BioTwin electron microscopes operated at 80 and 60 kV respectively.

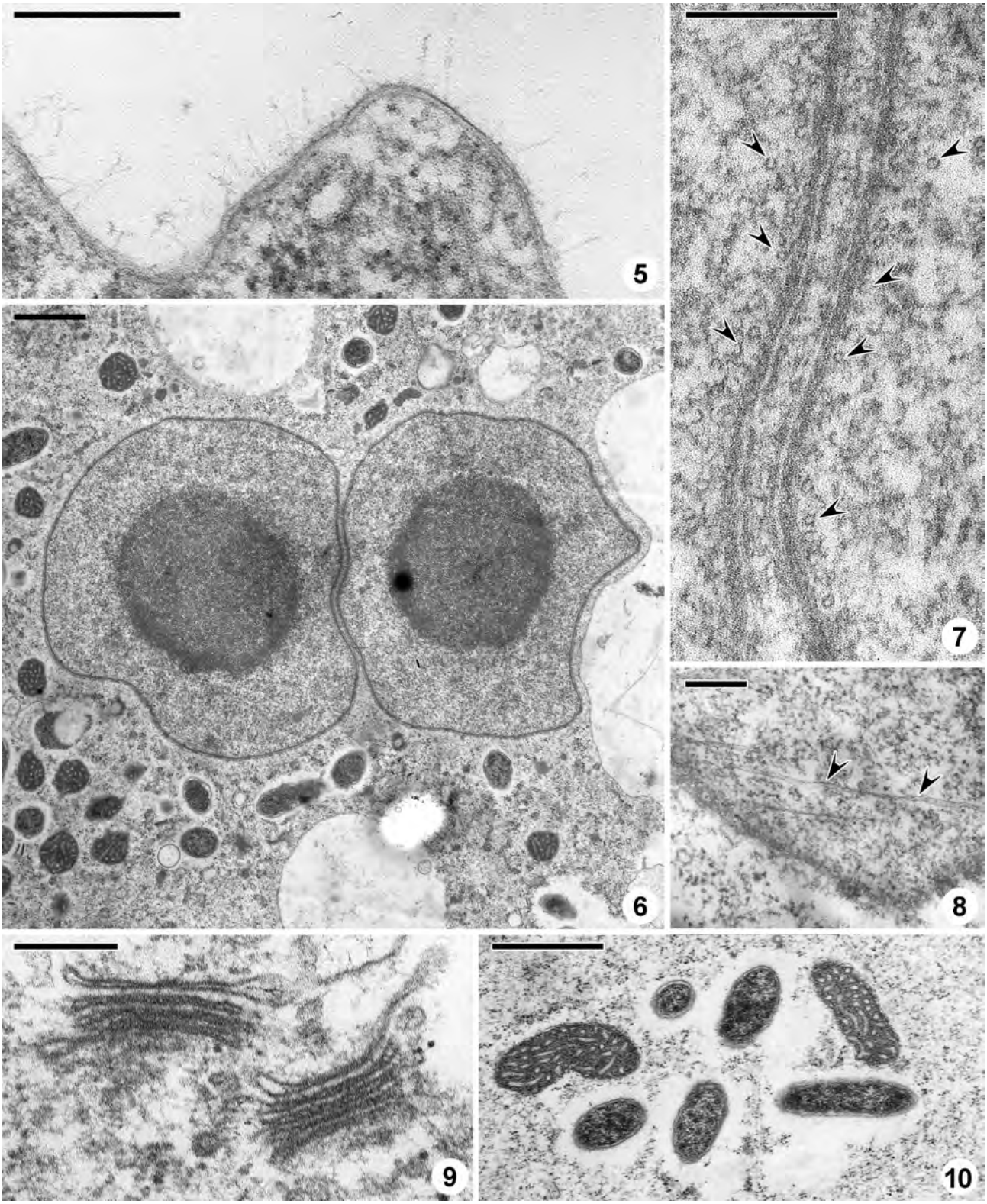
RESULTS

The investigated strain showed the typical habitus of the genus *Sappinia* with two vesicular nuclei (diameter about 4 μm) including a large central nucleolus each and a large anterior hyaline area occupying approximately 1/2 of the cell during locomotion (Fig. 2). The locomotive forms had a lingulate morphotype (Fig. 2). The newly described species resembles *S. diploidea* in its general form (Michel *et al*. 2006) but has a larger size. The amoebae are oblong (57–76 × 23–38 μm, n = 10) and have a length/breadth ratio of about 2.0. The emerging cysts typically start from two closely apposed individuals showing two nuclei each (Fig. 3). Cysts of the newly described species (diameter 23–30 μm, n = 10) are enclosed by a double wall (Fig. 4) and are smaller than cysts of *S. diploidea* (30–34 μm, Michel *et al*. 2006). Cysts of *S. pedata* are slightly smaller (about 20–25 μm, Dangeard 1896; Brown *et al.* 2007), even if larger cysts (50–55 μm) have been reported (Cook 1939). The differentiation into endocyst and ectocyst is clearly visible.

The electron microscopical observations of the cell coat (Fig. 5) showed a typical plasma membrane covered with a loose layer of irregular, bent hair-like structures reaching up to 60–120 nm above the plasma membrane. They did not show a typical glycocalyx as found by Michel *et al*. (2006). The nuclei of trophic cells were irregularly rounded in sections and closely adjacent to each other (Fig. 6). The nuclear envelope was underlain by a layer of dense material ca. 25 nm thick (Fig. 7). Numerous microtubules were located in the area of contact of the two nuclei beneath the nuclear envelope (Figs 7, 8). Numerous dictyosomes were clearly visible in sections scattered throughout the granuloplasm (Fig. 9). They had a typical shape of stacks consisting of 4–6 flattened cisternae. Numerous prokaryotic endocytobionts were visible as single cells or dividing stages (Figs 6, 10). These organisms were located freely in the cytoplasm, without any surrounding vacuoles, and a halo of transparent cytoplasm was seen around every endocytobiont. The mitochondria appeared rounded or oval in sections, with branching



Figs 2–4. *Sappinia platani* sp. nov. strain PL-247, CCAP 1575/4. Light micrographs. **2 –** trophozoite with clearly visibly hyaloplasm at the anterior part of the cell and one pair of nuclei (marked by arrows); **3 –** initiating development of cyst. Four nuclei (arrows) and the cyst wall are visible; **4 –** young cyst with two amoeba cells separated by a border. The differentiation in endocyst and ectocyst is clearly visible (arrows). Scale bars: 20 μm.



Figs 5–10. *Sappinia platani* sp. nov. strain PL-247, CCAP 1575/4. Electron micrographs. **5 –** detail of the plasma membrane and cell coat; **6 –** nuclei and part of the cytoplasm surrounding them; **7 –** area of contact between two nuclei. Note microtubules (arrowheads) beneath the nuclear envelopes; **8 –** microtubules (arrowheads) inside the nucleus associated with nuclear envelope; **9 –** dictyosomes; **10 –** mitochondria and bacteria in the cytoplasm. Scale bar: 1 μm in Figs 6, 10; 0.25 μm in other figures.

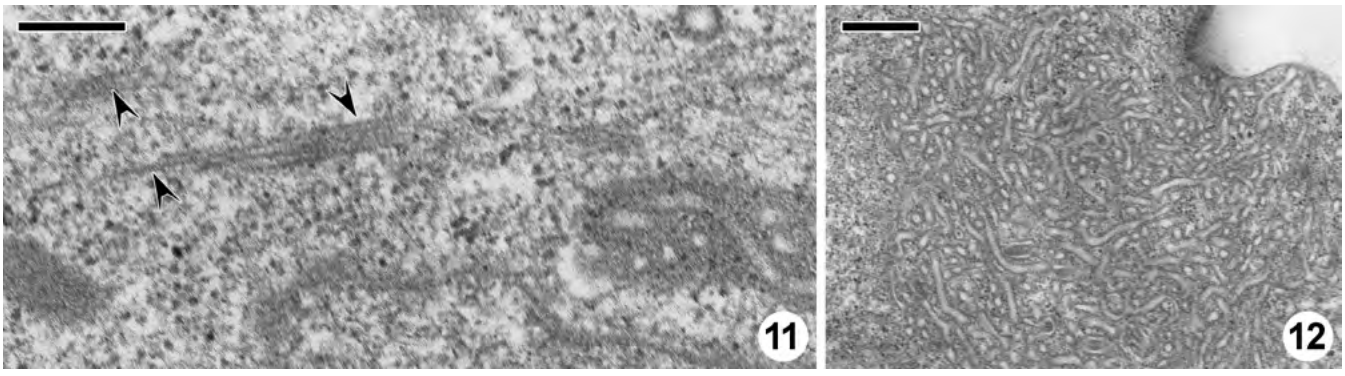
tubular cristae sometimes in parallel arrays (Fig. 10). Some areas of the cytoplasm contained dense arrays of membranous tubules (Fig. 11) that resembled a spongiome, but did not seem to be specifically associated with contractile vacuoles in all cases, therefore could also be interpreted as cisternae of smooth endoplasmic reticulum. Numerous bundles of microfilaments were often seen close to the plasma membrane and inside the granuloplasm (Fig. 12).

DISCUSSION

Until the use of molecular markers to investigate *Sappinia* isolates, the genus has been regarded to include only two species. However, the SSU rRNA gene sequence of *Sappinia* strain PL-247 (CCAP 1575/4) did not show close relationship to the two known species *S. diploidea* and *S. pedata* and permitted a conclusion that this was a new species of this genus based on the gene sequence data (Wylezich *et al*. 2009). The present study supports this hypothesis with ultrastructural data.

The most astonishing result of our survey is the presence of dictyosomes in the cytoplasm of *S. platani*. Such an organelle could not be found in any of the previous electron microscopical investigations of *S. diploidea* (Goodfellow *et al*. 1974, Michel *et al*. 2006). Although there are always chances that this structure was overlooked in sections, due to either imperfect preservation, or non-complete sectioning, this does not seem to be the case here. The preservation quality of the cytoplasm in both studies was adequate and comparable to the data we provide here, and in both previous works the absence of dictyosomes is explicitly stated so that there is no reason to doubt that the number of screened sections of *S. diploidea* was large enough to not miss the dictyosome. The strain PL-247 (*S. platani*) had also been included in the former EM study of Michel *et al*. (2006) in order to compare the cell coat of different *Sappinia* strains with each other. In the course of that incomplete investigation no dictyosome had been found in strain PL-247. Because of these varying results we can not exclude that the existence of dictyosomes depends on the trophic status of the amoeba cells during investigation. However, we hypothesize that the presence of dictyosomes is a specific character of *S. platani* sp. nov.

Another character that allows a distinction of *S. diploidea* and *S. platani* is the cell coat, although it also shows slight structural variations between different strains of *S. diploidea* (Goodfellow *et al*. 1974, Michel *et al*. 2006). Whereas amoebae studied by Goodfellow *et al*. (1974) had a continuous, electron-dense basal layer of glycocalyx and a more flocculent layer on top of it, the strain Sd-plat-4/02 (*Sappinia diploidea*) possesses a continuous glycocalyx consisting of an electron-dense fuzzy layer separated from the plasma membrane by a transparent space crossed by the vertical structures, and the cell coat of strain Busnog (*Sappinia* sp.) consists of only single elements (Figures 9 and 10 in Michel *et al*. 2006). However, none of the previously studied strains shows a structure similar to a loose filamentous layer of low electron density that we demonstrate in the species described here. Although a previous electron microscopical study of the cell coat in strain PL-247



Figs 11–12. *Sappinia platani* sp. nov. Transmission electron micrographs, continued. **11 –** bundles of microfilaments in the cytoplasm (arrowheads); **12 –** agglomeration of membranous tubules (presumably endoplasmic reticulum) in the cytoplasm. Scale bar: 0.25 μm in Fig. 11 and 0.5 μm in Fig. 12.

(Figure 11 in Michel *et al*. 2006) did not show the same structure as we demonstrated here, it might be that the discontinuous glycocalyx structure illustrated by Michel *et al*. (2006) is limited only to special functional areas of the plasma membrane. This hypothesis is supported by the observation that this special glycocalyx was not detected in all sections in the former study of Michel *et al.* (2006) (R. Michel, unpublished results). Unfortunately, electron microscopical studies on *S. pedata* are not available for comparison; however, this species clearly differs from *S. platani* sp. nov. in having a narrower anterior hyaline area of the locomotive form (Brown *et al*. 2007). Standing cells were very seldom observed in the species described here. Gene sequence data also clearly separate these two species (Fig. 1).

Furthermore, endocytobiotic bacteria are abundant in the cytoplasm of all *Sappinia* species. Their identification may help to clarify the evolutionary history of these amoebae and perhaps could also be used to discriminate between species. Indeed, preliminary results based on the molecular characterization of endocytobionts, through 16S rDNA analysis, indicate that *S. platani* as well as *S. diploidea* and *S. pedata* harbour species-specific and distinct *Pedobacter* and *Flavobacterium* spp. (Corsaro *et al*. manuscript in preparation).

Diagnosis

Phylum Amoebozoa Lühe, 1913 (em. Cavalier-Smith 1998)

Subphylum Lobosa Carpenter, 1861 (em. Cavalier-Smith 2009, Smirnov *et al*. 2011)

Class Discosea Cavalier-Smith, 2004 (em. 2011)

Subclass Longamoebia Cavalier-Smith and Smirnov, 2011

Order Thecamoebida Smirnov and Cavalier-Smith, 2011

Family Thecamoebidae Schaeffer, 1926 (em. Smirnov *et al.* 2011)

***Sappinia platani* sp. nov. Wylezich, Walochnik, Corsaro, Michel et Kudryavtsev, 2015**

Description: Locomotive forms oblong, of a lingulate morphotype; often irregular, length 57–76 μm (n = 10), breadth 23–38 μm (n = 10). Two nuclei in each trophic cell; nuclei vesicular, with large, central nucleoli. Cysts formed by two individual cells, 23–30 μm in diameter. Cell coat 60–120 nm thick, consisting of a loose layer of bent, filamentous structures. Standing forms as typical for *S. pedata* were extremely seldom observed.

Type material: The type strain PL-247 is deposited with CCAP under the accession number 1575/4.

Type locality: Specimen of the type strain was collected at the bark of sycamore tree in Koblenz, Germany.

Type sequence: EU881943 (SSU rRNA gene, Wylezich *et al*. 2009).

Etymology: The specific epithet is derived from the sycamore tree *Platanus occidentalis* (Plantae, Angiospermae, Proteales) from which the type strain was isolated.

Differential diagnosis: The species presented here differs from *S. diploidea* in having slightly larger locomotive forms and smaller cysts, presence of a dictyosome, and a different type of a cell coat. *S. platani* differs from both known species based in SSU rRNA gene sequences.

Remarks: The granuloplasm of the strain investigated here contains dictyosomes and endocytobionts of the genus *Pedobacter* (Bacteroidetes, Sphingobacteria).

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