First isolation of *Leptomyxa* (Amoebozoa, Leptomyxida) from endozoic conditions: an interaction between two common soil organisms

M. MRVA ©, T. BOROVIČKOVÁ, & M. GARAJOVÁ ©*

Department of Zoology, Faculty of Natural Sciences, Comenius University in Bratislava, Bratislava, Slovak Republic

(Received 29 June 2022; accepted 31 August 2022)

Abstract
We report the first isolation of a widely distributed free-living soil amoeba *Leptomyxa* (Amoebozoa, Leptomyxida) from endozoic conditions. The amoebae were detected after 14 days following inoculation of the intestine of the earthworm *Lumbricus terrestris* on an agar plate with *Escherichia coli*. The earthworm was collected from Calcaric Fluvisol (pH 7) in the Upper Váh region, north-west Slovakia. Observed amoebae were uninucleate, polypodial with typically branched pseudopodia, and morphologically resembled *Leptomyxa australiensis*. This study enlarges the range of amphizoic tendency in leptomyxid amoebae and indicates their interactions with Oligochaeta.

Keywords: Endozoic conditions, earthworm, amphizoic free-living amoeba, intestine, Leptomyxida

Introduction
Free-living amoebae (FLA) able to reproduce and occur in environmental in addition to endozoic conditions are termed amphizoic amoebae. Some species of FLA, such as *Acanthamoeba* spp., *Balamuthia mandrillaris*, *Neagleria fowleri* and *Sappinia pedata*, have caused numerous cases of severe infections in humans and in animals, frequently with fatal consequences (e.g. Foreman et al. 2004; Schuster & Visvesvara 2004; Qvarnstrom et al. 2009; Moussa et al. 2015; Siddiqui et al. 2016; Cope et al. 2020). Information about the isolation of other freshwater and soil amoebae from endozoic conditions is not so extensive, but their pathogenic potential was proved by findings of symptomatic and asymptomatic infections in animals, particularly in fishes (Dyková et al. 1997, 2005).

A recent comprehensive phylogenetic and morphological analysis of the order Leptomyxida Pussard and Pons, 1976 has led to the reclassification of several species along with the description of new species of the genera *Leptomyxa* Goodey, 1915; *Rhizamoeba* Page, 1972; and *Flabellula* Schaeffer, 1926 (Smirnov et al. 2017). At present, the family Leptomyxidae Pussard and Pons, 1976 includes 10 valid species of the genus *Leptomyxa* Goodey, 1915 (Siemensma 1987; Del Valle et al. 2017; Smirnov et al. 2017; Smirnov 2018; Glotova et al. 2021), which have been isolated from river and lake sediments (Mrva 2003; Smirnov et al. 2009), moss (Matis & Mrva 1998), leaf litter (Mrva & Matis 2000), permafrost-affected soils (Shmakova et al. 2013), and plant roots (Ramirez et al. 2010; Smirnov et al. 2017). Species of this genus usually feed on bacteria, but fungi, yeasts and small amoebae have also been observed to be a food source (Del Valle et al. 2017; Smirnov et al. 2017).

Although it is known that FLA are an important part of the microbial community of intestinal contents in earthworms (Monroy et al. 2008) and may play a significant role in their nutrition (Bonkowski & Schaefer 1997), the data on the presence of FLA in internal organs of earthworms is scarce. Some experiments with artificial introduction of amoebae

*Correspondence: M. Garajová, Department of Zoology, Faculty of Natural Sciences, Comenius University in Bratislava, Mlynská dolina, Ilkovičova 6, Bratislava 842 15, Slovak Republic. Email: maria.garajova@uniba.sk

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into the digestive tract of *Lumbricus terrestris* and *Eisenia fetida* have been performed (Rouelle 1983). However, only a single record of a free-living amoeba, *Thecamoeba quadrilineata*, isolated from an earthworm intestine was reported (Borovičková et al. 2019).

In this study, we present the first isolation of amoebae of the genus *Leptomyxa* from the intestine of the common earthworm *Lumbricus terrestris* and, at the same time, the first isolation of this genus from endozoic conditions.

**Materials and methods**

Individuals of *Lumbricus terrestris* Linnaeus, 1758 were examined for the presence of amoebae in their internal organs. Ten specimens of the earthworm were collected from Calcaric Fluvisol (Šály 2000) (pH 7) in the Upper Váh region, north-west Slovakia (49°11′30″N, 18°30′17″E) and stored in a glass vessel filled with the soil (up to ¾ of the volume), in a refrigerator at 6°C. The day after collection, an autopsy in sterile conditions with repeatedly sterilised dissecting instruments was performed. Before the autopsy, the surface of each annelid was sterilised with ethanol. The anterior and posterior end of each annelid was pinned in a dissecting dish; a cut into the medial part of the ventral side from posterior to anterior end followed. The seminal vesicles, intestine and all its contents were removed, smeared with a sterile needle on an agar surface and cultivated.

The organs of *L. terrestris* were inoculated separately in Petri dishes with 2% non-nutrient agar (NNA) and *Escherichia coli* providing a food source for amoebae. After this, 50 µL of Modified Neff’s amoeba saline (AS) solution was added (Page 1991). The cultures were obtained only from the intestine and were incubated at laboratory room temperature (22°C) and under undirected light.

The cultures were examined on a daily basis. Observation and documentation of isolated amoebae were performed with a Leica DM 2500 light microscope and Canon EOS 70D camera equipment. Morphology and locomotion of trophozoites were noted using the method of hanging drops, which provided appropriate conditions for amoeba movement (Page 1991). Cell shape, anterior and posterior end of trophozoites, shape and number of pseudopodia, hyaloplasm, type of nucleus and localisation and number of vacuoles were noted. The length (L), breadth (B), L/B ratio and size of hyaloplasm and organelles were measured in AxioVision Release 4.6.3.0 (Carl Zeiss, Jena, Germany) software. For identification, the works of Page (1983, 1991), Smirnov and Goodkov (1999), Smirnov and Brown (2004), Smirnov et al. (2009, 2011, 2017), Del Valle et al. (2017), Smirnov (2018) were used.

**Results and discussion**

A leptomyxid amoeba culture was obtained from a single specimen of *L. terrestris*, from its intestine which showed normal anatomy. The amoebae were detected on an agar surface after 14 days of cultivation. Initial mixed cultures of leptomyxid amoebae and acanthamoebae were massively contaminated by unidentified hyphae of moulds originating from the earthworm intestinal contents. Therefore, attempts to purify and isolate the amoebae were performed. However, the establishment of monoxenic cultures was not successful. The moulds persisted and successively destroyed the cultures after several days in each established subculture.

We identified isolated amoebae as members of the genus *Leptomyxa* Goodey, 1915. The amoebae were polypodial, with branch-extended pseudopodia and slow locomotion (Figure 1a–c), or formed an expanded, flattened irregular form (Figure 1d). Monopodial forms were not observed. The length of the cells ranged from 47.0 to 80.2 µm (average 65.4 µm), the breadth from 5.9 to 63.0 µm (average 39.0 µm). The pseudopodia frequently branched on their anterior end and individual branches extended from 12.5 to 29.7 µm. The shape of pseudopodia varied from expanded and shorter with almost digitiform outline, to narrow and long which formed a branched trophozoite shape (Figure 1a–c). Pseudopodia never formed anastomoses. A hyaline zone was situated on their rounded apical end, with maximum size of 3.9 µm. The posterior end of the cells formed a mass from which pseudopodia radiated. Although no uroidal filaments were noted, the uroidal part of the cells was occasionally covered with attached debris. During locomotion, not all the pseudopodia adhered well to the glass surface; some protruded towards the water column and adhered afterwards. Adhesion of trophozoites on a cover glass was observed after 90 minutes in a wet chamber.

Several small contractile vacuoles, which did not exceed 2.5 µm, were located in various parts of the cell cytoplasm (Figure 1a, b). The size of the single vesicular nucleus reached about 3.2 µm (Figure 1b, c). Multinucleate cells were not detected. No cytoplasmic inclusions were present. Typical cysts belonging to the species of the genus *Leptomyxa* were not observed. Monoxenisation and purification of mixed cultures was not successful due to the high abundance of acanthamoebae.
On the basis of the overall morphology, and in comparison with the data in publications on leptomyxid amoebae, the amoebae were identified as *Leptomyxa* sp. Observed trophozoites were polypodial with typically branched pseudopodia, or formed an expanded, flattened irregular form. Monopodial forms were not observed. Generally, species of the genus *Leptomyxa* form polypodial, branched to monopodial cells (Smirnov et al. 2017) and therefore they are classified as branched and temporarily also to the monotactic morphotype (Smirnov & Brown 2004). The morphology of the present amoebae clearly differed from that of polypodial species from the order Euamoebida including the genera *Amoeba*, *Chaos*, *Polychaos*, *Parachaos* and *Deuteramoeba* which bear several cylindrical or subcylindrical pseudopodia that never take on an expanded or flattened irregular form (Smirnov et al. 2011). Therefore, these genera were excluded from the identification.

As the cytoplasm of the present amoebae contained a single nucleus, exclusively multinucleate *Leptomyxa* species can be excluded from the identification. From the other described species, the present amoeba is morphologically most similar to the soil species *Leptomyxa australiensis*. According to published descriptions, this species reaches 30–180 µm in size and more than 95% of the cells are uninucleate (Page 1991; Matis & Mrva 1998). The size of the present amoebae cultivated from the intestine of the earthworm was within the size range of *L. australiensis*. Similarity was noted also in the shape of the polypodial or flattened irregular forms. However, a detailed comparison revealed several differences. In our trophozoites we did not observe the formation of a monopodial form, which is typical for *L. australiensis*, and also the adhesive uroidal filaments were not detected. However, this may also be the result of poor adherence of the cells to the glass surface.

Among other uninucleate *Leptomyxa* species the present amoeba resembles, to a certain extent, three other species that take on flattened and/or polypodial forms. *Leptomyxa neglecta* (Smirnov et al. 2009) differs in the polypodial flattened form, which possesses distinctly shorter pseudopodia that narrow towards the tips and consist mostly of the hyaloplasms. The polypodial form of the species *L.*
valladaresi, depicted in micrographs and a video sequence by Del Valle et al. (2017), differs in having a lower number of pseudopodia which are also shorter than in the present amoeba. Moreover, both of the abovementioned species are typically found with monopodial limax-shaped locomotive forms, which are rather frequent during the locomotion. In this sense we can almost certainly exclude the species L. ambiguа due to its dominant monopodial locomotive form (Smirnov 2018).

Findings of amoebae in invertebrates are not numerous. The presence of amoebae has been reported in crustaceans (Mullen et al. 2005; Nowak et al. 2010) and sea urchins (Dyková et al. 2007) using molecular methods. Patsyuk (2017) isolated FLA from molluscs. Moreover, Mortazavi et al. (2010) experimentally infected locust Locusta migratoria with Acanthamoeba spp. and observed the death of individuals up to 17 days after infection. Amoebae were present in the muscles, brain lesions, fat tissue and hemolymph of locusts.

The intestine of the earthworms is well studied, and conditions such as water content, organic compounds and pH were proved to change and to be different in comparison with the soil (Horn et al. 2003). Information has been published about bacteria (Singleton et al. 2003; Meier et al. 2018) and the effect of digestive enzymes on ciliates (Piearce & Phillips 1980). There is evidence that the transit through the intestine of earthworms could have a positive effect on the density of FLA (Monroy et al. 2008). However, except for a single record of Thecamoeba quadrilineata (Borovičková et al. 2019), amoebae in the digestive tract of Lumbricus terrestris have not been studied so far. Experimental inoculation of trophozoites of Acanthamoeba, Saccamoeba and Thecamoeba into Lumbricus terrestris and Eisenia fetida intestine was performed, but intestine section revealed only Acanthamoeba cysts three hours after inoculation (Rouelle 1983).

In Figure 2 we summarise the present knowledge about a relatively broad spectrum of habitats from

![Figure 2. Known habitats of Leptomyxa spp. The life cycle of Leptomyxa spp. illustrated in the centre of the figure comprises trophozoite and cyst stages.](image-url)
which *Leptomyxa* amoebae were isolated. Although *Leptomyxa* amoebae have been isolated from natural habitats such as cold permafrost-affected soils (Shmakova et al. 2013), the survival of these amoebae in diverse environments and at various temperature levels is not well clarified. Ramirez et al. (2010) successfully tested the survival of *Leptomyxa* sp. isolated from the root of water hyacinth *Eichhornia crassipes* (now *Pontederia crassipes*), even at 30°C. Although a wide range of thermotolerance is considered an important precondition for pathogenicity potential, the pathogenicity of *Leptomyxa* spp. has not been studied yet and none of the described species has been detected as a pathogen. However, within the order Leptomyxida, the genus *Leptomyxa* is related to the genus *Flabellula* Schaeffer, 1926, also detected from soil habitats (Esteban et al. 2006). Some strains of *Flabellula* have also been isolated from fish tissues (Dyková et al. 2008). At present we may assume that although species of the genus *Leptomyxa* do not represent a risk for man or homeothermic animals, the poikilothermic vertebrates and invertebrates may host them under certain conditions. The finding of *Leptomyxa* sp. in the earthworm intestine opens further questions. The majority of *Leptomyxa* species form cysts and we can also suppose these form in the present species; however, in this case they were not detected. Detection of *Leptomyxa* sp. in the earthworm gut may be explained not only by the ability of trophozoites to survive in endozoic conditions, but also by a passage of amoeba cysts through the earthworm intestine.

In conclusion, the present work enlarges our knowledge on the habitat spectrum of leptomyxid amoebae. Furthermore, it shows that an amphizooic tendency in FLA is common and we can expect further species isolated from endozoic conditions from various invertebrate and vertebrate taxa. Considering the available information, this is the first isolation of *Leptomyxa* sp. from the intestine of *Lambicus terrestris* and also the first isolation of amoebae of the genus *Leptomyxa* from endozoic conditions.

**Funding**

This work was supported by the Slovak Research and Development Agency under contract nos. APVV-15-0123 and APVV-19-0056; and by the Ministry of Education, Science, Research and Sport of the Slovak Republic agency Vedecká Grantová Agentúra MŠVVaŠ SR a SAV under grant VEGA 1/0389/19.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

**ORCID**

M. Mrva [http://orcid.org/0000-0003-4993-2549](http://orcid.org/0000-0003-4993-2549)

M. Garajová [http://orcid.org/0000-0003-3043-3096](http://orcid.org/0000-0003-3043-3096)

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