

From Environmental Sequences to Morphology: Observation and Characterisation of a Paulinellid Testate Amoeba (*Micropyxidiella edaphonis* gen. nov. sp. nov. Euglyphida, Paulinellidae) from Soil using Fluorescent in situ Hybridization

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High microbial diversity is revealed by environmental DNA surveys. However, nothing is known about the morphology and function of these potentially new organisms. In the course of an environmental soil diversity study, we found for the first time environmental sequences that reveal the presence of Paulinellidae (a mostly marine and marginally freshwater family of euglyphid testate amoebae) in samples of forest litter from different geographic origins. The new sequences form a basal, robust clade in the family. We used fluorescent in situ hybridization (FISH) to detect the organisms from which these sequences derived. We isolated the cells and documented them with light and scanning electron microscopy. Based on these observations, we described these organisms as *Micropyxidiella edaphonis* gen. nov. sp. nov. The organisms were very small testate amoebae (generally less than 10 μm) with an irregular proteinaceous test. This suggests an unknown diversity in testate amoebae, and calls for extending this type of investigations to other protist groups which are known only as environmental DNA sequences.

Key words: Cercozoa; molecular diversity; soil; novel clades; evolutionary transitions.

Introduction

Recent culture and isolation independent environmental DNA surveys based on sequencing the small subunit ribosomal RNA gene of eukaryotes

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have revealed several new clades whose existence was unsuspected (Lara et al. 2010; Lopez-Garcia et al. 2001; Massana et al. 2004), bringing a more complete picture of the overall diversity of domain Eukaryota (Epstein and Lopez-Garcia 2008). However, the existence of these “orphan” sequences remains of limited interest if no further information is obtained on the morphology, lifestyle and traits of the organisms. The correspondence between environmental sequences and protist morphotypes is therefore an important goal in modern protistology (Pawlowski 2013). One of the most common approaches to link environmental sequences and organisms consists in designing a specific FISH probe to be hybridized selectively to the cells from which the sequence of interest derived. Once the organism is located, this method is generally coupled to other approaches to document cell morphology such as light or scanning electron microscopy. In this way, cells of the uncultured environmental MAST-12 were documented from a sub-oxic enrichment culture. These cells were demonstrated to have and showed typical heterokont morphology and to be bacterivorous (Kolodziej and Stoeck 2007). Also, novel deep sea Acantharea and their different life-stages have been detected and documented (Gilg et al. 2010). This approach opens therefore the way for a better knowledge of uncultured microbial eukaryotic forms.

Paulinellidae are a family of filose testate amoebae that belong to the larger clade Euglyphida (Cercozoa, Rhizaria). To date, it comprises only two genera, *Paulinella* and the monospecific *Ovulinata* (Adl et al. 2012; Howe et al. 2011). While the first genus includes species harbouring self-secreted silica scales on their tests, thus presenting a typical Euglyphida morphology, *Ovulinata* secretes a hyaline proteinaceous test (Anderson et al. 1996, 1997). To date, Paulinellidae have been found mostly in marine environments (Hannah et al. 1996; Nicholls 2009; Vørs 1993), and marginally in freshwater (Hasler et al. 2008; Pankow 1982), but never in soils. *Paulinella chromatophora* is by far the best studied species because of its symbiotic association with a cyanobacterium, considered as the only reported case of recent primary endosymbiosis (Marin et al. 2005). In the course of an environmental survey on the diversity of Euglyphida in forest litters, we obtained unexpectedly sequences belonging to Paulinellidae in samples originating from environments as different as Switzerland, Southern Morocco and West Coast Canada. Because testate amoebae are large and conspicuous protists in comparison to e.g.

nanoflagellates and naked amoebae, the possibility that these organisms remained unnoticed seemed unlikely. Therefore, and in order to document better the diversity of forms and features of this group, we aimed at revealing these organisms at their features using fluorescent in situ hybridization (FISH).

Results

Clone sequences related to paulinellids branched robustly within this family, which comprised the two described species (*Paulinella chromatophora* and *Ovulinata parva*), as well as several environmental clones, mostly from marine sediment except MPE2_30 (AB695524), which was retrieved from submerged freshwater mosses from Antarctica. The three soil sequences branched robustly together, forming a relatively deep clade within family Paulinellidae (Fig. 2). These sequences have been deposited in GenBank under the accession files KP892886-KP892888.

At the described conditions for hybridization, cells from the close-related species *Ovulinata parva* showed only a weak Cy3 signal (Fig. 3, subfig. 3b), if any, as the probe binding site had a single mismatch. In contrast, small cells present in the filters gave a strong signal (Figs 3, 4-7), indicating a specific hybridization. DAPI counterstain showed a large nucleus in *O. parva* situated at the back of the cell (Fig. 3, subfigs 2a and 3a). Hybridization of the environmental samples revealed fluorescing small testate amoebae cells in samples from both litter and moss, however the high level of autofluorescence in soil samples did not allow to take good pictures; only samples derived from mosses are shown here (Fig. 3, subfigs 4a-b, 5a-b, 6a-b, 7a-b).

In the soil Paulinellids, nucleus was less conspicuous than in *O. parva*, perhaps because of the small size and possibly a different test composition. Cells were also visualised under classical light microscopy, which permitted us to detect similar organisms in the sample suspension extracts.

Discussion

M. edaphonis is the first member of family Paulinellidae found in a strictly edaphic environment (sampling site was far from any river or pond, and was situated on a slope). Life in soils demands a series of adaptations for a protist, including the capacity of forming resistant structures (cysts) against desiccation or frost. As a result, it has been evidenced that soil communities are significantly

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CH2_2_11      GCTTCAAT-GGTCTGGTAATGGGAATGAGTGTATTTTAAATCCCTATGCG 418
CD3_3         GCTTCAAT-GGTCTGGTAATGGGAATGAGTGTATTTTAAATCCCTATGCG 404
Ma_44         GCTTCAAT-GGTCTGGTAATGGGAATGAGTGTATTTTAAATCCCTATGCG 404
NA2_1B8 EF526891 GCTTTTTT-GGTCTGGTAATGGGAATGAGTGTAAATTTAAATCCCTATGCG 544
DSGM_59 AB275059 GCTTTTTT-GGTCTGGTAATGGGAATGAGTGTAAATTTAAATCCCTATGCG 490
RM1_SGM47_AB505504 GCTTTTTAT-GGTCTGGTAATGGGAATGAGTGTAAATTTAAATCCCTATGCG 486
P. chromatophora FJ456918 GCTTTTTAC-GGTCTGGTAATGGGAGTGAGTGTATTTTAAATCCCTATGCG 544
P. chromatophora X81811 GCTTTTTAT-GGTCTGGTAATGGGAATGAGTGTAAATTTAAATCCCTATGCG 545
YPF501_AB453002 GCTTTTTAC-AGTCTGGTAATGGGAATGAGTGTAAATTTAAATCCCTATGCG 525
O. parva CCAP 1554 HQ121432 GGCTTTTACAGTCTGGTAATGGGAATGAGTGTAAATTTAAATCCCTATGCG 403
CYSGM_17AB275100 GCCTTTTAC-GGTCTGGTAATGGGAATGAGTGTAAATTTAAATCCCTATGCG 490
Saccharomyces EU011664 GCCCATTCGGTCTTGTAAATGGGAATGAGTACAATGTAATACCTTAACG 483
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Figure 1. Alignment of the sequences close to clone CH2_2_11 (i.e. *Micropxydiella edaphonis*). The short sequence used to define the FISH-probe was 5'- GAGTGTATTTTAAAT – 3'. Environmental clones Ma_14 and B1_2_29 grouped in others clusters close to the cluster of CH2_2_11.

different from their aquatic counterparts in ciliates (Foissner 1998). Other groups, such as dinoflagellates, are totally absent from soils (Foissner 1991). These examples emphasize the importance of the

frontier that separates aquatic and soil protist communities, and the considerable evolutionary step achieved by the (most probably aquatic) ancestors of *Micropxydiella edaphonis*. The relatively long

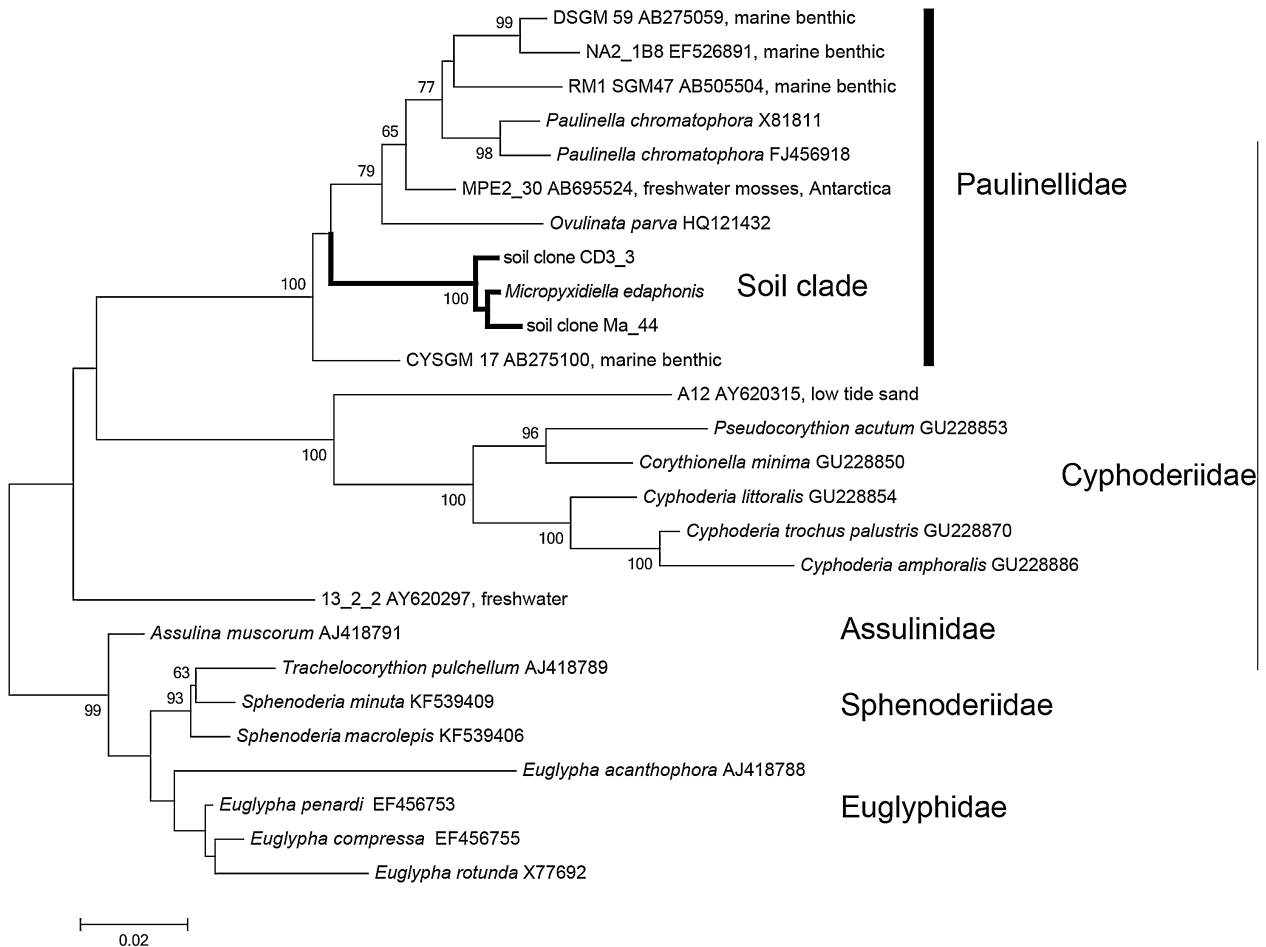


Figure 2. Maximum likelihood phylogenetic tree showing the position of *Micropxydiella edaphonis* and the soil clones affiliated to family Paulinellidae with respect to other Euglyphida. Bootstrap values above 70% are indicated at the nodes (1000 replicates). Sequences from Euglyphidae, Assulinidae and Sphenoderiidae have been used to root the tree.

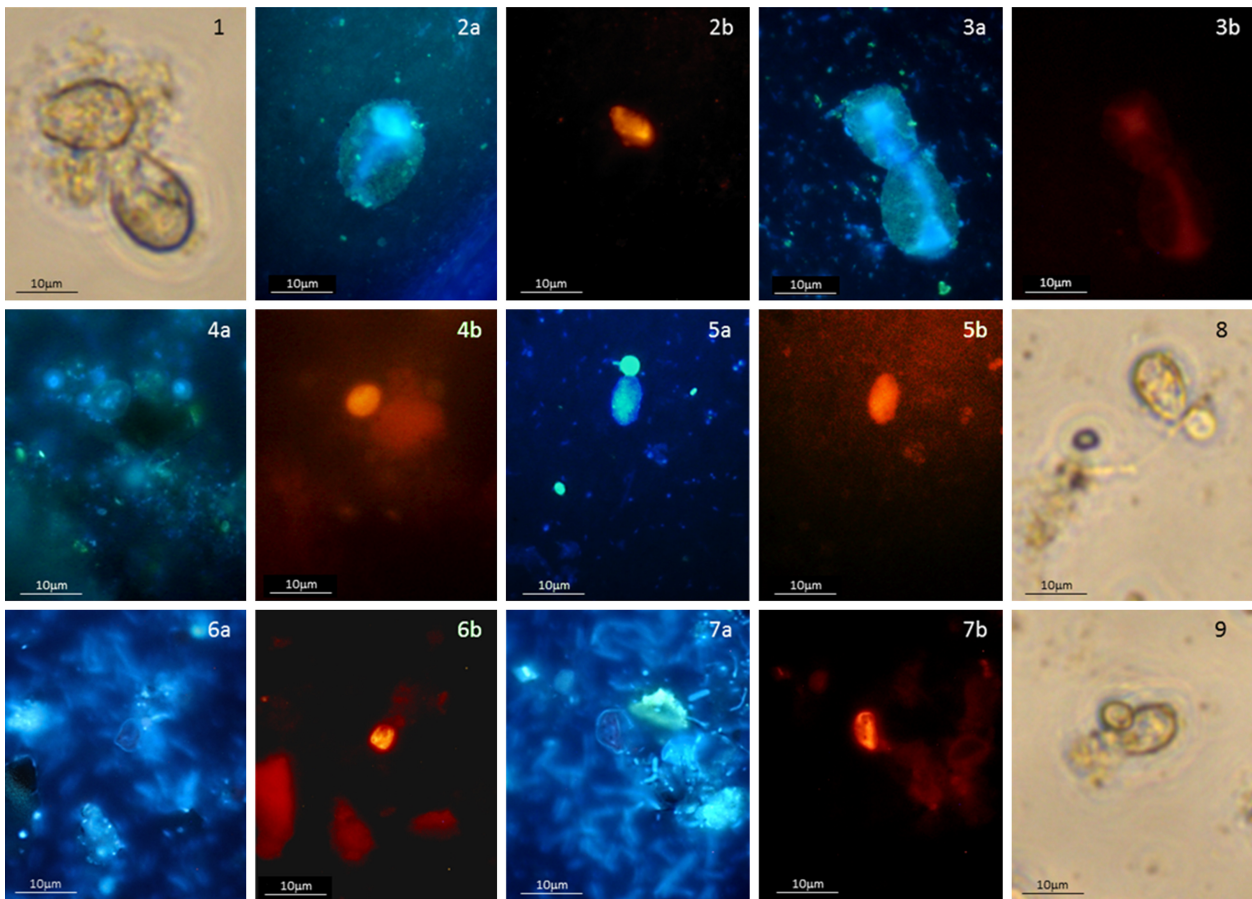


Figure 3. Light microscopy observations of *Ovulinata parva* pure culture and of moss environmental extracts. Pictures with same number (e.g. 2a and 2b) referred to a same image taken a) with DAPI filter and b) with CY3 filter. **1)** *O. parva* under visible light, **2a** and **3a)** *O. parva* stained with DAPI, **2b)** *O. parva* hybridized with Eukaryote FISH probe, **3a)** OP hybridized with the probe defined in this study. **4** to **7)** picture from environmental moss sample a) marked with DAPI and b) marked with the specific probe defined in this study. **8** and **9)** Light picture of the amoeba found in the moss extracts that matched with the morphology and size observed with FISH experiment. These cells were collected for SEM observation (Fig. 4).

branch that separate soil paulinellid sequences from the aquatic members of the family suggests also a strong evolutionary pressure exerted by the transition towards a new environment.

Filose testate amoebae which do not incorporate mineral elements in their shells were unknown until recently in Euglyphida (Meisterfeld 2002), but have been found in Thecofilosea (Cavalier-Smith and Chao 2003) and, more recently, in the Labyrinthulomycetes (Gomaa et al. 2013). Recently, *Ovulinata parva* with its proteinaceous shell has been assigned to euglyphids using molecular data (Howe et al. 2011), and remained until now an exception in this clade where all members use self-secreted silica scales to reinforce their tests. Scale-forming *Paulinella chromatophora* has a derived position compared to both *Micropoxydiella edaphonis* and

Ovulinata parva within family Paulinellidae. As a consequence, scale forming ability has either been invented several times (at least once in genus *Paulinella* and once for the rest of euglyphid genera), or pre-existed in the ancestral euglyphid and has been lost independently several times. If the scale-bearing flagellated thaumatomonads and euglyphids are indeed sister groups as suggested by Cavalier Smith and Chao (Cavalier-Smith and Chao 2003), silica mineralization would be an ancestral character in Silicofilosea and therefore represents a secondary loss in both *Ovulinata parva* and *Micropoxydiella edaphonis*.

The small size and the transparent shell that characterize *M. edaphonis* are responsible for its inconspicuous appearance. This is the most probable reason why *Micropoxydiella edaphonis* has

never been detected before. It is one of the smallest known euglyphids, together with some other members of family Paulinellidae (Nicholls 2009); other even slightly smaller testate amoebae with an agglutinated test and filamentous pseudopodia do exist, but their phylogenetic affiliation still remains to be determined (Kiss et al. 2009). The existence of “dwarf” undescribed euglyphids suggests that the whole diversity of this clade is still under-evaluated. Furthermore, we suggest that a substantial part of the undescribed protist diversity is composed by small and inconspicuous organisms that have not been detected by traditional surveys.

Taxonomic Appendix

Rhizaria Cavalier-Smith, 2002

Cercozoa Cavalier-Smith, 1998

Imbricatea Cavalier-Smith, 2003 (sensu Cavalier-Smith, 2011)

Silicofilosea Adl, et al. 2005 (sensu Adl, et al. 2012)

Euglyphida Copeland, 1956 (sensu Cavalier-Smith, 1997)

Paulinellidae de Saedeller, 1934 (sensu Adl et al., 2012)

Micropxydiella g. nov. Tarnawski and Lara, 2015 **Diagnosis:** Genus of testate amoebae with filamentous pseu-

dopodia, entirely organic test (without self-secreted scales or mineral particles). Large round nucleus (about 20% of shell length).

Type species: *Micropxydiella edaphonis* (Monotypic)

Etymology: Micro=small and pyxis=small box in ancient Greece used by women to hold cosmetics, trinkets or jewellery. ella = diminutive (a reference to its shape and small size).

Micropxydiella edaphonis sp. nov. Tarnawski and Lara, 2015

Diagnosis: Very small testate amoebae (length: 8-10 μm ; width: 5-6 μm), with an ovoid transparent test, comparable to the related species *Ovulinata parva*. These organisms were most often associated to organic particles, presumably feeding on bacteria. Shell with a pointed end, reminding of certain members of genus *Diffflugia* such as *D. acuminata*, clearly visible under scanning electron microscopy (Fig. 4), but not under light microscopy. Nucleus of about 2 μm .

Etymology: Genus and species name from ancient Greek:

Species name: edaphos= soil, edaphonis = from soil (reference to its habitat).

Type material: One SEM stub with a specimen is deposited at the Natural History Museum of Neuchâtel (Ref. Nr.: UniNe-EM-6).

Type locality: Organisms were collected from a forest litter sample taken near Les Ponts de Martel, 47°00'N 6°44'E, Switzerland.

Methods

Sampling, DNA extraction and sequencing: Forest litter samples came from, respectively, a palm tree forest (Morocco, near Marrakesh; 31° 36' 47" N; 08°01' 07" W), a coniferous

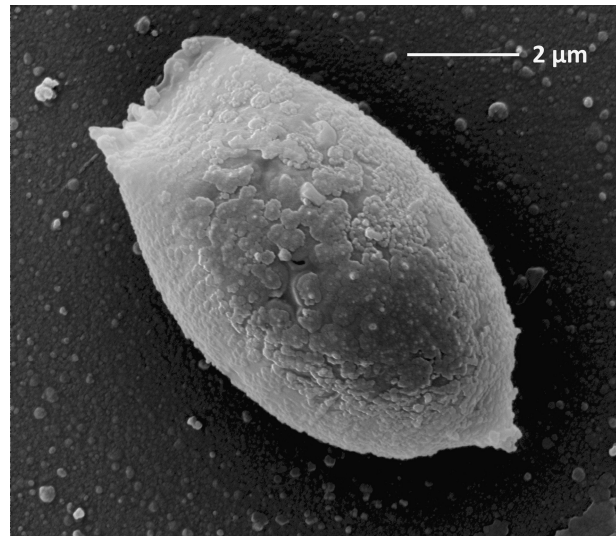


Figure 4. SEM images of the organism detected with FISH (figure 4), located on the filter and subsequently isolated.

temperate forest (Canada, Vancouver, 49° 18' 16" N; 123° 08' 37" W) and a mixed broadleaf-coniferous forest (Switzerland, near Les Ponts de Martel, 47°00'N 6°44'E). DNA was extracted using a MoBio Power Soil™ kit (Carlsbad, USA) following the manufacturer's instructions. The SSU rRNA gene amplification was obtained after a semi-nested protocol; a first PCR was achieved with the specific primers EuglySSUF (5'-GCGTACAGCTCATTATATCAGCA-3') and EuglyLSUR (5'-GTTTGGCACCTTAACCTCGCG-3'), the latter primer placed on the LSU rRNA gene. Cycling profile was the following: an initial denaturation at 94 °C for 5 minutes, followed by 40 cycles with 94 °C for 15 s, 62 °C for 15 s with a touchdown of 1 °C per cycle in the eight first cycles and 72 °C for 150 s. A final step of 10 minutes at 72 °C for elongation was performed. A second, PCR was done on the first PCR product using again EuglySSUF in combination with EuglySSUR (5'-GCACCACCATAGAATCWAGAAAGATC-3'), with the following amplification program: initial denaturation at 94 °C for 3 minutes, then 30 cycles with a denaturation step at 94 °C for 30 seconds, an annealing step at 59 °C for 30s and an elongation step at 72 °C for 60 seconds, followed by a final elongation at 72 °C for 10 minutes. Amplicons were cloned into pCR2.1 Topo TA cloning vector (Invitrogen) and transformed into *E. coli* TOP10' One Shot cells by heat shock (Invitrogen). Inserts from the right size were sequenced in an ABI PRISM 3700 DNA Analyzer (PE Biosystems, Genève, Switzerland) using a BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems). Amongst all clone sequences obtained, paulinellid sequences were placed in a maximum likelihood phylogenetic tree comprising sequences taken from different euglyphid families, as well as several related environmental clones found in GenBank. The tree was built using MEGA 6 (Tamura et al. 2013) under a General Time Reversible model and a gamma distribution of variation among sites (5 categories). 1000 bootstrap replicates were used to estimate node robustness.

Design of the specific FISH probe: Environmental euglyphid 18S rRNA gene sequences retrieved from GenBank were aligned using the biological sequence alignment editor BioEdit

v.7 (Tom Hall, Ibis Biosciences). FISH probe was planned to match specifically clones affiliated to Paulinellidae. A conserved target region was identified and also match with close Paulinellidae affiliated clones (Fig. 1). *In silico* specificity analysis was done with nucleotide blast from NCBI online program using blastn algorithm. A 5' Cyanine 3 labeled probe (5'- [Cy3] ATTTAAAATACACTC - 3') complementary to the 18S rRNA identified sequence was synthesized (Microsynth AG, Balgach, Switzerland) to verify the presence of cells in environmental samples.

Environmental sample preparation and fixation: Samples containing mosses and forest litter were taken in March 2012 exactly at the same microsite from where the Swiss paulinellid sequence was found, in order to maximise the chances to detect the organisms. Around 1-2 g of fresh sample of each type (soil and moss) was mixed with 30 mL of Phosphate Sodium Buffer (PSB, 0.1 M, pH 7) in a 50 mL glass bottle and placed under average agitation during 40' to release microorganisms. The whole mixture was then passed through a 100 µm mesh membrane to eliminate large particles. Half volume of this filtrate was passed through a 50 µm mesh membrane. 500 µL of each filtrates (100 and 50 µm) were fixed in 1:1 (v/v) Bouin's solution (Sigma-Aldrich, St Gallen, Switzerland) during 1 hour at RT. 100 to 300 µL of the fixed cells were diluted in 5 mL PSB and collected on a 0.2 µm nitrocellulose ISOPORE filter (Millipore, Billerica, USA) by gentle pumping. Filters were rinsed 4 times with 10 mL of PSB removed by filtration, then wash 2 times with sterile water, and finally passed through successive Ethanol baths at 50%, 80%, 100%, for 3 min. each. Filters were stored at 4 °C or used directly for hybridization.

Optimization of fluorescence in situ hybridization (FISH) protocol: The protocol of the fluorescence *in situ* hybridization was optimized according to (Pernthaler et al. 2001) using *Ovulinata parva* CCAP 1554/1 monoprotistan cultures as negative controls. Optimal hybridization temperature was fixed at 46 °C with no formamide addition in hybridization solution. Briefly, filter hybridizations with fluorescent probe were performed on slides in a hybridization oven (reference) at 46 °C for 1h30 in 80 µL of hybridization buffer (900 mM NaCl, 20 mM Tris/HCl, 0% formamide, 0.01% SDS) containing the probes at a final concentration of 5ng/µL. After hybridization, filters with fixed cells were rinsed twice in pre-heated washing buffer (900 mM NaCl, 20 mM Tris/HCl, 5 mM EDTA, 0.01% SDS) at 46 °C for 15 min, then quickly rinsed in distilled water and air dry on blotting paper. For counterstaining, filters were recovered with 50 µL of DAPI solution (50ng/µL) during 3 min. then wash in 80% ethanol to remove unspecific staining, rinsed in distilled water and well air-drying. Sections of filter were mounted in 4:1 mix of Citifluor (Citifluor Ltd. London, UK) and Vecta Shield (Vector Laboratories, Inc., Burlingame, U.S.A) and observed with an inverted microscope (Leica DMI 3000 B) under UV light using microscope filters at 450 nm for DAPI and 550 nm for Cy3. As samples derived from litter presented too many autofluorescing particles, we focussed only on samples from moss.

Scanning electron microscopy (SEM) analysis: Same moss and litter extracts used for FISH experiment were observed under light microscope in order to visualise cells looking like the one evidenced with FISH experiments in size and morphology. Cells were individually collected with a drawn pipette and were placed on a microscope slide. They were rinsed in distilled water and dried in a desiccator. After being coated with gold, the samples were examined with a Phillips XL 30 scanning electron microscope with an acceleration voltage of 10 kV.

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