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Guillaume Reboul,1 David Moreira,1 Paola Bertolino,1Alexandra Maria Hillebrand-Voiculescu2,3 and Purificación López-García 1

1 Unité d'Ecologie; Systématique et Evolution, CNRS, Université Paris-Sud, Université Paris-Saclay, AgroParisTech, bâtiment 360, 91400 Orsay, France.
2 Department of Biospeleology and Karst Edaphobiology, Emil Racovita Institute of Speleology, Bucharest, Romania.
3 Group for Underwater and Speleological Exploration, Bucharest, Romania.

Summary
Movile Cave is a small system of partially inundated galleries in limestone settings close to the Black Sea in Southeast Romania. Isolated from the surface for 6 million years, its sulfidic, methane and ammoniarich waters harbour unique chemosynthetic prokaryotic communities that include sulphur and ammonium-metabolizing chemolithotrophs, methanogens, methanotrophs and methylotrophs. The cave also harbours cave-dwelling invertebrates and fungi, but the diversity of other microbial eukaryotes remained completely unknown. Here, we apply an 18S rRNA gene-based metabarcoding approach to study the composition of protist communities in floating microbial mats and plankton from a wellpreserved oxygen-depleted cave chamber. Our results reveal a wide protist diversity with, as dominant groups, ciliates (Alveolata), Stramenopiles, especially bicosoecids, and jakobids (Excavata). Ciliate sequences dominated both, microbial mats and plankton, followed by either Stramenopiles or excavates. Stramenopiles were more prominent in microbial mats, whereas jakobids dominated the plankton fraction of the oxygen-depleted water column. Mats cultured in the laboratory were enriched in Cercozoa. Consistent with local low oxygen levels, Movile Cave protists are most likely anaerobic or microaerophilic. Several newly detected OTU clades were very divergent from cultured species or environmental sequences in databases and represent phylogenetic novelty, notably within jakobids. Movile Cave protists likely cover a variety of ecological roles in this ecosystem including predation, parasitism, saprotrophy and possibly diverse prokaryote-protist syntrophies.

Introduction
The Movile Cave harbours a unique underground aquatic ecosystem that has been isolated from the surface for almost 6 million years (Lascu, 1989). Located in a limestone area close to the Black Sea in Southeast Romania, it encompasses several inundated galleries fed by thermal (21_C) sulfidic waters. The first explorations of these galleries showed that some of them contained oxygendepleted air pockets ('airbells') and floating whitish microbial mats apparently formed of bacteria and fungi (Sarbu et al., 1994; Sarbu et al., 1996). Early stable isotope labelling experiments showed that this subsurface ecosystem is chemosynthetic (Sarbu et al., 1996). Subsequent studies uncovered a wide diversity of prokaryotes and revealed the presence of sulphur- and ammonium-based chemolithotrophy (Chen et al., 2009) but also an important contribution of methanogenesis, methanotrophy and methylotrophy to the carbon cycle in this cave ecosystem (Hutchens et al., 2004; Wischer et al., 2015; Kumaresan et al., 2018). Methanogenic archaea were indeed isolated from floating biofilms (Ganzert et al., 2014) and anoxic sediment (Schirmack et al., 2014).

The chemolithoautotrophic C fixation sustains not only microbial communities but also a variety of obligate cavedwelling invertebrates, from which more than 30 species are endemic (Sarbu et al., 1996; Fiser et al., 2015). Amphipods are particularly diverse. Species of the prevalent Niphargus genus are tightly associated to Thiothrix sulphur-oxidizing ectosymbiotic bacteria (Flot et al., 2014). Prokaryote-eukaryote symbioses are widespread in oxygen-depleted ecosystems (Dubilier et al., 2008; Nowack and Melkonian, 2010; Edgcomb, 2016). This type of symbioses, essential for adaptation to these ecosystems and source of evolutionary innovation, and are particularly widespread in anaerobic microbial eukaryotes (Nowack and Melkonian, 2010;

Lopez-Garcia et al., 2017) and might also be prevalent in protists from the suboxic Movile ecosystem. However, the diversity of microbial eukaryotes in this cave is practically unknown. Only a recent, culture-based study provided information about the diversity of culturable fungi in Movile samples (Novakova et al., 2018). This situation mirrors that of other cave ecosystems, which have traditionally attracted interest either on the prokaryotic communities (Northup and Lavoie, 2001) and/or the diversity and specific adaptations of the, very often, endemic animal species (Juan et al., 2010; Casane and Retaux, 2016), while leaving protist diversity largely unexplored.

With the aim to fill this knowledge gap and characterize microbial eukaryotic communities in the chemosynthetic Movile ecosystem, we carried out a study based on highthroughput 18S rRNA gene amplicon sequencing (metabarcoding) of microbial mat and plankton samples from an oxygen-depleted 'airbell' compartment. Our results revealed a considerable diversity of likely anaerobic and/or microaerophilic protists, several of which represent divergent groups from known taxa.

Results and discussion

Movile Cave is a small cave (~ 250 m length) developed in Sarmatian limestone partially flooded with mesothermal (22–23°C) sulfidic (H2S, 0.3 mM) water enriched in CH4 (0.2 mM) and NH4 + (0.3 mM). The dissolved oxygen ranges between 9 and 16 μM at the water surface and less than 1 μM below the upper 3–4 cm of the water column, which becomes anoxic towards the bottom (Sarbu et al., 1994; Chen et al., 2009). We collected water and microbial mat samples from the second Movile Cave chamber, more remote from the entry, containing an airbell ('AirBell2'). The atmosphere of this chamber was oxygen-poor (8%–10% O2) and contained high relative concentrations of CO2 (2.5%) and CH4 (2%). As previously described, whitish microbial mats were observed floating on the water surface, sometimes retaining bubbles of reduced gases coming from below (Fig. 1). We collected a fraction of this mat (Mov6, surface of ca. 20 cm2), which was fixed in ethanol for subsequent DNA purification and 18S rRNA gene metabarcoding analysis (see Supporting Information). A water sample of 0.8 l collected below the surface was prefiltered through a 200 μm mesh to eliminate large particles and the planktonic biomass was retained in 0.2 μm pore size filters (Mov4). We also included in our study a sample of a microbial mat collected in AirBell2 two months earlier and maintained in culture in a sealed cave water-containing bottle in the laboratory (Mov2).

After DNA purification, we amplified 18S rRNA gene fragments of approximately 550 bp length comprising the hypervariable V4 region using primers EK-565F (50-GCAGTTAAAAAGCTCGTAGT-30) and 18S-EUK- 1134-R-UNonMet (50-TTTAAGTTTCAGCCTTGCG-30) tagged with different molecular identifiers for each sample. After mixing the products of several independent PCR reactions to minimize amplification biases, we purified, pooled, and sequenced amplicons using MiSeq paired-end (2 x 300 bp, chemistry v3) Illumina technology. We merged and treated paired-end sequence reads using an in-house bioinformatic pipeline to check quality and eliminate primers and molecular identifiers. We also eliminated potentially chimeric sequences (see Supporting Information). We then dereplicated the resulting clean merged reads (CMRs) and used them to define operational taxonomic units (OTUs) at 97% identity cut-off (see Supporting Information). We chose this cut-off value as a good compromise offering a reasonable operational approximation to the genus-species level diversity while producing a manageable number of OTUs to be included in specific phylogenetic trees (see below). Collectively, this yielded a total of 7,454 OTUs, including shared OTUs among samples, but most of them were singletons and were discarded for the rest of the analysis. In total, we retained 652 OTUs (some of them shared between samples) (Table 1). We assigned these OTUs to known taxonomic groups based on their similarity with sequences of a local database that included sequences from cultured/described organisms and environmental surveys retrieved from SILVAv128 (Quast et al., 2013) and PR2v4.5 (Guillou et al., 2013). We further refined the phylogenetic assignation by the phylogenetic placement of our OTU sequences in a reference phylogenetic tree (Supporting Information).

We retrieved OTUs belonging to all major super-groups of microbial eukaryotes including Amoebozoa, Opisthokonta (including apusomonads), Excavata, Archaeplastida, and the SAR clade (Stramenopiles, Alveolata, Rhizaria), as well as sequences of uncertain classification or

belonging to groups of unresolved phylogenetic placement such as haptophytes, katablepharids and telonemids, sometimes referred to as Hacrobia (Okamoto et al., 2009) (Fig. 2). 'Fresh' samples harboured most of the diversity with 372 and 297 OTUs for, respectively, the biofilm Mov6 and the plankton sample Mov4 (Table 1). In both samples, alveolate (ciliate, in particular) sequences dominated (ca. 70%–80%; Fig. 2A) although, in general, OTUs from other groups collectively accounted for a larger diversity (Fig. 2B). However, whereas Stramenopiles, followed to some extent by Amoebozoa, were the subsequent most prevalent groups in the microbial mat Mov6, Excavata were the more relatively abundant in the planktonic Mov4 sample. A small fraction of OTUs was shared by the plankton and the microbial mat samples, highlighting their different community composition (Fig. 2C). The most abundant shared OTUs belonged to Stramenopiles and Amoebozoa (Supporting Information Table S1). As expected, Mov2, the biofilm sample that was maintained in culture for two months in the laboratory, was less diverse and had a different community composition as compared to the 'fresh' sample Mov6. Interestingly, although Mov2 had similar proportions of OTUs across taxa (Fig. 2B), the relative abundance of reads was very different from Mov6 (Fig. 2A). This implies that, although the phylogenetic diversity of OTUs was maintained in mat cultures over time (Fig. 2B), the relative proportion of the different taxa considerably shifted (Fig. 2A). In particular, Rhizaria, and more specifically members of the Cercomonadida, opportunistically proliferated under laboratory conditions. Mov2 and the other two samples shared very few OTUs (Fig. 2C).

In general, OTU sequences retrieved from Movile samples resembled more sequences retrieved from environmental surveys than sequences from cultured/described species, as shown in divergence plots (Fig. 3). These plots also show that, on average, Excavata and Amoebozoa included the most divergent 18S rRNA gene sequences as compared to those existing in databases. Although some ciliate sequences were also divergent, most of them had closer relatives in databases. In order to explore better the phylogenetic diversity within the dominant and most diverse protist groups identified in Movile Cave, we reconstructed phylogenetic trees for Alveolata, Stramenopiles and Excavata. Because our amplicon sequences were relatively short and contained limited phylogenetic information, we first built an alignment of taxon-specific near full-length reference 18S rRNA gene sequences including the closest blast hit sequences to our OTUs with Mafft-linsi v7.38 (Katoh and Standley, 2013) and trimmed gaps and ambiguously aligned positions (Capella-Gutierrez et al., 2009) before building reference trees. Subsequently, we included our OTU sequences to the corresponding alignments using the Mafft-linsi 'addfragments' option. We then reconstructed maximum likelihood phylogenetic trees using IQ-TREE v1.6.5 (Nguyen et al., 2015) applying a GTR model of sequence evolution with a Gamma law and taking into account invariant positions (see Supporting Information).

The vast majority of alveolate OTUs corresponded to ciliates, but three OTUs clustered within the Apicomplexa, corresponding most likely to parasites of protists or animals (Fig. 4). The most relatively abundant of them (OTU7443) was distantly related to gregarines (e.g., Ancora spp.). We also detected a few OTUs related to the parasitic perkinsids, as well as several dinoflagellate OTUs (22), all of them in very low abundances (Fig. 4 and Supporting Information Fig. S2). Dinoflagellates are typically photosynthetic, although many have lost photosynthesis and become bacterivorous (Boenigk and Arndt, 2002). Many of our OTUs were very similar to environmental sequences from oxygen-deprived settings or deep marine sediments, suggesting that they may be actually heterotrophic (Supporting Information Fig. S2). Other OTUs were more closely related to typical photosynthetic species, and we cannot discard the possibility that they infiltrated from marine waters, given the proximity of the Black Sea, or are low-frequency contaminants introduced during diving (through diving equipment). At any rate, most alveolate sequences were scattered in various ciliate classes (Fig. 4). Three of them contained clades of Movile OTUs that were particularly abundant. The first of them was the class Armophorea, which includes anaerobic and microaerophilic ciliates from diverse environments (Vdacny et al., 2018), often containing prokaryotic endosymbionts (Nowack and Melkonian, 2010). Armophorea encompassed two clades of relatively abundant OTUs that seem related to metopids, a family of anaerobic ciliates, MOV-AL-1 and MOV-AL-2. MOV-AL-2 appeared also forming a clade with metopids but branched at the base of the group and had a longer branch, suggestive of a potential parasitic lifestyle (Fig. 4). The class Phyllopharyngea comprised a clade of nine related OTUs, MOV-AL-3, which was by far the most represented in Movile Cave. MOV-AL-3 likely

represents a new ciliate clade, being divergent with respect to their closest relative, a sequence from a hydrothermal deposit in the Mariana Trough. Finally, the class Oligohymenophorea encompassed the largest diversity of OTUs. Many of them were scattered in the class, having as closest relatives sequences retrieved from anoxic or suboxic settings, such as the Cariaco Basin (Edgcomb et al., 2011), the Guaymas hydrothermal sediment (Edgcomb et al., 2002) or the Framvaren fjord (Behnke et al., 2006), and microbialites from alkaline lakes (Couradeau et al., 2011), displaying similar physico-chemical conditions to those of karstic systems. The most diverse clade, MOV-AL-4, comprised 93 OTUs together with one environmental sequence and Uronema nigricans, an opportunistic marine parasite of animals (Crosbie and Munday, 1999).

The stramenopiles were also diverse, but most of the OTUs clustered in three major groups, which were also relatively abundant, MOV-ST-1 (bicosoecids, 156 OTUs), MOV-ST-2 (labyrinthulids, 25 OTUs) and MOV-ST-3 (chrysophytes, 20 OTUs) (Fig. 5 and Supporting Information Fig. S3). Although many ochrophytes are photosynthetic (e.g. diatoms or chrysophytes), reversion to heterotrophy has occurred several times independently within this group. Although some diatom and chrysophyte sequences in low frequency might be photosynthetic contaminants introduced during diving, some clades such as MOV-ST-3, and the diatom clades MOV-ST-4 and MOVST- 5, relatively abundant and related to sequences retrieved from deep-sea or freshwater sediments (Fig. 5), likely correspond to heterotrophic lineages that dwell in the cave ecosystem. However, many other OTUs belong to clear heterotrophic clades, such as the MAST-12 and MAST-3 clades, the saprophytic labyrinthulids or bicosoecids. By far, the most diverse abundant clade, which also included one environmental sequence retrieved from a shallow subtropical lake, was MOV-ST- 1. It comprised three major subclusters of OTUs amounting a total of 156 OTUs (Fig. 5 and Supporting Information Fig. S3). In agreement with the local physicochemical conditions of the cave, and as in the case of alveolates, most of the closest environmental sequences to the Movile OTUs were retrieved from oxygen-depleted habitats or correspond to microaerophilic or anaerobic species.

Excavates comprised very divergent OTUs, with average 18S rRNA gene similarities of approximately 70%– 75% (Fig. 3). Many OTUs, in particular the clades MOVEX- 1 and MOV-EX-2, are associated to the family Stygiellidae, which encompasses the genera Stygiella and Velundella. This is a highly diverse jakobid family whose members typically inhabit anoxic, sulfide- and ammonium-rich marine habitats worldwide (Panek et al., 2015). Stygiella incarcerata contains hydrogenosomes, mitochondria-related organelles typical of many anaerobic protists (Leger et al., 2016). However, the most diverse and relatively abundant clade, MOV-EX-3, comprised 76 OTUs and formed an independent lineage with some affinity to jakobids (Fig. 6). This group likely represents either a new jakobid family or a novel euglenozoan lineage.

In addition to alveolates, stramenopiles and excavates, several other taxa were represented in our samples. The most divergent of them corresponded to Amoebozoa (Fig. 3) and were member of the Lobosa or were unassigned (Supporting Information Table S2). This is not surprising given that amoeba have often fast-evolving 18S rRNA sequences and contain insertions. Anaerobic amoeba are relatively poorly known and some of them are so divergent that are usually classified as incertae sedis (Taborsky et al., 2017). Among Opisthokonta, we detected OTUs affiliating to apusomonads, metazoans (calcareous sponge), Ichthyosporea, choanoflagellates and various fungal and fungi-related taxa (Supporting Information Table S2). Within Rhizaria, we detected one acantharian member and several cercozoan OTUs, notably in the Mov2 sample. This suggests that cercozoa are opportunistic predators that developed better in the laboratory conditions. Finally, a few OTUs represented by few sequences belonged to Archaeplastida, breviates, prymnesiophytes, telonemids and katablepharids (Supporting Information Table S2). Some of these might be local inhabitants of the cave belonging to the rare biosphere; others, notably those potentially photosynthetic, might be dispersal forms infiltrated from oceanic waters or human-introduced contaminants (e.g. through diving suits).

Our results show that Movile Cave harbours a wide diversity of protists belonging to most major eukaryotic super groups, with ciliates (alveolates), stramenopiles and jakobids (excavates) being the dominant and most varied groups. However, while stramenopiles are more abundant in the floating microbial mats, jakobids seem clearly planktonic, thriving in the oxygen-deprived water column. By contrast, mats cultured in the laboratory for several weeks show protist community shifts, with cercozoans becoming dominant community members. Most of the diversity

observed correspond to lineages that have as closest relatives anaerobic or microaerophilic protists or, else, environmental sequences coming from oxygen-deprived habitats. This strongly suggests that Movile Cave protists are mostly anaerobic or microaerophilic. It also seems that protists in the Movile Cave might have both, freshwater and marine, origins. Indeed, the diversity found in this chemosynthetic ecosystem bears resemblance with that of protists found in sulfurous lakes and lagoons, including karstic sites (Triado-Margarit and Casamayor, 2015). At the same time, many of the closest relatives of the Movile OTUs have been identified in anoxic seawater columns (Edgcomb et al., 2011) or sediments (Edgcomb et al., 2002). Given that many of these protists seem anaerobic, it is likely that prokaryote-protist symbioses are prevalent in this chemosynthetic ecosystem. Like in other oxygendepleted ecosystems (Edgcomb, 2016), Movile Cave protists are thus likely important members of this chemosynthetic microbial ecosystem, covering a range of ecological functions from predation, saprotrophy and parasitism to more subtle hubs of metabolic exchange through syntrophy.

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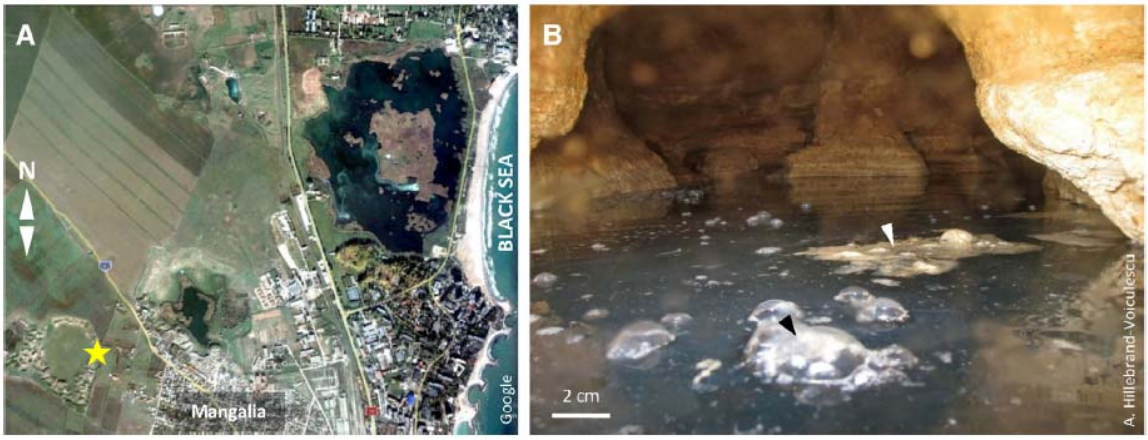

Fig. 1. Sampling at Movile Cave. A. Location of Movile Cave in the vicinity of Mangalia village and the Black Sea. The entrance of Movile Cave is indicated by a yellow star. B. sampling site at 'airbell 2'. The sampled floating biofilms are indicated by a white arrowhead. The black arrowhead points at methane bubbles accumulating at the surface of the cave water.

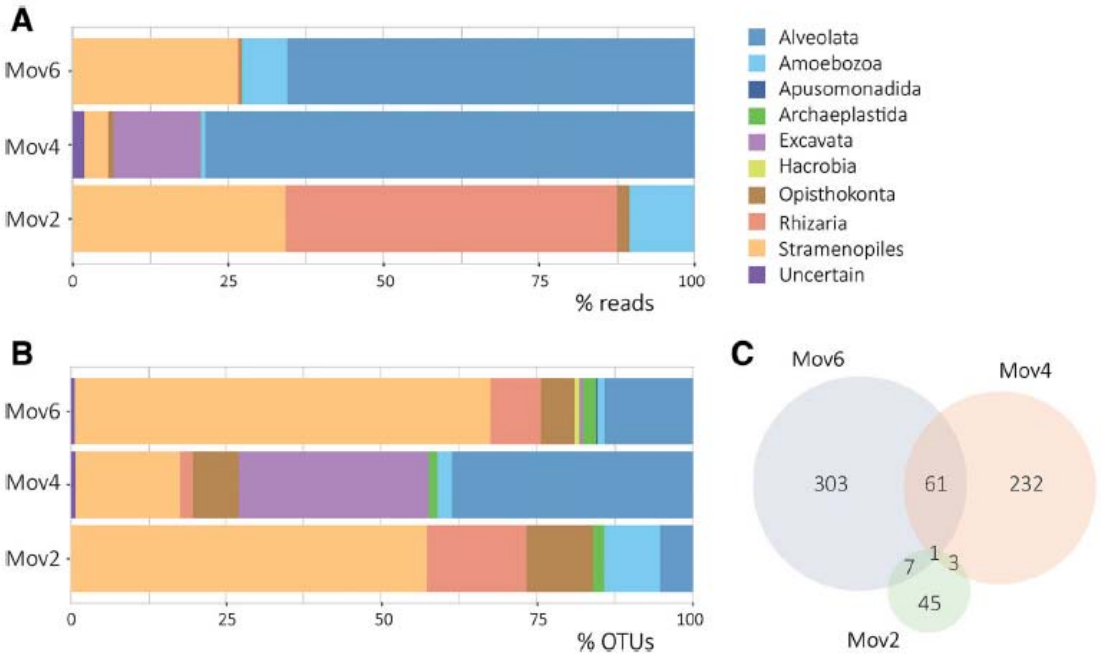

Fig. 2. Relative abundance of microbial eukaryotes in Movile Cave samples. A. Relative abundance of 18S rRNA gene amplicon reads. B. Relative abundance of operational taxonomic units (OTUs). C. Venn diagrams showing specific and shared OTUs among samples.

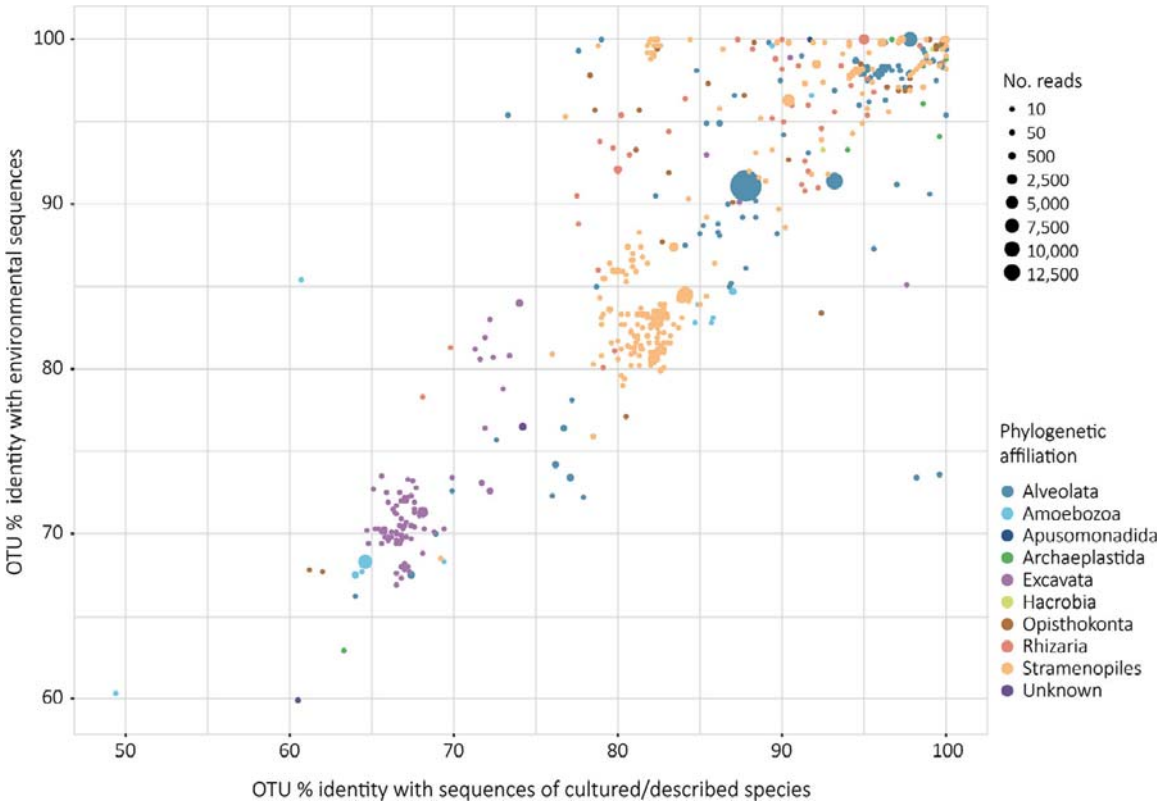

Fig. 3. Divergence plots of eukaryotic OTUs from the Movile Cave with respect to 18S rRNA gene sequences of cultured/described protists and environmental surveys. The size of the dots is proportional to the number of reads. Their colour indicates their phylogenetic affiliation.

Fig. 4. Maximum likelihood (ML) phylogenetic tree of partial 18S rRNA gene sequences showing the position of OTUs affiliating to Alveolata. The number of reads per OTU or group of OTUs as well as the number of reference sequences (rs) in the case of nodes that have been collapsed (triangles) is indicated. A total of

1,603 unambiguously aligned positions and 284 sequences were used to reconstruct the tree. Bootstrap values higher than 50% are given at nodes. The scale bar represents the number of estimated substitutions per position for a unit branch length.

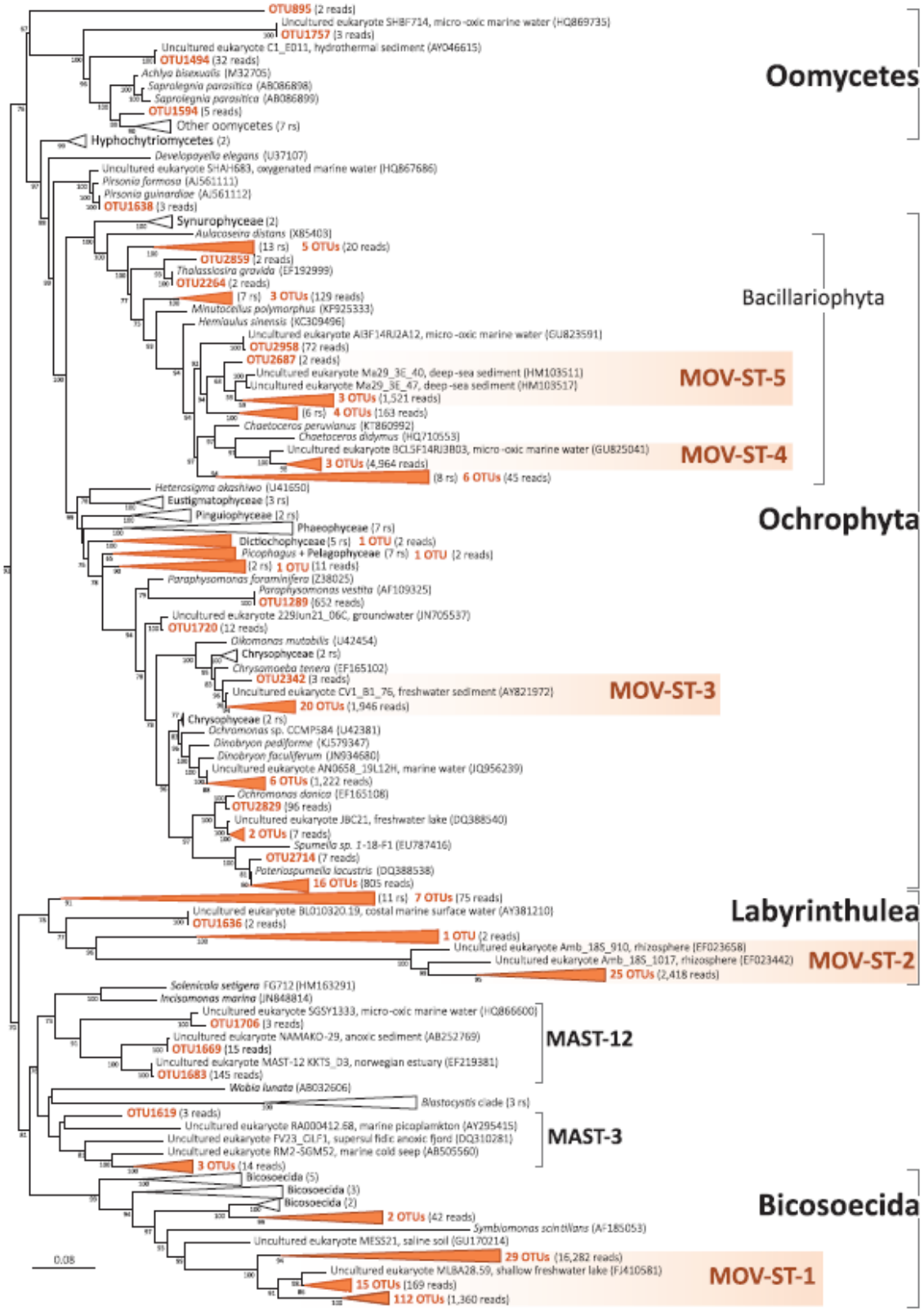

Fig. 5. ML phylogenetic tree of partial 18S rRNA gene sequences showing the position of OTUs affiliating to stramenopiles. The number of reads per OTU or group of OTUs as well as the number of reference sequences (rs) in the case of nodes that have been collapsed (triangles) is indicated. A total of 1,311

unambiguously aligned positions and 446 sequences were used to reconstruct the tree. Bootstrap values higher than 50% are given at nodes. The scale bar represents the number of estimated substitutions per position for a unit branch length.

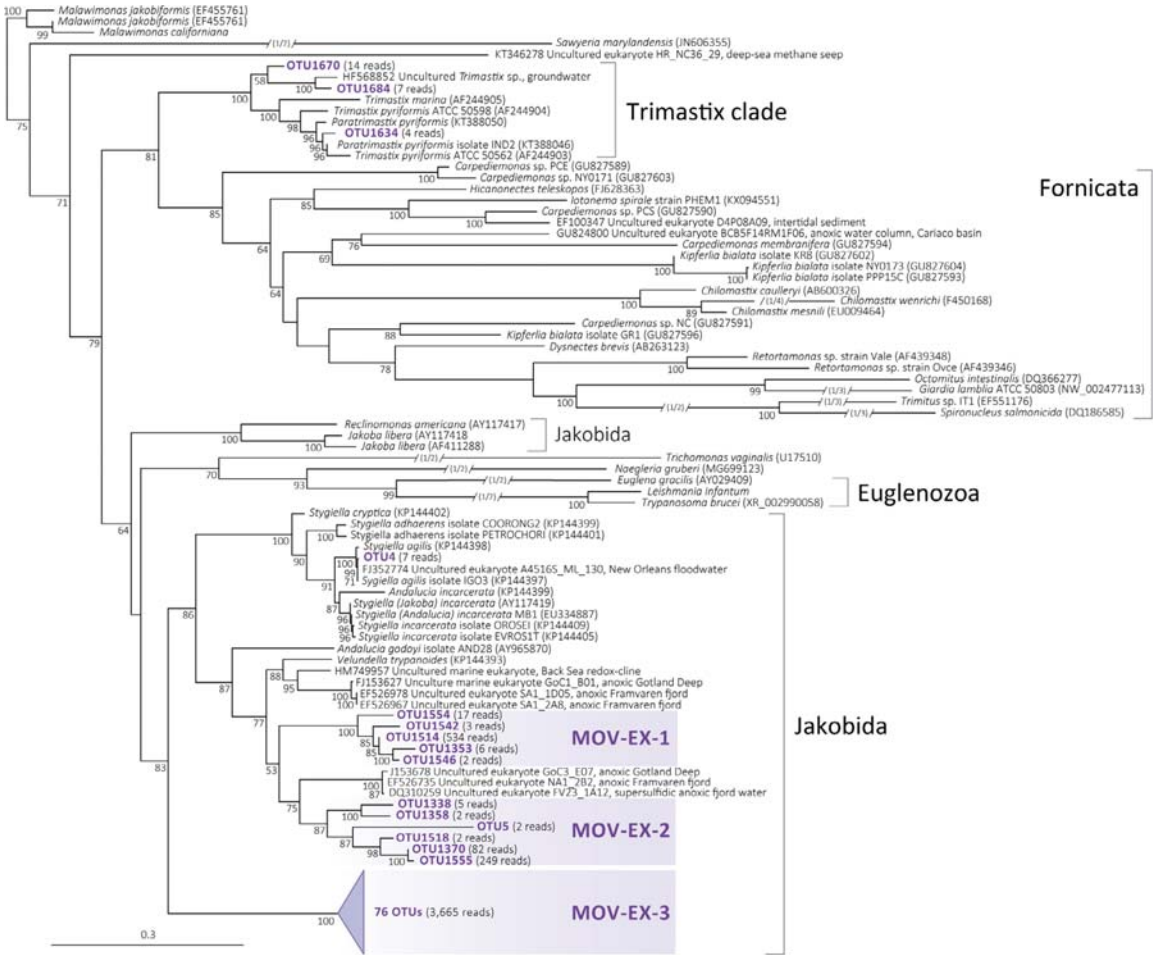

Fig. 6. Approximate ML phylogenetic tree of partial 18S rRNA gene sequences showing the position of OTUs affiliating to excavata. The number of reads per OTU or group of OTUs (triangles) is indicated. A total of 521 unambiguously aligned positions and 153 sequences were used to reconstruct the tree. The scale bar represents the number of estimated substitutions per position for a unit branch length.