



# *Thecamoeba quadrilineata* (Amoebozoa, Lobosa) as a new member of amphizoic amoebae—first isolation from endozoic conditions

Terézia Borovičková<sup>1</sup> · Martin Mrva<sup>1</sup> · Mária Garajová<sup>1</sup>

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## Abstract

A free-living soil amoeba *Thecamoeba quadrilineata* (Carter, 1856) Lepš, 1960 (Amoebozoa: Thecamoebidae) was isolated from endozoic conditions for the first time. Presence of amoebae was detected after 4 days following inoculation of the gut of the earthworm *Lumbricus terrestris* on agar plate with *Escherichia coli*. On the basis of our isolation, we consider *T. quadrilineata* as further amphizoic amoeba species. This study enlarges the range of amphizoic tendency in members of the genus *Thecamoeba* and stresses the need for further research on the pathogenic potential of *Thecamoeba* species.

**Keywords** Amphizoic tendency · Earthworm · Free-living amoeba · Gut · Thecamoebidae

## Introduction

Amphizoic amoebae are defined as free-living ubiquitous organisms which can occur in environmental as well as in endozoic conditions. Pathogenic strains of free-living amoebae *Acanthamoeba* spp., *Naegleria fowleri* Carter, 1970; *Balamuthia mandrillaris* Visvesvara, Schuster & Martinez, 1993; and *Sappinia pedata* Hartmann & Nägler, 1908 cause serious diseases in humans as well as in animals (Schuster and Visvesvara 2004). Data about isolations of other amphizoic amoebae species from endozoic conditions are not numerous. Strains of *Vermamoeba vermiformis* (Page, 1976) Cavalier-Smith & Smirnov, 2011, *Hartmannella* sp., *Vahlkampfia* sp., and *Vannella* sp. have been isolated from eyes of human keratitis patients (Michel et al. 2000; Lorenzo-Morales et al. 2007; Niyyati et al. 2010). Furthermore, symptomatic and

asymptomatic infections of fish have been documented, e.g., species of the genus *Paramoeba* are causative agents of the Amoebic Gill Disease (AGD) (Dyková and Kostka 2013).

Species of the family Thecamoebidae Schaeffer, 1926 occur in soil, freshwater, and marine environments (Page 1983, 1991; Kudryavtsev and Hausmann 2009). Trophozoites usually graze on bacteria, but many species feed on algae, fungi, or small amoebae (Page 1977). So far, from the genus *Thecamoeba* Fromentel, 1874, 18 species were described; of them, ten are valid (Page 1983, 1991; Kudryavtsev and Hausmann 2009). The species *Thecamoeba quadrilineata* (Carter, 1856) Lepš, 1960 is well known and widely distributed in various types of habitats such as freshwater, soil, moss, and leaf litter (Smirnov and Brown 2004). Recent studies focused on *T. quadrilineata* provide information about presence of endocytobionts and parasites (Scheid 2014). Intranuclear parasites identified as members of the genus *Nucleophaga* belonging to Opisthosporidia clade, can cause rupture and destruction of the invaded cells (Michel 2008; Michel et al. 2012).

Nevertheless, no evidence of endozoic occurrence of *Thecamoeba quadrilineata* was detected up to the present day. In this study, we present the first isolation of *Thecamoeba quadrilineata* from endozoic conditions from the gut of the common earthworm *Lumbricus terrestris* and consider it as an amphizoic amoeba species for the first time.

Terézia Borovičková and Martin Mrva contributed equally to this work.

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✉ Martin Mrva  
martin.mrva@uniba.sk

<sup>1</sup> Department of Zoology, Faculty of Natural Sciences, Comenius University in Bratislava, Mlynská dolina, Ilkovičova 6, 84215 Bratislava 4, Slovak Republic

## Material and methods

### Sample collection and preparation

Amoebae were isolated from the gut of *Lumbricus terrestris* collected in garden soil in Upper Váh region, northwest Slovakia (49°11'30"N, 18°30'17"E). The earthworm was stored in glass vessel with perforated lid and filled with garden soil (brown earth, pH 7) up to  $\frac{3}{4}$  of its volume. The vessel with annelid was deposited in fridge at 6 °C for 3 days, afterward autopsy was performed. The anterior and posterior ends of the earthworm were pinned down on the dorsal side, a cut of abdominal side in medial part from aboral to oral end followed. All the steps were realized in sterile conditions, continual disinfection of dissecting instruments was essential. The seminal receptacles, seminal vesicles, ventriculus, gut, and all its contents were removed, smeared with sterile needle on agar surface, and cultivated.

### Cultivation and microscopy

The organs of *L. terrestris* were inoculated in Petri dishes with 2% non-nutrient agar (NNA) and *Escherichia coli* providing a food source for amoebae. On the top, 50 µl of Modified Neff's amoeba saline (AS) solution was added (Page 1991).

The cultures which were obtained only from the gut were incubated at laboratory temperature and undirected light. The cultures were examined on daily base. Living amoebae found in the cultures were observed and documented with Leica DM2500 light microscope with differential interference contrast (DIC) optics and Canon EOS 70D camera equipment. For characterization and identification, cell shape, number and shape of pseudopodia, hyaloplasm, anterior and posterior ends of cell, type of nucleus, and number and localization of vacuoles were studied (Smirnov and Brown 2004). Length, breadth, L/B ratio, and size of organelles and hyaloplasm were measured in AxioVision Release 4.6.3.0 (Carl Zeiss, Jena, Germany) software. For identification of amoebae, keys and descriptions by Page (1977, 1991) and Smirnov and Brown (2004) were used.

## Results and discussion

Amoebae cultures were obtained only from the gut. The gut showed macroscopically normal morphology, no distinguishable perforation was present. After 4 days of cultivation of the gut, presence of amoebae in agar plate was detected. The monoxenization was not successful and amoebae in mixed cultures with *Acanthamoeba* sp. vanished several weeks after isolation.

Isolated amoebae were identified as *Thecamoeba quadrilineata* (Carter, 1856) Lepš, 1960. The trophozoites

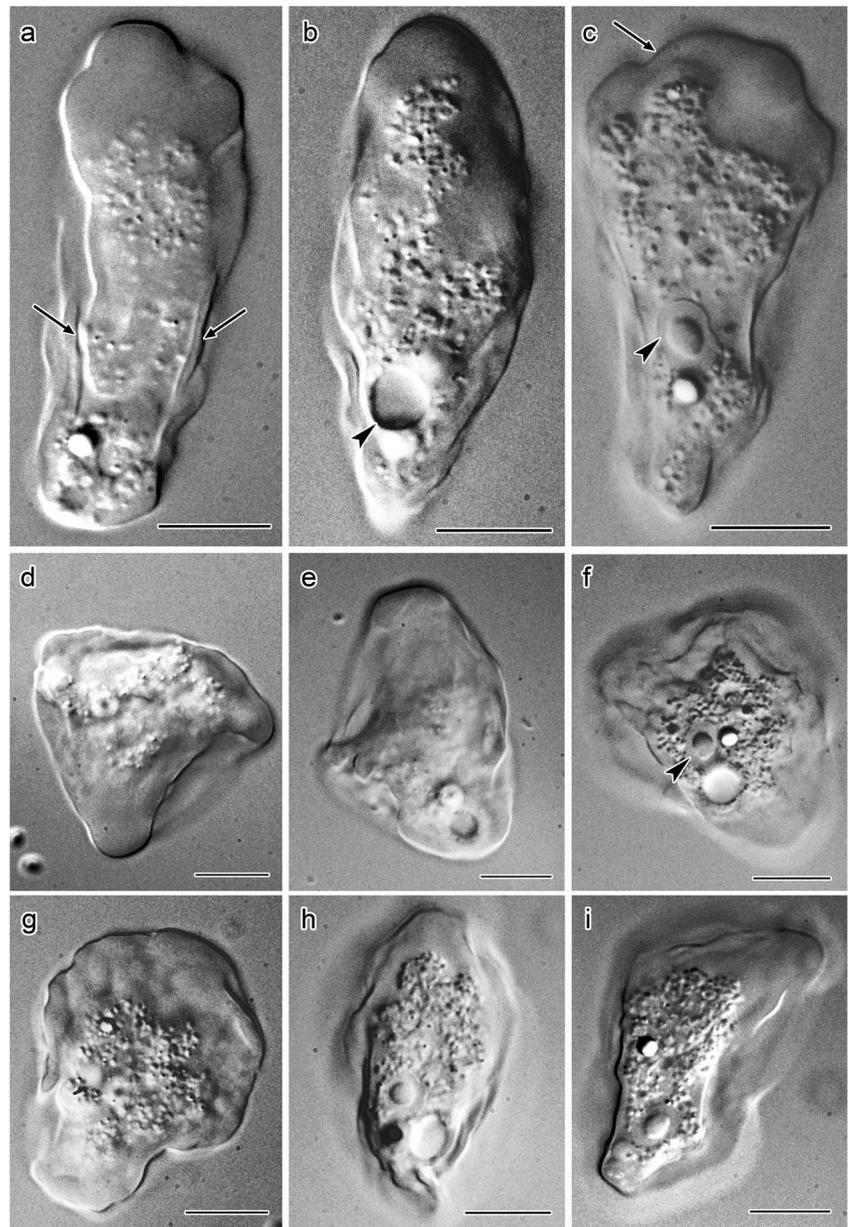
adhered to the cover glass after 35 min in wet chamber. Well adhered but not numerous, mostly smooth, cells showed slow, although steady locomotion. Typical elongated shape (Fig. 1a–c) varied from oval (Fig. 1e) to circular (Fig. 1f) form while changing direction of locomotion. The anterior end of trophozoite sometimes altered from smooth (Fig. 1b) to slightly folded (Fig. 1c). The hyaloplasm, located as anterolateral crescent, varied in size during the movement from 4.7 to 8.0 µm. Subpseudopodia were never present. The posterior end displayed narrower than the anterior end with smooth, round shape, without uroid (Fig. 1a–c). Dorsal parallel longitudinal folds (Fig. 1a) were formed on cell surface, laterally situated. According to morphological features, amoebae belonged to striate morphotype (Smirnov and Brown 2004). The length of trophozoites ranged from 39.2 to 43.5 µm (average 42.0 µm), the breadth from 14.5 to 19.7 µm (average 17.0 µm). L/B ratio was 2.5. The floating forms were not observed.

The single vesicular nucleus was located in the posterior half of the trophozoite (Fig. 1c), its size reached about 5 µm. Central nucleolus was well distinguishable (Fig. 1c, f) with a diameter of about 2.8 µm. A single contractile vacuole was located in the posterior fourth (Fig. 1b), with a maximum size of no more than 5.0 µm. No cytoplasmic inclusions were present. The cysts or indications of encystation were not detected.

There is no doubt that isolated amoebae belong to the genus *Thecamoeba*. Nuclear diversity and surface of cells are characteristic morphological features of *Thecamoeba* identification even to the species level. Therefore, most of them can be well differentiated using light microscopy (Smirnov and Brown 2004). Our isolate of *Thecamoeba quadrilineata* completely corresponds to the striate morphotype. So far, the other six *Thecamoeba* species are listed in striate morphotype of which three are fresh-water amoebae—*Thecamoeba striata* (Penard, 1890) Schaeffer, 1926, *Thecamoeba similis* (Greeff, 1891) Lepš, 1960, and *Thecamoeba sparolata* Fishbeck & Bovee, 1993 (Smirnov and Brown 2004).

Morphology of identified *Thecamoeba quadrilineata* corresponds unambiguously to known descriptions which refer cell surface and margin of trophozoite as smooth, with several anterior parallel folds. Published information noted length from 35 to 100 µm, in giant forms up to 170 µm. L/B ratio is 1–2.5 (mean 1.7). Single nucleus, with one central smooth nucleolus, measures from 7.5 to 11 µm, up to 15 µm in giant forms (Page 1977). Our isolated trophozoites did not reach more than 43.5 µm and L/B ratio came up to 2 but these values remain within the size range of published descriptions. Despite the size of the nucleus reaching not more than 5 µm, this value is close to the stated information by the above-mentioned authors. Thus, the smaller proportions of the nucleus do not question the identification of isolated amoebae. The single species, which appears to be

**Fig. 1** Light microscopy of single trophozoite of *Thecamoeba quadrilineata* during locomotion in vivo, DIC. **a–b** Elongated trophozoite with parallel longitudinal folds (arrows) and single contractile vacuole in posterior end (arrowhead). **c** Elongated trophozoite with slightly folded anterior end (arrow) and well-differentiated vesicular nucleus (arrowhead). **d–g** Trophozoite of irregular or oval shape in slow locomotion without longitudinal folds and with well-differentiated nucleus in **f** (arrowhead). **h–i** Transformation of trophozoite from circular to elongated shape. Scale bar = 10  $\mu$ m



morphologically similar to *T. quadrilineata* is *Thecamoeba aesculea* Kudryavtsev & Hausmann, 2009 isolated from the surface of the bark of the tree *Aesculus hippocastanum*. Despite the similarity of nucleus, much bigger size of the isolate, presence of numerous surface wrinkles, higher number of especially well-pronounced longitudinal folds and presence of uroid in *T. aesculea* (Kudryavtsev and Hausmann 2009) definitely exclude this species.

The number of amoebae in the culture was very scarce and the multiplication was not observed. Although *T. quadrilineata* may be considered as easily culturable species, the viability of our strain was very low which resulted in its loss. Therefore, molecular analysis of our isolate was not performed. The described members of the genus *Thecamoeba* are

relatively well identifiable by using the morphological criteria and ultrastructure. However, the morphological identification in the majority of amoebae species is limited and at present, for the ultimate result, 18S rDNA analysis is needed. Even a morphologically good defined species may represent actually a group of cryptic species.

Data about isolations of free-living amoebae from invertebrates are rare (Nowak et al. 2010) and the presence of amoebae in the digestive tract of earthworms was not studied so far. Only few publications on presence of bacteria in earthworm gut (Singleton et al. 2003) and the effect of digestive tract enzymes on ciliates (Pierce and Phillips 1980) are known. Survival of free-living amoebae in gut of *Lumbricus terrestris* was documented by Rouelle (1983) where these amoebae,

including the genera *Thecamoeba* and *Acanthamoeba*, were artificially injected into the digestive tract. After 3 h, there were no active trophozoites in a gut section, only *Acanthamoeba* cysts were present. In comparison, we detected *Thecamoeba* trophozoites in earthworm gut. Nevertheless, in known *Thecamoeba* species, cysts were not described (Page 1991; Kudryavtsev and Hausmann 2009). We also did not observe cysts or indications of encystation in cultures. Therefore, our cultivated living trophozoites from earthworm gut clearly demonstrate the ability of amoebae to survive in endozoic conditions. Moreover, environment of the earthworm gut was proved to differ from soil considerably, e.g., twofold greater water content in gut, higher nitrogen, and organic carbon contents in gut, higher content of organic compounds such as glucose and maltose in gut, and less variable pH in the gut (Horn et al. 2003). Thus, to the best of our knowledge, we propose *Thecamoeba quadrilineata* as amphizoic amoeba for the first time.

These findings point out considerable tolerance of *T. quadrilineata* to a range of diverse environmental factors and urge to discuss the *Thecamoeba* pathogenic potential. However, there is more to be understood about the ecology and distribution of various thecamoebids as *Thecamoeba* sp. amoebae were isolated also from water-hyacinth root *Eichhornia crassipes* and successfully cultivated at 37 °C (Ramirez et al. 2010). Amoebae, able to survive and reproduce at this temperature, are considered to be thermotolerant which is one of the preconditions for pathogenicity (Schuster and Visvesvara 2004).

There are also evidences of *Lumbricus terrestris* as vectors of *Escherichia coli* and the effect of these annelids on microbial diversity in soil (Williams et al. 2006). Based on the survival of *T. quadrilineata* trophozoites from the gut of the earthworm, we assume that *L. terrestris* could participate also in amoebae distribution in soil.

The single known pathogenic species from the genus *Thecamoeba* is freshwater amoeba *Thecamoeba hoffmani* Sawyer, Hnath & Conrad, 1974 which is insufficiently described and needs a redescription. Damage of the gill tissue by these amoebae caused respiratory impairment and severe mortalities in salmonid fish species *Oncorhynchus mykiss*, *Oncorhynchus kisutch* and *Oncorhynchus tshawytscha* (Sawyer et al. 1974; Hoffman 1999). Furthermore, also free-living marine species *Thecamoeba hilla* Schaeffer, 1926 was isolated from the gills of turbot, *Scophthalmus maximus* (Dyková et al. 2008). However, no symptoms of disease were stated in this case. According to molecular evidence, members of the genus *Thecamoeba* are related to the pathogenic *Acanthamoeba* spp. (Smirnov et al. 2005). Within the family Thecamoebidae, a study by Brown et al. (2007) revealed strong phylogenetic affinity of the genus *Thecamoeba* with pathogenic species *Sappinia pedata* which caused the only one case of *Sappinia*-related granulomatous encephalitis in

man up to the present time (Gelman et al. 2003). These isolations, phylogenetic affinity to the pathogenic *S. pedata* and *Acanthamoeba* spp. and proved thermotolerance of *Thecamoeba* sp., imply pathogenic potential and possibility of further endozoic findings of amphizoic *Thecamoeba* species. The number of infections caused by free-living amoebae, considered among emerging infectious diseases (EID) is still rising and the continuous progress of research in this field can reveal new causative agents.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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