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ORIGINAL ARTICLE

Mycamoeba gemmipara nov. gen., nov. sp., the First Cultured Member of the Environmental Dermamoebidae Clade LKM74 and its Unusual Life Cycle

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Keywords
Budding; Discosea; eukaryotic diversity; fungus; high throughput sequencing; Longamoebia; ribosomal genes; serial dilution; yeast.

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ABSTRACT

Since the first environmental DNA surveys, entire groups of sequences called "environmental clades" did not have any cultured representative. LKM74 is an amoebozoan clade affiliated to Dermamoebidae, whose presence is pervasively reported in soil and freshwater. We obtained an isolate from soil that we assigned to LKM74 by molecular phylogeny, close related to freshwater clones. We described *Mycamoeba gemmipara* based on observations made with light- and transmission electron microscopy. It is an extremely small amoeba with typical lingulate shape. Unlike other Dermamoebidae, it lacked ornamentation on its cell membrane, and condensed chromatin formed characteristic patterns in the nucleus. *M. gemmipara* displayed a unique life cycle: trophozoites formed walled coccoid stages which grew through successive buddings and developed into branched structures holding cysts. These structures, measuring hundreds of micrometres, are built as the exclusive product of osmotrophic feeding. To demonstrate that *M. gemmipara* is a genuine soil inhabitant, we screened its presence in an environmental soil DNA diversity survey performed on an experimental setup where pig cadavers were left to decompose in soils to follow changes in eukaryotic communities. *Mycamoeba gemmipara* was present in all samples, although related reads were uncommon underneath the cadaver.

OUR vision of protist diversity has been radically challenged since the introduction of observation-independent environmental DNA surveys. Large and deep-branching groups of eukaryotes have been discovered with classical cloning/sequencing strategies, thus overtaking our estimations on eukaryotic environmental diversity. Unsuspected alveolate clades appeared to be extremely diverse in marine systems (López-García et al. 2001), and were found later to be exclusively composed by parasitoids (Guillou et al. 2008). Likewise, many new stramenopile lineages were discovered in the early 2000s in marine systems (Massana et al. 2004). These organisms, which include nowadays 25 different lineages spread all across the tree of stramenopiles, were found to be the most diverse and numerous bacterivores in the sunlit part of oceans (Massana et al. 2014), and thus vesting them with a prominent role in the oceanic microbial loop (Azam et al. 1983). In deeper waters, a particular group of excavates, the diplonemids revealed an immense diversity (Lara et al. 2009; Lukes et al. 2015). New massive sequencing technologies also revealed a large diversity in opisthokonts (del Campo et al. 2015).

Other systems like soils, however, have been by far not as deeply studied as the ocean. The high prevalence of fungal, plant, and metazoan sequences has been for a long time a major hindrance for studies on soil protist diversity (Lesaulnier et al. 2008). Still, previously unsuspected deep branching clades have also been discovered in soils, like the Opisthosporidia (also known as Rozellomycota) (Karpov et al. 2013; Lara et al. 2010). Recently, the development of high throughput sequencing has allowed obtaining high numbers of phylotypes, showing

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promising results in terms of overall microeukaryotic diversity (Geisen et al. 2015). Relationships among organisms have been inferred using sound experimental designs and approaches such as co-occurrence networks (Lentendu et al. 2014). However, strong conclusions on the organisms' morphology and function can only be provided by direct observation or, even better, culturing. Therefore, a current challenge in eukaryotic microbiology is to identify the organisms hiding behind these environmental clades and to infer their ecological function. For this purpose several approaches have been used. Fluorescence in situ hybridization (FISH) in combination with scanning electron microscopy has been recently applied to the characterization of Paulinellida (Euglyphida testate amoebae) living in forest litter (Tarnawski and Lara 2015). Bulk soil protists remain, however, widely inaccessible to FISH probes because of the large amount of soil particles unless organisms are large enough to be isolated individually (e.g. ciliates, macroscopic mycetozoa, and testate amoebae). Typically, naked amoebozoans are numerous in soils (Geisen et al. 2015), and harbour a wide array of lifestyles and morphologies (Shadwick et al. 2009).

The amoebozoan environmental clade LKM74, named after the first clone encountered in an environmental DNA survey (van Hannen et al. 1999) is quite abundant and well distributed in soils (Corsaro and Venditti 2013), but also present in freshwaters (Di Filippo et al. 2015; Richards et al. 2005) and peat bogs (Lara et al. 2011). This clade has been repeatedly placed in the vicinity of *Dermamoeba algensis* in small subunit ribosomal gene trees (18S rRNA) (Corsaro and Venditti 2013; Kudryavtsev and Pawlowski 2015), although this relationship remained weakly supported. Despite its pervasive presence in many environments, the organisms have never been kept in culture and their morphology remains unknown. In this study, we describe a tiny naked amoeba isolated from the bulk soil of a coniferous forest by serial dilutions. We affiliated it to LKM74 based on 18S rRNA gene sequences, characterized its complex life cycle and feeding strategy, and documented its ultrastructure. Furthermore, we demonstrated that this species is a typical soil inhabitant by following related sequence reads in an environmental eukaryotic DNA survey of soils. This study was conducted in an experimental setup where pig cadavers were left to decompose and samples were taken at regular intervals to follow modifications of the microbial eukaryotic communities in the underlying soil during the process of decay.

MATERIALS AND METHODS

Sample collection and identification

The original sample from which the species was described has been taken from a coniferous forest (dominated by *Picea abies*) near Neuchâtel, Switzerland (47°00.853′N; 6°55.959′E) in August 2011. Soil was suspended into phosphate buffer, and serially diluted into a low nutrient medium in 96 well plates as described in (Lara et al. 2007). Active amoebae were transferred into culture flasks containing Page's Amoeba Saline medium amended with 1 g per 100 ml of Tryptone Soy Broth and *Escherichia coli* as food organism. Amoebae were subcultured regularly to obtain pure, monoprotistan strains and to lower the proportion of environmental bacteria. Cultures were kept at 12 °C.

Cultures were observed using Utermöhl's plankton chambers with an inverted microscope (Olympus IX81). Cells were measured at different life stages, which were morphologically documented and photographed with light microscopy. Different life stages were also documented using Methyl blue ($C_{37}H_{27}N_3Na_2O_9S_3$) to stain cell walls and condensed cytoplasm.

We also used a full flask containing active and coccoid life stages for transmission electron microscopy (TEM). In that purpose, we pelleted cells from a thriving culture. Fixation, staining, and mounting were achieved as described in (Lara et al. 2006). Observations were made on a Philips CM 100 transmission electron microscope.

Molecular analyses

In addition, a flask containing 10 ml of a thriving culture was used for DNA extraction. Cells were removed from the flask bottom with a cell scraper, and the resulting supernatant was placed into a Falcon tube and centrifuged at maximum speed during 20 min. The obtained pellet was placed into 200 µl of Guanidine thiocyanate buffer and nucleic acids were extracted following a protocol (Chomczynski and Sacchi 1987) adapted after (Lara et al. 2007). We amplified the 18S rRNA gene using primers EK 82F (GAAACTGCGAATGGCTC) and EK 1498R (CACCTACGGAAACCTTGTTA) in a total volume of 30 µl with an amplification profile consisting of 4 min at 95 °C followed by 35 cycles of 30 s at 94 °C, 30 s at 55 °C, and 1 min 30 s at 72 °C with a final elongation of 10 min at 72 °C. Sequencing was carried out using a BigDyeTM Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems, Genève, Switzerland) and analysed with a ABI-3130XL DNA sequencer ABI PRISM 3700 DNA Analyzer (PE Biosystems). The sequence was deposited in GenBank with the following accession number: KX687875.

Phylogenetic analysis

The obtained sequence was placed in an alignment containing various sequences from Discosea (with an emphasis on Longamoebia as defined in Smirnov et al. 2011b) derived either from isolated cells or cultures, or from environmental clone sequences. The root was placed on Vannellida. The alignment is available from the authors upon request. We build a maximum likelihood phylogenetic tree using the RAxML algorithm (Stamatakis et al. 2008) as implemented on the web server "http://embnet.vital-it.ch/raxml-bb", and evaluated the robustness of the nodes by bootstrapping.

Screening of high throughput sequencing data

The experimental setup from which the soil samples were retrieved has already been published in (Szelecz et al.

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2014). Briefly, three pig (*Sus scrofa*) carcasses were left to decompose during 1,051 d in a forest and soil was sampled at different time steps (21 samples in total). Three control soils (without pig) situated at a few meters of distance were sampled at the same time (24 samples). This experiment was performed in the context of a forensic study aiming at improving the estimation of the post mortem interval based on the observation of soil eukaryotic communities. DNA extraction, PCR of environmental DNA and sequencing of the V9 region of the 18S rRNA gene were performed as in (Seppey et al. 2016). The obtained reads were pretreated as in (de Vargas et al. 2015). The environmental sequences were aligned against a database constructed from publicly available V9 sequences of clones related to *Mycamoeba gemmipara* as determined with BLAST (GenBank: AY919786.1, AY919722.1, GQ861575.1, GQ861565.1, GQ861560.1), plus the sequence derived from the *M. gemmipara* culture. We determined empirically a threshold based on e-values to assess if a given environmental read belongs to the *M. gemmipara* group. The selected reads were then clustered into phylotypes using the SWARM v: 1.2.5 clustering algorithm (Mahé et al. 2014). To determine if the abundance of reads related to *Mycamoeba* changed significantly between control and cadaver plots, we performed Wilcoxon test on the data. All statistical analysis was done with R (R Development Core Team 2011).

RESULTS

Light/electron microscopy observations

Active trophozoites were extremely small (maximum 7 μm in length when moving towards a single direction, and 2 μm width), had a flattened shape and lobose pseudopodia. When moving in a single direction, trophozoites had the typical lingulate shape observed in other Dermamoebidae (Fig. 1A), and conical-shaped pseudopodia can be observed when the amoeba changes direction (Fig. 1B). The stationary shape (Fig. 1C) showed small pseudopods radiating in several directions. The hyaline zone seemed to be generally only restricted on the extensive pseudopodia and was not visible laterally. No uroid or surface ridges were visible.

The life cycle of *M. gemmipara* comprised four stages (Fig. 2). (i) Active trophozoites were observed moving and feeding on *E. coli*. At this stage, no cell division could be observed. (ii) Cells then became rounded-elongated and smooth (coccoid phase), and stopped moving and feeding (Fig. 2B). Cytoplasm became highly condensed, and a cell wall was built. (iii) After about 24 h, the coccoid cells started budding several times successively, in a modular growth manner (Fig. 2C, 3A). The resulting structures grew, reaching up to 200 μm or more in length (Fig. 2C'). These structures in which cells cling together in chains correspond to a pseudomycelium as it has been described in several yeasts and bacteria. Old modules were devoid of cytoplasm, which probably migrated towards newly developed structures (Fig. 3B); still, cell walls without

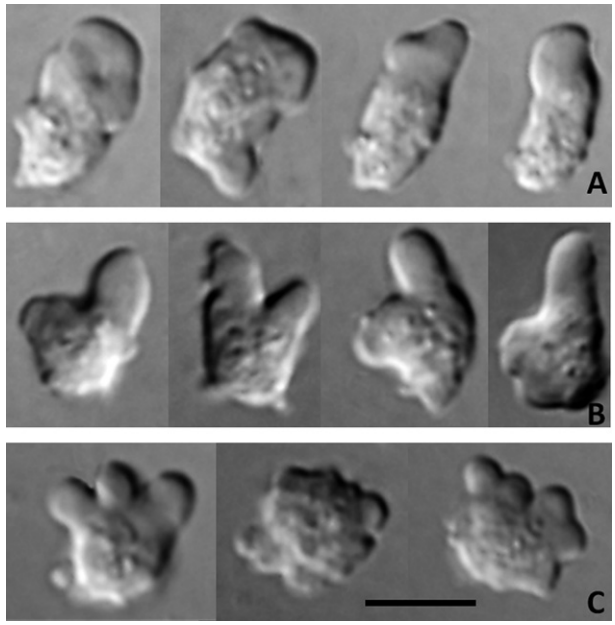

Figure 1 Different morphologies of active trophozoites. (**A**) Trophozoites showing a typical lingulate shape. (**B**) Trophozoites when changing direction. (**C**) Stationary form. Scale bar: A–C = 5 μm.

cytoplasm remained up to several months. Spherical dispersal cysts (Fig. 2D, 3B), were formed at the tip of the branched structures, the latter being finally degraded (iv). Dispersal cysts could then germinate into active amoebae immediately, but will do so most often when fresh medium is provided, thus closing the cycle. A movie (Movie S1) where the first steps of budding in coccoid cells can be observed is available on the journal website.

Transmission electron microscopy pictures did not show any ornamentation on the cellular membrane (Fig. 4). Chromatin in the nucleus appeared inhomogeneous and presented several conspicuous zones with higher density within the nucleus. These zones were situated against the nuclear membrane, as well as in the centre of the nucleus, and a large nucleolus could be observed. Mitochondrial cristae were tubular. Several phagocytosis vacuoles could be seen containing bacteria at different stages of digestion. Small vesicles, probably containing digestive enzymes could be seen surrounding the bacterial cells.

Molecular phylogeny

Mycamoeba gemmipara branched within a group that comprised the original LKM74 environmental clone plus several other clones and unidentified isolates (Fig. 5). This clade, named genus *Mycamoeba* hereafter, received a moderate support (bootstrap value (BV) = 65). Furthermore, it branched robustly (BV = 87) at the base of a clade comprising only freshwater forms, the B1 clade sensu Corsaro and Venditti (2013). The monophyly of family Dermamoebidae was recovered with high statistical support (BV = 92), including genera *Dermamoeba*, *Paradermamoeba* and, now, *Mycamoeba*.

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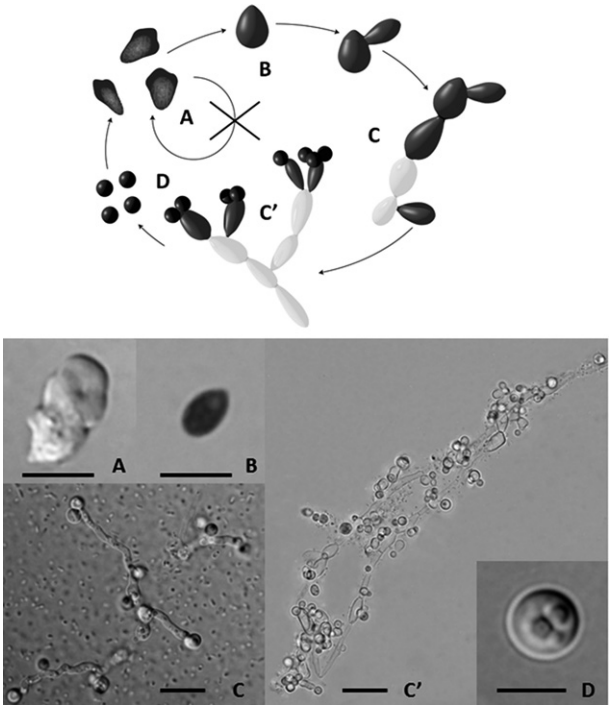

Figure 2 Life cycle of *Mycamoeba gemmipara* including its four developmental stages. (**A**) Trophozoites. (**B**) Coccoid stage, stained with methyl blue. (**C–C'**) bud like ramification. (**D**) Cysts released from branches of the pseudomycelium ramification. Scale bar: A–B, D = 5 μm, C–C' = 10 μm.

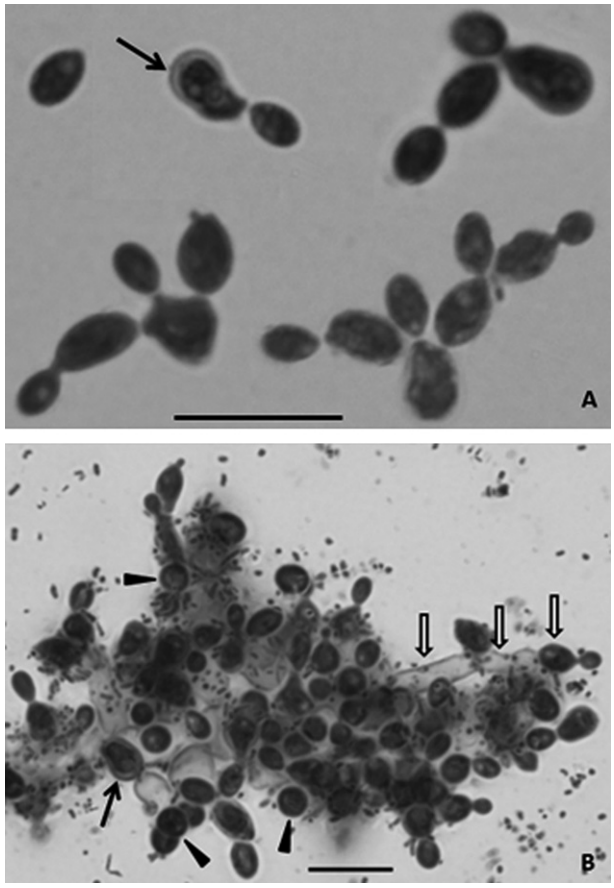

Figure 3 Development of the coccoid cells. (**A**) Budding of coccoid (ovoid shaped) cells. Black arrow indicated cell wall. (**B**) Mature fructifications. Black triangles indicate mature cysts (spherical shape); a young coccoid cell filled with cytoplasm is indicated with a black arrow; old (empty) coccoid cells are shown with white arrows. Scale bar: A–B = 10 μm. All structures have been stained with methyl blue coloration.

Presence of *Mycamoeba* reads in environmental DNA surveys

Our screen through the Illumina reads obtained from the soil from the forensic experiment allowed assigning 7,482 sequences to genus *Mycamoeba* (using an e-value alignment threshold below $1e^{-45}$) out of 25,579,257 environmental sequences, thus representing a total of 0.03% of all reads. These reads were present in all the 45 samples. The clustering of the environmental sequences resulted in a single Operational taxonomic unit (OTU) corresponding at 100% of identity with the original sequences of *M. gemmipara*. Figure 6 shows the sequences abundances distributions according to the control group and pig treatment. Number of reads is significantly lower in the pig treatment samples than in the control group (Wilcoxon test: p-value < 0.05).

DISCUSSION

Mycamoeba gemmipara is the only described representative of environmental clade LKM74. Its size, clearly below 10 μm, places it among the smallest known amoebozoans. *Parvamoeba rugata* (Rogerson 1993) *Paravanella minima* (Kudryavtsev 2014) and *Sapocribrum chiconteaguense* (Lahr et al. 2015) may be shorter in average but the thinner and flattened (lingulated) shape of *M. gemmipara* gives it a smaller biovolume. Coccoid forms measure a little above 3 μm. The size of *M. gemmipara* and its inconspicuous aspect, plus the fact that the amoeboid stage lasts a relatively short amount of time is probably the reason why it had never been detected previously. This small size may be characteristic of the whole LKM74 clade, as the isolates observed by Corsaro and Venditti (2013) were also smaller than 10 μm. Recently, there has been an increasing number of descriptions of nano-sized Amoebozoa, summarized in (Kudryavtsev and Pawlowski 2015). As their 18S rRNA genes are often fast-evolving like in many other Amoebozoa, they remain undetected in DNA-based environmental diversity surveys, either because "general eukaryotic" primers fail in amplifying their 18S rRNA, or because their sequences cannot be assigned with confidence to a given group of eukaryotes. In that sense, application of systematic cultural approaches to soils and sediments are promising in revealing new lineages of Amoebozoa.

Our DNA survey demonstrated that *M. gemmipara* was a genuine forest soil inhabitant, as it has been found in all

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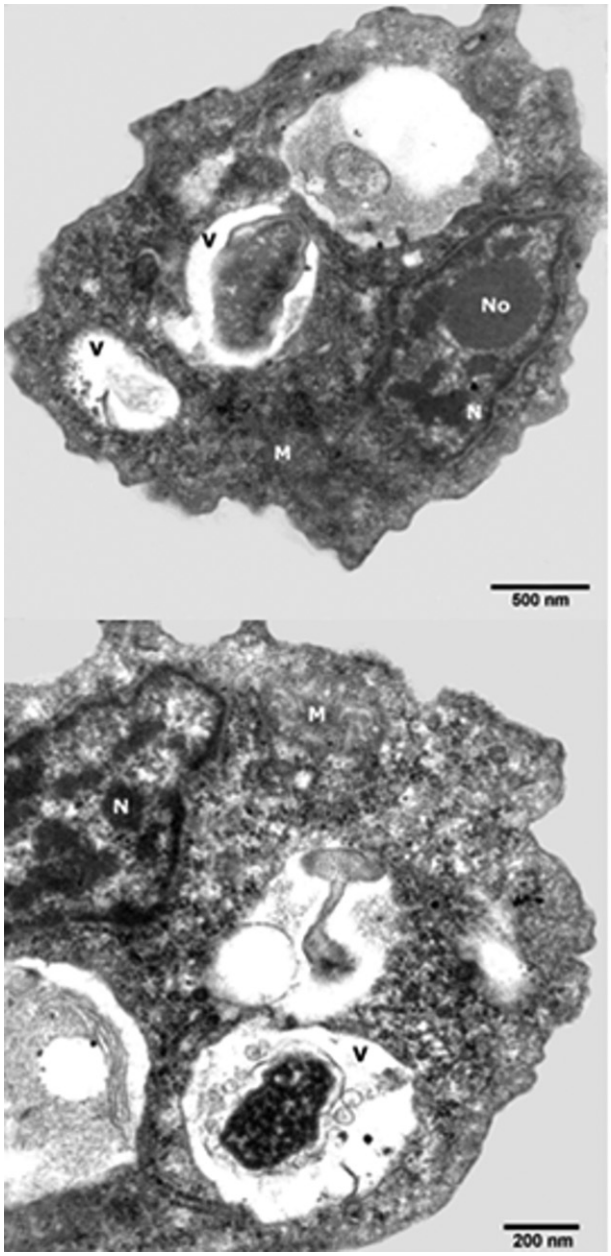

Figure 4 TEM sections of *Mycamoeba gemmipara* showing: "M" the tubulicristate mitochondria; "N" the nucleus with the particular arrangement of the chromatin and "No" the nucleolus. Phagocytosed bacterial cells are visible in the vacuoles "V", where lysosomes can be noticed in the process of releasing digestive enzymes.

samples taken in the control group and under the pig carcasses. The site where *M. gemmipara* was isolated was located a few kilometres away from the experimental setup (Szelecz et al. 2014), and we can therefore suppose that this amoeba is widespread and probably abundant in the forests surrounding Neuchâtel (Switzerland). Nevertheless, it branched at the base of a clade comprising exclusively species from aquatic environments (Corsaro and Venditti 2013). Other fruiting amoebae, such as mycetozoa and "protosteliales" were also recovered from aquatic environments, where trophozoites are probably active (Lindley et al. 2007). Therefore, it cannot be excluded that *M. gemmipara* lives in both freshwater sediments and soils. Its ability to build bud like ramifications in a liquid medium differs from most fruiting amoebae, although instances of myxogastrids completing their whole life underwater have been reported (Gottsberger and Nannenga-Bremekamp 1971; Kappel and Anken 1992). Moreover, this organism could be used as an indicator of certain environmental perturbation as it responded significantly to the effect of the cadaver decomposition (release of high concentration of nutrient, anoxic conditions; Fig. 6).

Order Dermamoebida has been first suggested by Cavalier Smith et al. (2004). It hosted a single family, Thecamoebidae, with genera *Thecamoeba* and *Dermamoeba*. Further analyses based on 18S rRNA gene sequences suggested a separated grouping for *Thecamoeba* (Kudryavtsev et al. 2005), which formed the order Thecamoebida including also genera *Sappinia* (Brown et al. 2007), *Stenamoeba* (Smirnov et al. 2007), and *Vermistella* (Tekle et al. 2016). A new, emended order Dermamoebida comprised genera *Dermamoeba* and *Paradermamoeba* (Dermamoebidae), and *Mayorella* (Mayorellidae). To our knowledge, the monophyly of order Dermamoebida has never been recovered using 18S rRNA genes, as the grouping of genus *Mayorella* with Dermamoebidae remained either weakly supported (Pawlowski and Burki 2009) or not at all (Corsaro and Venditti 2013). Its inclusion within order Dermamoebida has been suggested on the base of the presence of a cell coat without wrinkles (Smirnov et al. 2011b); however, a recent multigene tree analysis suggested that *Mayorella* was rather related to the mainly marine amoebozoan clade Dactylopodida (Tekle et al. 2016).

The general lingulate shape of the locomotive form and the conical pseudopodia of *M. gemmipara* are typically found in all other members of Dermamoebidae (Kudryavtsev et al. 2011). Chromatin patterns in the nucleus of *M. gemmipara* are very distinctive, makes condensed granules all through the nucleus and especially near the borders of the nuclear membrane. All other known Dermamoebidae possess homogeneous chromatin in their nucleus with the exception of a large nucleolus. This includes *D. algensis* (Smirnov et al. 2011a) and *Paradermamoeba* (Smirnov and Goodkov 2004). Genera placed within Dermamoebidae are characterized by a conspicuous cell coat. *Dermamoeba* and *Paradermamoeba* have a thick, highly structured cell coat (Smirnov et al. 2011a), either cuticle-like or consisting analogous glycostyle-like structures. In contrast, *M. gemmipara* does not possess any structure around its cell membrane, at least during its trophozoite life stage.

The peculiar life cycle of *M. gemmipara* has no known equivalents in Amoebozoa. While trophozoites actively ingest bacteria (as shown in TEM images; Fig. 4), the cell walls that are formed around coccoid cells preclude any phagocytosis. Nevertheless, the organisms undergo a

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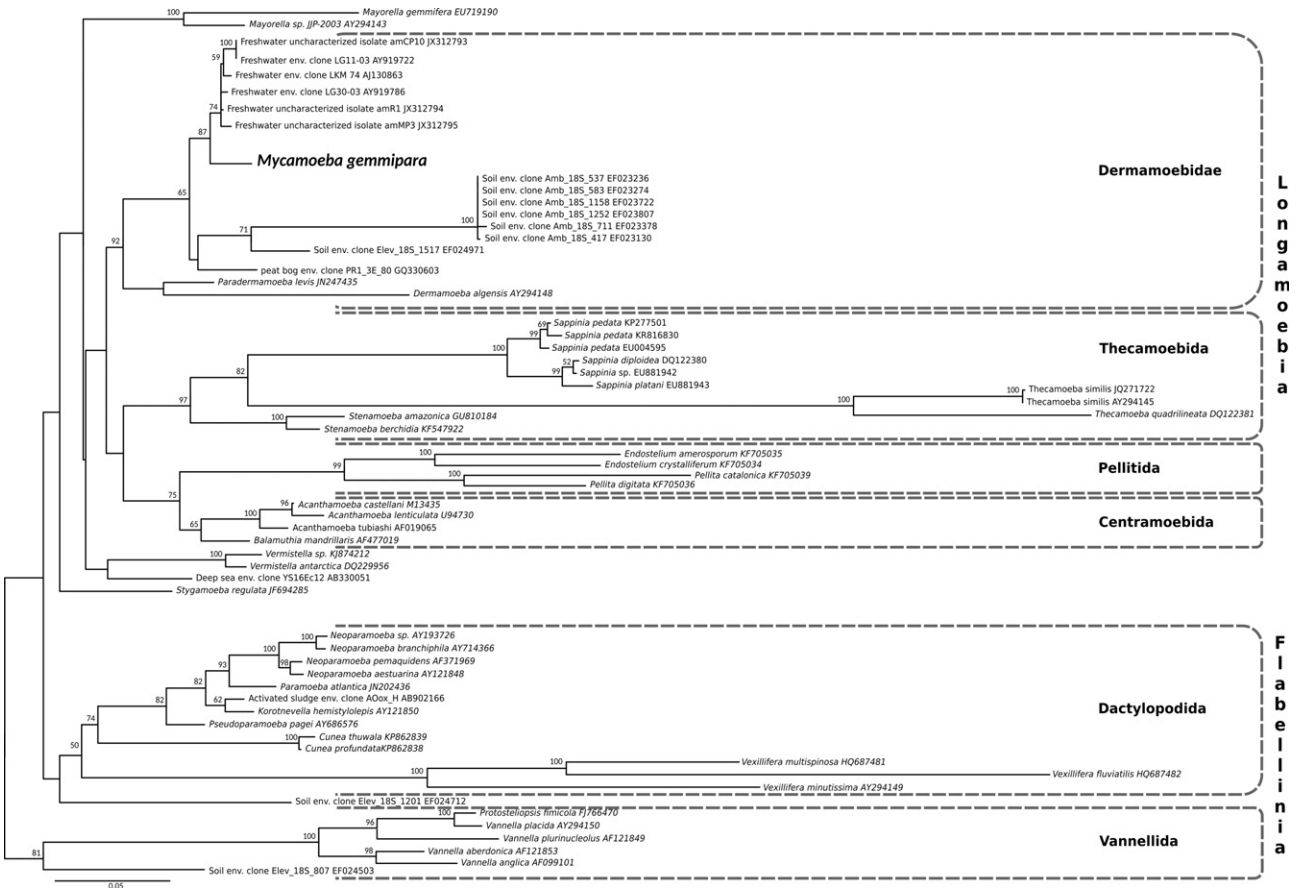

Figure 5 Maximum likelihood phylogenetic tree based on complete 18S rRNA sequences of several Longamoebida, showing the position of *Mycamoeba gemmipara* within Dermamoebidae. The tree was built using the RAxML algorithm (Stamatakis et al. 2008).

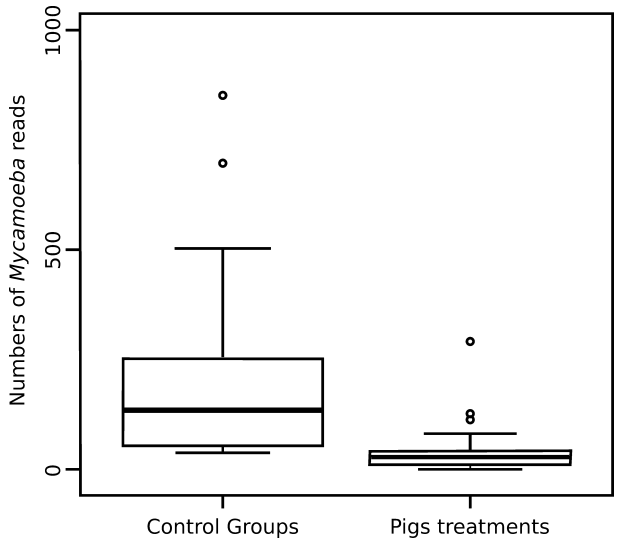

Figure 6 Boxplot showing the number of V9 18S rRNA reads related to *Mycamoeba gemmipara* in control treatments and under the decomposing pig, suggesting a negative effect of cadaveric fluids on the amoebae.

considerable biovolume increase during pseudomycelium formation. Biomass incorporation can therefore only occur by osmotrophy. Its principle may remind the polyphyletic "protosteliales" (Shadwick et al. 2009), where a prespore stage (without cell wall) precede the formation of a stalk, which is used for spore dispersal (Olive 1967). However, the simple shape of protostelids stalks differs considerably from the branched formations observed in *M. gemmipara* pseudomycelium (Fig. 2, 3). Moreover, in protostelids, stalks are formed from cysts when environmental conditions are degrading, whereas active amoebae (trophozoites) undergo cell division (Dykstra and Keller 2000). In *M. gemmipara*, we did not observe any cell division at the trophozoite stage, which suggests that multiplication occurs only during coccoid budding process. Globally, the structures produced by *M. gemmipara* resemble those observed in Fungi. The reproductive mode by budding reminds strongly of yeasts such as *Saccharomyces cerevisiae*, and many fungi revert from yeasts to mycelial growth in a single organism (Rippon 1982). As *M. gemmipara* original culture was regularly subcultured for about five years in the laboratory (thus generating dozens of replicates), we rule out the possibility that budding coccoid

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cells and pseudomycelia could be originated by fungal contaminants, which should have logically either disappeared or invaded our cultures. The main difference between the pseudomycelium observed in *M. gemmipara* and a classical mycelium is that its structures are not perennial and degrade once the cysts are released. *M. gemmipara* combines thus two life strategies: phagotrophy in a first stage of its life cycle (probably accumulating enough biomass to enter its next life stage) and osmotrophy afterwards. This strategy appears successful in freshwater and soil environments, and suggests a similar evolutionary pathway as in the Nucletmycea. These latter also evolved from amoeboid organisms, such as *Nuclearia*, towards mycelial growing organisms such as the true Fungi (Brown et al. 2009), which may have occurred also in soils or freshwater.

CLASSIFICATION SUMMARY

Taxonomic summary: *Mycamoeba gemmipara* nov. gen. nov. spec

Amoebozoa
Eudiscosea
Longamoebia
Dermamoebidae
Mycamoeba
gemmipara

Genus *Mycamoeba* gen. nov

Description: flattened amoebae with lobose or conical pseudopodia and a lingulate shape. Mitochondrial cristae are tubular. No glycocalyx or other ornaments on the cell surface. Possess a complex life cycle where active cells transform into coccoid stages, which undergo subsequent buddings, eventually turning into ramified structures (pseudomycelia) with spherical cysts in a terminal position on the ramifications. These structures disappear and cysts are released prior to germinating into active trophozoites.

Etymology: From ancient Greek: myces; mould, fungus, in reference to the fungal-like structures appearing in the osmotrophic stage of its life-cycle, as well as its peculiar mode of reproduction, atypical for an amoeba, but reminding strongly yeasts. Sole species: *Mycamoeba gemmipara*.

Mycamoeba gemmipara sp. nov

Description: Small amoeboid cells up to 7 μm. Chromatin is not distributed in a homogeneous manner: there are conspicuous condensed regions pressed against the nuclear membrane and also in the centre of the nucleus.

Ecology: has been detected to date only in forest soils. Seems to avoid important amounts of nitrogen (release of cadaveric fluids) or other perturbations generated by cadaver decomposition.

Hapantotype: a culture has been deposited at the culture collection "Culture Collection of Algae and Protozoa". 18S rRNA gene sequence of *Mycamoeba gemmipara* (1,738 bp) was deposited in GenBank (KX687875).

Etymology: *gemmipara*, as a reference to the reproduction mode (pario, giving birth in latin) through budding (gemma, a bud in latin).

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

Movie S1. Time-lapse showing the development of a ramification of *Mycamoeba gemmipara*.

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