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Thecamoeba aesculea n. sp. (Amoebozoa, Thecamoebidae), a Terrestrial Amoeba with Affinities to *Th. sphaeronucleolus* (Greeff, 1891)

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Summary. *Thecamoeba aesculea* n. sp. was isolated and described from the surface of the bark of *Aesculus hippocastanum* and from terrestrial mosses growing on it. This amoeba is superficially similar to *Thecamoeba sphaeronucleolus*, but comparison of the newly isolated strain with the photographs and video records of the type strain of this species reveals differences which show that the two strains do not belong to the same morphospecies. The data obtained indicate the necessity of further comparative studies on the diversity of thecamoebian 'morphospecies' to outline clearer borders between them.

Key words: Amoebae, morphology, morphospecies, taxonomy, *Thecamoeba*, ultrastructure.

INTRODUCTION

The genus *Thecamoeba* Formentel, 1874 comprises ten valid and easily recognizable species as well as six poorly studied species inhabiting freshwater, soil and marine habitats (Page 1991). Most of these species can be distinguished from each other using light microscopy (Smirnov and Brown 2004), a fact which gives the impression that species identification within this genus is relatively easy, and that it can be done even when only a few individuals from a natural sample are available. However, the diversity of the *Thecamoeba* morphospecies is probably higher than previously thought, and some of them may represent complexes of morphologically similar, yet distinguishable, species. Here we present a description of one such species which is very close in morphology and ultrastructure to a well-known soil amoeba *Thecamoeba sphaeronucleolus* (Greeff, 1891), but which demonstrates a number of differences from its previously studied strains. These differences justify not assigning the studied strain to *Th. sphaeronucleolus*.

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MATERIAL AND METHODS

Amoebae were isolated from the epiphytic mosses and bark surface of *Aesculus hippocastanum* collected in the city center of Saint-Petersburg (59º56′30″N, 30º18′05″E), northwestern Russia, in January 2005. Samples were inoculated in Petri dishes with (1) Prescott and James (PJ) medium (Prescott and James 1955) and a grain of wheat, and (2) 1.5% non-nutrient (NN) agar prepared with PJ medium, without liquid overlay. Amoebae were found in both media,

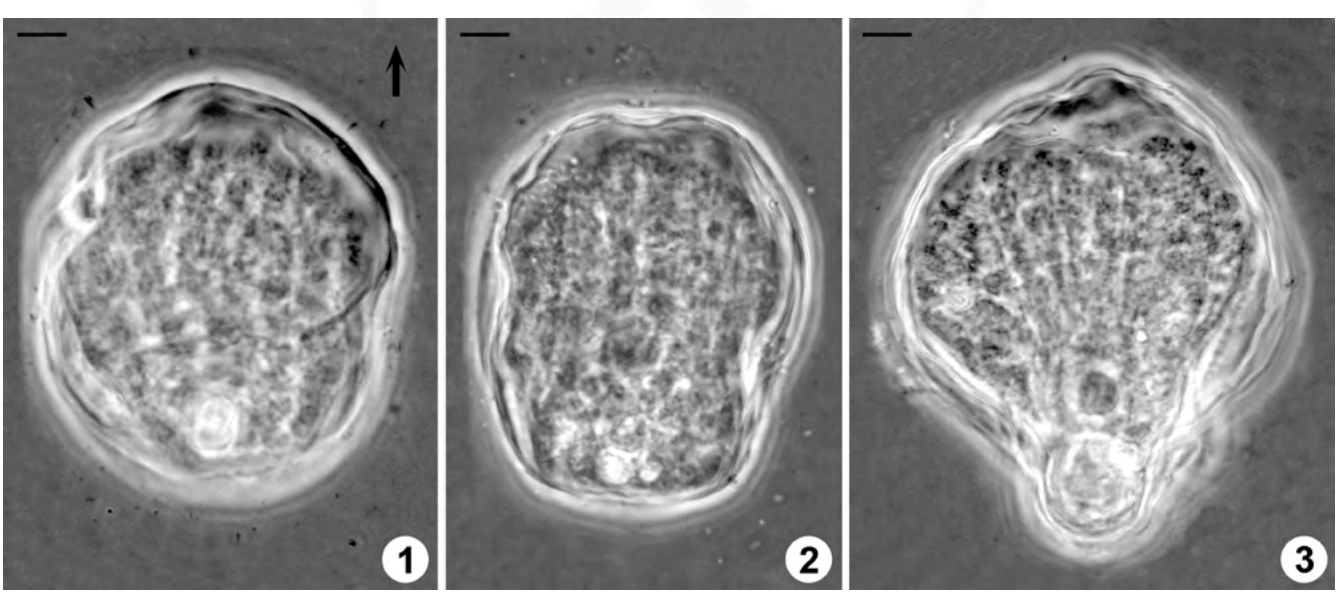
but the stable clonal cultures could be established and maintained only on NN agar. Living amoebae were observed and measured using a Zeiss Axiovert 200 light microscope with phase contrast and DIC optics. In total, several hundred living cells were observed and 60 measured. For transmission electron microscopy, amoebae were fixed at room temperature with (1) 0.5% osmium tetroxide in 0.05M cacodylate buffer (pH 7.4) for 5 min. followed by 2.5% glutaraldehyde in the same buffer for 40 min., and then post-fixed with 1% osmium tetroxide in the same buffer for one hour; (2) 1% osmium tetroxide in 0.05M cacodylate buffer (pH 7.4) for one hour. Specimens were washed with buffer three times for five minutes each time, between fixation steps and prior to dehydration. Specimens were dehydrated in a graded ethanol series followed by epoxypropane, and embedded in Araldite epoxy resin (Serva). Sections were double-stained with 6% aqueous uranyl acetate (30 min.) and Reynolds' lead citrate (10 min.), and then examined with a Philips EM 208 electron microscope.

RESULTS

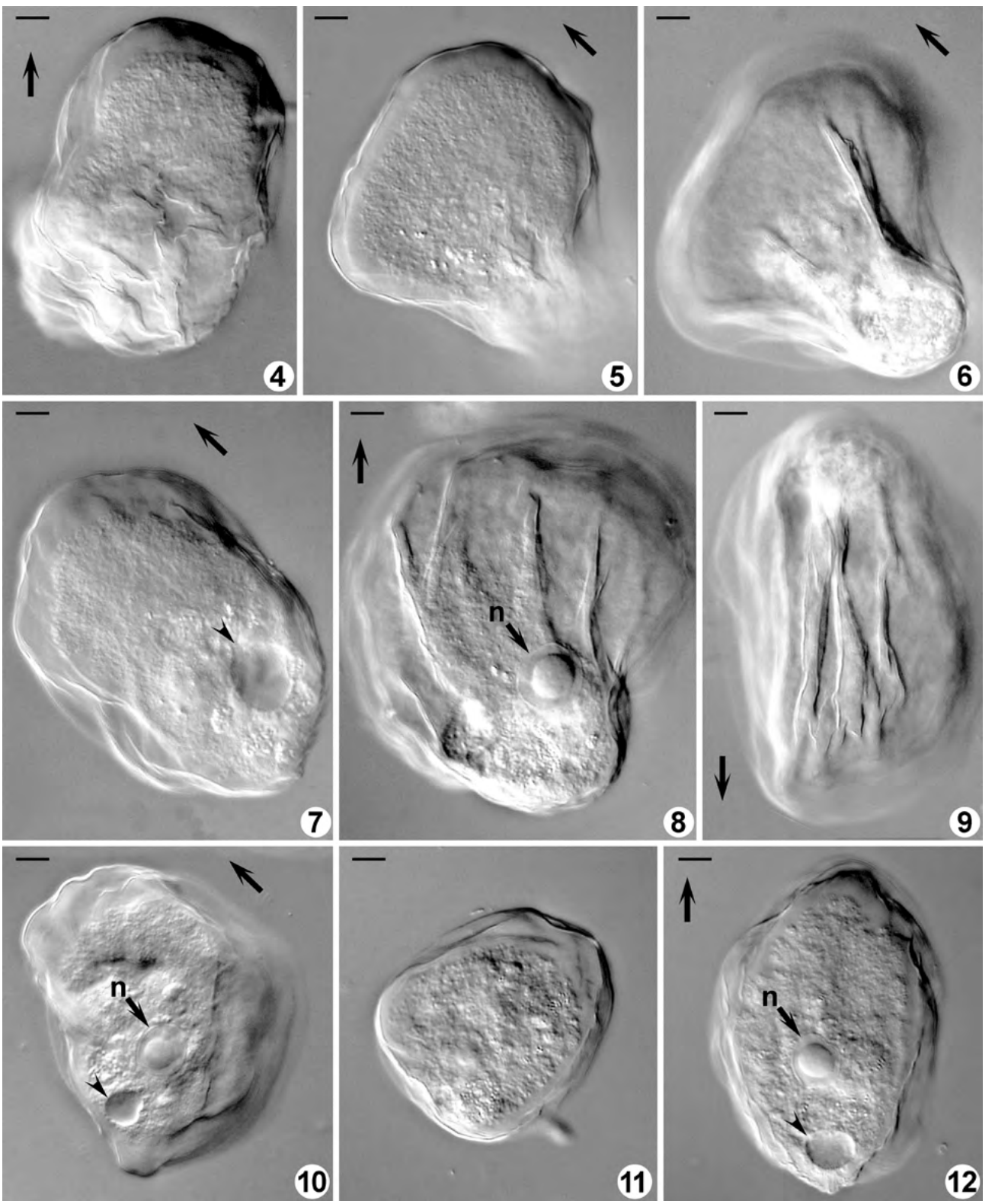
Light microscopy

Most of the observed amoebae readily demonstrated adhesion to and locomotion on agar (Figs 1–3); by contrast, when placed on glass surface, the amoebae remained irregularly shaped and usually failed to adhere to the substratum. The locomotive forms were adopted rarely, yet, their shape was the same as on agar surface (Figs 4–10). During locomotion amoebae were broadly spatulate (Figs 1–2, 4, 7, 10) to circular and sometimes even fan-shaped, with a slightly tapering uroid (Figs 3, 5–6, 8). The cell showed irregular lateral wrinkles and 2–5 distinct dorsal longitudinal folds on its surface. The folds were especially well pronounced in extended amoebae with a high length : breadth ratio (Figs 8–9). All these features correspond to the "rugose" morphotype of Thecamoebidae as described by Smirnov and Brown (2004). The hyaloplasm occupied anterior and lateral position as a narrow crescent (Figs 1–3, 4–5, 7). An uroid was usually blunt and carried numerous ventral wrinkles produced by the contraction of the cytoplasm in this region (Fig. 4). Sometimes an anterior part of highly elongated amoebae detached from the substratum; in this case longitudinal folds were also formed on the ventral surface. The locomotion of this amoeba was accompanied by a typical polyaxial flow of the granuloplasm and rolling movement of the plasmalemma that could be seen from the movement of particles adhering to the cell surface. Amoebae displaying non-directed movement and stationary amoebae were irregularly shaped and wrinkled, but without pronounced large folds (example in Figs 11–12). They often detached from the substratum and started to float, maintaining their irregular form.

The amoebae had a single spherical or ovoid nucleus with a thick envelope and a large central nucleolus



Figs 1–3. Locomotive forms of *Thecamoeba aesculea* on the surface of NN agar, Phaco. Arrow indicates direction of movement of all three cells. Scale bar: 10 μm.



Figs 4–12. Light micrographs of *Thecamoeba aesculea* on glass surface, DIC. **4–9** – locomotive forms (**5**, **6** – the same amoeba in ventral and dorsal views, respectively; **9** – dorsal view of a greatly elongated amoeba showing longitudinal folds); **10** – amoeba changing its direction of movement; **11** – amoeba in non-directed movement; **12** – nucleus and contractile vacuole in a slowly moving cell. In all figures: n – nucleus, arrowheads – contractile vacuoles, arrows in **4–10**, **12** – direction of movement. Scale bar: 10 µm.

(Figs 8, 12). The nucleus was located in the posterior third of the cell. Just posterior to the nucleus there was a single contractile vacuole (Figs 7, 10, 12) and one or two large food vacuoles. The anterior part of the granuloplasm was mostly filled with fine spherical inclusions, rather uniform in size (Figs 5, 7). There were no crystals.

In our cultures amoebae never encysted. They fed on various small accompanying organisms, both bacteria and eukaryotes. Trophozoites and cysts of a small amoeba resembling *Stenamoeba stenopodia* co-isolated in the same culture appeared to be the main food source.

Electron microscopy

The plasma membrane of the cell was about 6–7 nm thick. It was covered with a stratified glycocalyx consisting of a basal electron-dense layer 8–10 nm thick and a less dense outer layer about 10–15 nm thick, separated from the basal layer by an electron-transparent 8–10 nm space (Fig. 13). The maximal total thickness of the glycocalyx was about 33 nm. Other ultrastructural features of trophic amoebae were typical for *Thecamoeba* and mostly resembled those illustrated by Houssay and Prenant (1970). The nucleus was rounded, with a typical envelope about 30 nm thick underlain with a nuclear lamina about 0.3 μm thick (Figs 14–16). The tangential sections of the nuclear lamina showed that this structure consisted of curved interlacing filaments (Fig. 14). The nuclear membrane contained numerous pores 60–70 nm in diameter. The nucleolus was rounded and non-homogeneous, consisting of a finely granular peripheral zone and several denser patches of material in the center (Fig. 15). Numerous rounded or ovoid mitochondria with tubular cristae and dictyosomes (consisting of about ten to twelve flattened cisternae) were scattered around the cytoplasm (Figs 17–18). Mitochondria and dictyosomes were often seen aligned along the border between granuloplasm and peripheral hyaloplasm, marked with the bundles of fine filamentous material similar in appearance to actin microfilaments (Fig. 17). Scarce cisternae of rough endoplasmic reticulum were sometimes seen between mitochondria (Fig. 17). The contractile vacuole was prominent in the sections; it was surrounded with a dense layer of rounded and elongated vesicles comprising, in all likelihood, a spongiome (Fig. 19). Occasionally, two or three contractile vacuoles of a smaller diameter and surrounded by the common spongiome layer were seen.

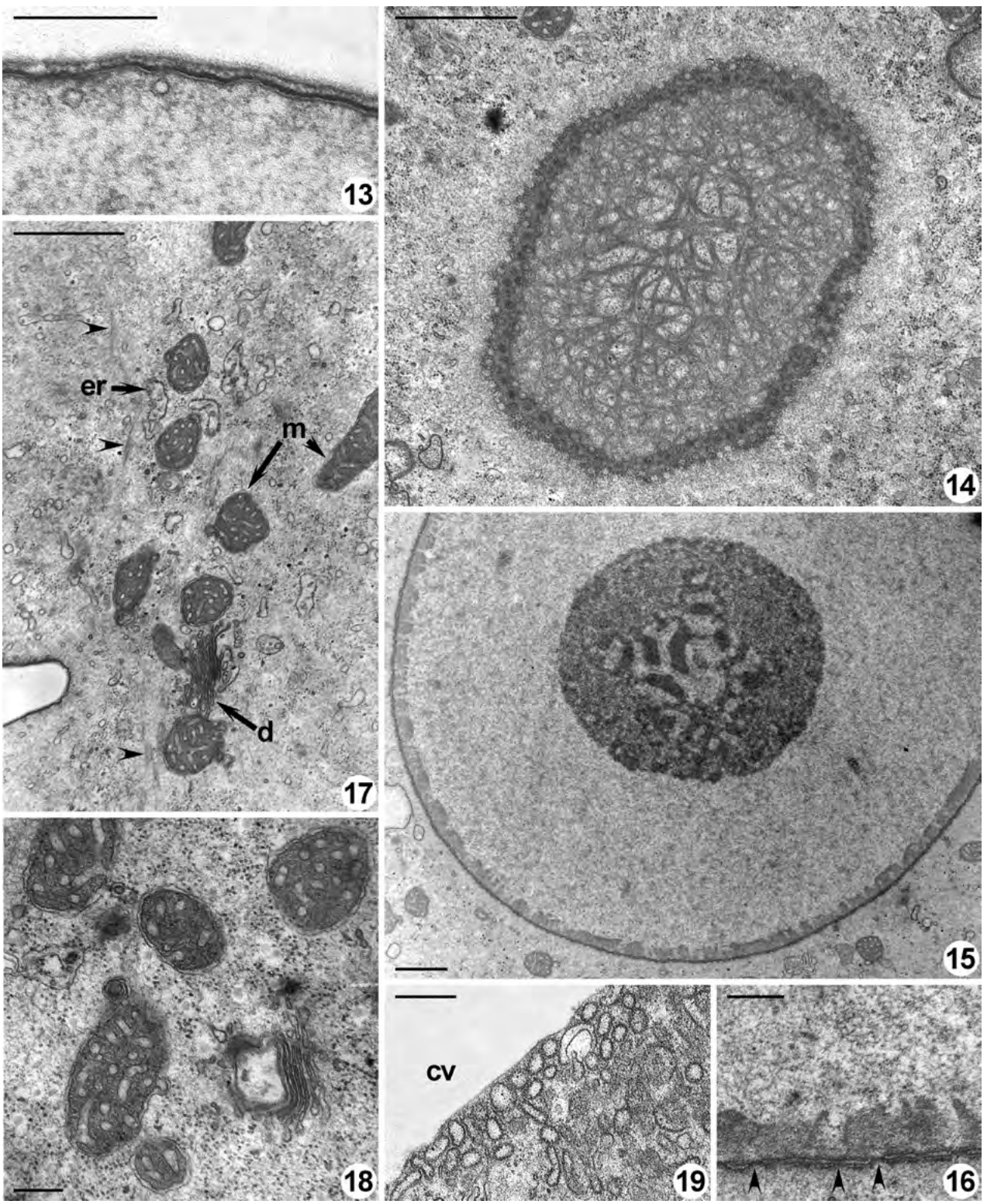
DISCUSSION

Identification

The described amoeba clearly belongs to the genus *Thecamoeba*, although an accurate assignment to one of the existing species is not possible. The strain is very similar to *Thecamoeba sphaeronucleolus* (Greeff, 1891) as re-defined by Page (1971, 1977; Page and Blakey 1979) in its size, some aspects of locomotion and in the ultrastructure of its nucleus and cytoplasm. We compared our strain with the type strain of *Th. sphaeronucleolus* (Page 1977), which until recently had been kept in the Culture Collection of Algae and Protozoa (CCAP), UK, with the accession No 1583/3, using videos and photographs made by Dr. Alexey Smirnov (St. Petersburg, Russia). A recent photograph of a CCAP strain amoeba is Fig. 25A in Smirnov and Brown (2004). The comparison reveals the following differences:

1. The locomotive form of the strain studied here had a stronger tendency to be broadly spatulate, circular or even fan-shaped, and unlike the CCAP strain of *Th. sphaeronucleolus*, was probably never elongated and drop-shaped with a pointed uroid (cf. Figs 3–4 of Page 1971); CCAP-strain amoebae had a wider hyaloplasm and a less wrinkled locomotive form.
2. The length : breadth ratio of our strain was generally lower than for the CCAP strain (an average of 1.3 and 1.5, respectively); amoebae with a length : breadth ratio of 1.0 or even less occurred more often. There were no amoebae with the length : breadth ratio less than 1.0 in Page's (1971) strain.

Generally, the shape of the locomotive form of the amoebae studied here is more similar to that of Page's *Thecamoeba similis* (Page 1977; Page and Blakey 1979), but they differ clearly in nuclear structure. The ultrastructural features of our strain are also very similar to those of other strains of *T. sphaeronucleolus* as described by Houssay and Prenant (1970) and Haberey (1973). However, our strain does not fit the former description in the structure of the cell coat. Amoebae studied by Houssay and Prenant (1970) had a dense cell coat about 20 nm thick with irregular extending outer filaments 70 nm long and 2 nm thick. Our results are in the same way inconsistent with the ultrastructural description of the cell coat by Page and Blakey (1979). The glycocalyx of their *Thecamoeba sphaeronucleolus* was about 50 nm thick and its outer layer looked less regular than that of our strain. Although the structure of



Figs 13–19. Transmission electron micrographs of *Thecamoeba aesculea*. **13** – plasma membrane and glycocalyx; **14** – tangential section of a nuclear envelope showing nuclear pores and lamina; **15** – cross section of a nucleus; **16** – enlarged view of the nuclear envelope in a transverse section showing a thick lamina and nuclear pores (arrowheads); **17** – mitochondria (m), rough endoplasmic reticulum (er) and a dictyosome (d) along the microfilamentous bundles (arrowheads) at the periphery of the cytoplasm; **18** – enlarged view of the mitochondria and a dictyosome; **19** – spongiome vesicles near the reservoir of a contractile vacuole (cv). Scale bar in Figs 13, 16, 18, 19: 0.25 µm, in other figures: 1 µm.

a glycocalyx may be prone to artifacts caused by fixation, it was rather constant in our strain, appearing the same both after fixation with osmium tetroxide-glutaraldehyde-osmium tetroxide and after osmium tetroxide alone (although the cytoplasm was very poorly preserved in the latter fixation). Another difference was the thickness of a nuclear lamina, which was only 0.3 μm thick in our strain and about 0.4 μm in the amoebae studied by Houssay and Prenant (1970).

Therefore, we cannot definitively assign the studied strain to any of the known species of *Thecamoeba* based solely on morphology and ultrastructure. *Thecamoeba sphaeronucleolus* might be the most closely related species, but our amoebae are not completely identical to its type strain. Comparison of the gene sequence data of the two strains could be useful, as this might provide an additional measurement of the phylogenetic distance between two strains and allow an assessment of whether the observed differences are really interspecific, or rather interclonal within the same morphospecies. However, no such data for *T. sphaeronucleolus* from CCAP are available at the moment, and the strain itself is lost. We propose therefore that the studied strain should be considered to be a new species of *Thecamoeba*, although further detailed studies of the morphospecies diversity in this genus, using both morphological and molecular methods, is needed to clarify the morphospecies concept in this group.

Diagnosis

Thecamoeba aesculea n. sp.

Measured length of the locomotive form 70–119 μm (average 101.5 μm) (n = 60); breadth – 56–117.5 μm (average 80.4 μm); length : breadth ratio – 0.81–1.93 (average 1.29). Spatulate during locomotion, sometimes slightly posteriorly tapering and almost fan-shaped. Dorsal surface with 2–5 longitudinal folds and numerous irregular wrinkles, especially in the uroidal part. Single vesicular nucleus 16–22 μm in diameter (average 18.5 μm); spherical central non-homogeneous nucleolus 7.5–14.4 μm in diameter (average 10.4 μm) (n = 35). Glycocalyx up to 33 nm thick, consisting of a basal electron-dense layer 8–10 nm thick and a less dense outer layer about 10–15 nm thick, separated from the basal layer by an 8–10 nm thick electron-transparent space.

Observed habitat: Dry epiphytic mosses and bark surface of *Aesculus hippocastanum* in Saint-Petersburg, northwestern Russia.

Type material: Type culture is deposited with the CCAP (Oban, UK), accession number 1583/12.

Etymology: *Aesculea*, from *Aesculus*, indicating the genus of a tree where the strain was found.

Differential diagnosis: Almost identical to *Th. sphaeronucleolus* in the size, ultrastructure of nucleus and cytoplasm, but differs from the former type strain of this species (CCAP 1583/3) in having a broader locomotive form, spatulate or fan-shaped, rather than elongated drop-shaped; also differs in the structure of the glycocalyx. Slightly resembles *Th. similis*, but differs from this species in the structure of the nucleus and glycocalyx.

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