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Short communication

Molecular Phylogenetics Evidence for a Novel Lineage of Amoebae Within Discosea (Amoebozoa: Lobosa)

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Abstract. Some amoebae were recovered from freshwater samples on agar plates. Due to a fungal contamination tightly associated with these amoebae, it was impossible to correctly characterize them on a morphological base, but sequences of the small subunit ribosomal RNA gene (SSU rDNA) were successfully obtained from three strains. Phylogenetic analysis performed on these SSU rDNA allowed to identify these amoebae as members of a new lineage, related to the Dermamoebida, which includes also several other environmental SSU sequences.

Key words: Amoebozoa, environmental 18S rDNA, small amoebae, Dermamoebida.

INTRODUCTION

In the past ten years, culture-independent surveys based on amplification and sequencing of 18S rRNA gene (18S rDNA), revealed an unexpected diversity of microbial eukaryotes in many types of habitats (Behnke *et al.* 2006, Dawson and Pace 2002, López-García *et al.* 2001, Moon-van der Staay *et al.* 2001, Richards *et al.* 2005, Slapeta *et al.* 2005). The majority of the obtained phylotypes could be assigned to well established groups or subgroups of eukaryotes (Berney *et al.* 2004), but some of them appeared however very divergent, forming likely novel high level taxa. The increasing record on 18S rDNA sequences from uncultured microbial eukaryotes, along with a re-analysis of reference strains and/or of new isolates, permitted also to elucidate some evolutionary relationships (Smirnov *et al*. 2008, 2009). We recovered from a previous study amoebae which appeared very small at direct observation. Two small-sized amoebae are already known, *Parvamoeba* (Rogerson 1993) and *Micriamoeba* (Atlan *et al*. 2012). The marine *Parvamoeba* is the smallest amoeba, < 6 μm, with discoidal cells, of unclear affinity (Cole *et al*. 2010), possibly related to Cochliopodiidae in the Flabellinia (Kudryavtsev 2012). *Micriamoeba* has slightly larger, 6–17 μm, vermiform cells. It was recently recovered from water treatment plant in France, and represents a new lineage of the Echinamoebida in the Tubulinea (Atlan *et al*. 2012). In previous molecular studies a few environmental 18S rDNA phylotypes formed an amoebozoan clade, LG-F, of unclear relationships (e.g., Richards *et al.* 2005, Slapeta *et al.* 2005,

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Kudryavtsev *et al.* 2011, Lara *et al.* 2011). We report herein molecular phylogenetic evidence that this clade forms a new lineage of amoebae, including several environmental sequences as well as our amoebae, closely related to Dermamoebida.

MATERIALS AND METHODS

Sample origin and DNA extraction

Amoebae were recovered from freshwater samples onto 1.5% non-nutritive agar (NNA) covered with *Escherichia coli*, during our former study aiming to search for chlamydiae in the environment (Corsaro and Venditti 2009). Amoebae showed rounded morphology with approximatively lengths of 6 × 7.5 µm. Strains am-R1 and am-CP10 originated from a river and a clear pond (North-Estern France) respectively, whereas strain am-MP3 originated from a mud pond (South Italy).

Amoebae were harvested from the agar plates, suspended in Page's Amoeba Saline (PAS) and rinsed three times in PAS at 200 × *g*. Whole DNA was extracted with the Wizard Genomic DNA kit (Promega) according to the manufacturer's recommendations. Amoebal 18S rRNA gene was amplified by using the eukaryotic primers 42F (5'-CTC AAR GAY TAA GCC ATG CA-3') and 1498R (5'-CAC CTA CGG AAA CCT TGT TA-3') (López-García *et al.* 2007), and 6F (5'-CCA GCT CYA AKA GCG TAT ATT-3) and 9R (5'-GTT GAG TCR AAT TAA GCC GC-3') (Corsaro *et al.* 2013), in reaction conditions of 5 min. at 94°C, followed by 35 cycles of 1 min. at 94°C, 1 min. at 56°C, and 2 min. at 72°C, with a final extension of 5 min. at 72°C.

Screening for chlamydiae and legionellae as endosymbionts was carried out by specific PCR, both directly from tiny amoeba extracts and after coculture in *Acanthamoeba* inoculated with tiny amoebae lysate (10 µl), as described previously (Corsaro and Venditti 2009; Corsaro *et al.* 2010a, 2010b).

Purified PCR products were sequenced with the same primer sets by using an automatic ABI DNA Sequencer (Applied Biosystems) with the BigDye Terminator Cycle kit. Sequences were edited by using BioEdit and analyzed through BLAST server to search for closest relatives. SSU rDNA sequences retrieved from GenBank were aligned by using MUSCLE v. 3.6. Molecular phylogenetic analyses were performed by applying distance (neighbor-joining, NJ) and maximum parsimony (MP) with MEGA5 (Tamura *et al.* 2011), and maximum likelihood (ML, GTR, G+I:4 model) with TREEFINDER (Jobb *et al.* 2004), with bootstrap values (BV) estimated after 1000 replications. Sequence similarity was calculated with BioEdit by pair-wise comparison, using all sites and indels but excluding introns, and by removing common and terminal gaps.

RESULTS AND DISCUSSION

Tiny amoebae were recovered on bacterized NNA tightly associated with contaminant fungi. Despite several attempts, we were unable to eliminate fungal contaminants and failed to provide satisfying morphological description. The only phenotypic trait available was their relative small size, < 10 µm.

PCR specific for chlamydiae and legionellae resulted negative for both tiny amoebae and inoculated *Acanthamoeba*.

We successfully amplified and sequenced SSU rDNAs from three distinct amoeba strains. At analysis, these strains resulted closely related each other, and showed at BLAST highest similarities (90–99%) with some uncultured eukaryotes and up to 93% with *Paradermamoeba levis* (Dermamoebida) and some other amoebozoans. We thus performed molecular phylogeny based on the nearly full SSU rDNA (Fig. 1), including these uncultured eukaryote sequences (> 1500 nt) and major representatives of Discosea, following the classification proposed by Smirnov *et al.* (2011).

By using members of Tubulinea as outgroup, the two classes of Tubulinea and Discosea were moderately supported (Fig. 1). Major orders within Tubulinea were highly supported, while in Discosea, the subclass Longamoebia emerged from the paraphyletic subclass Flabellinea. Within Longamoebia, Thecamoebida and Centramoebida were highly supported and emerged as sister-groups.

Dermamoebidae and Mayorellidae were each highly supported, but did not form an exclusive order Dermamoebida; rather these amoebae seemed to be intermixed into a larger cluster including also our strains, as well as *Stygamoeba* (Smirnov 1996, Lahr *et al.* 2011) and *Vermistella* (Moran *et al.* 2007). These two latter amoebae have been assigned to the same order Stygamoebida, in Flabellinia (Smirnov *et al*. 2011), on the basis of similar morphology. Nevertheless, both taxa appeared very unstable in 18S phylogenetic trees (Kudryavtsev *et al*. 2011, Lahr *et al.* 2011). By removing both *Stygamoeba* and *Vermistella*, Flabellinia was recovered as holophyletic (83% in ML), and Mayorellidae, Dermamoebidae and our strains clustered independently in a moderately supported clade (77% in ML) (Fig. 2). As suggested in some previous studies, *Vermistella* appears to be related to Dermamoebida and not specifically to *Stygamoeba*. The occasional clustering of the latter with these amoebae could be the result of an artifact. At present, both amoebae appear as *incertae sedis* and require further studies.

Most of the included uncultured eukaryotes cluster with our strains in a moderately supported lineage (74/83% BV in ML/NJ). The 'Amb-' and 'Elev-'

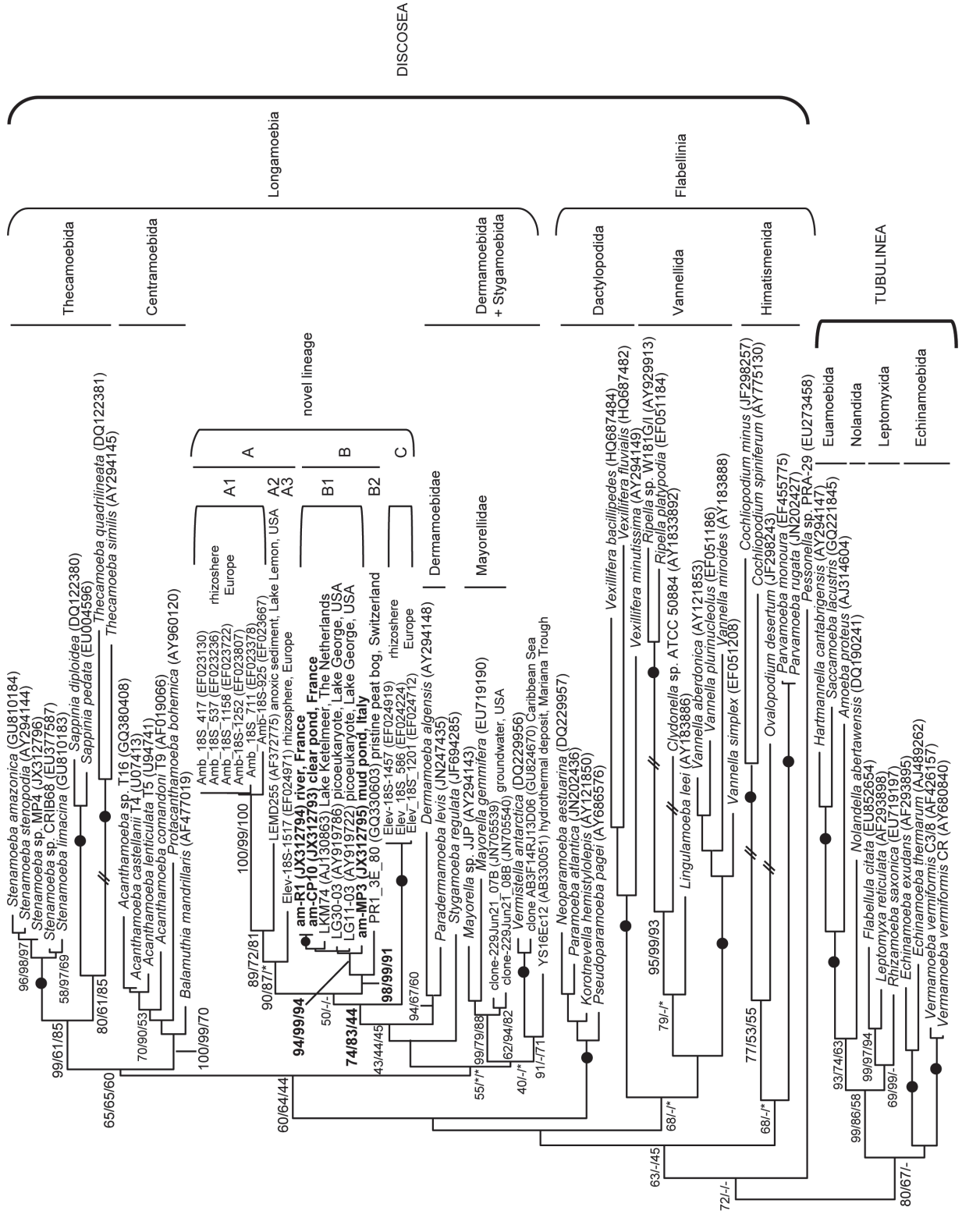

Fig. 1. Maximum-Likelihood tree based on SSU rDNA of major representatives of the subphylum Lobosa and the class Discosea, following the classification of Smirnov *et al.* (2011). Members of the class Tubulinea were used as outgroup. Subclasses and orders were indicated, and for Dermamoebida families also. Bootstrap values (BV) for ML/NJ/MP were presented at nodes; filled circles – 100% BV with all methods; * – node supported but BV < 40%.

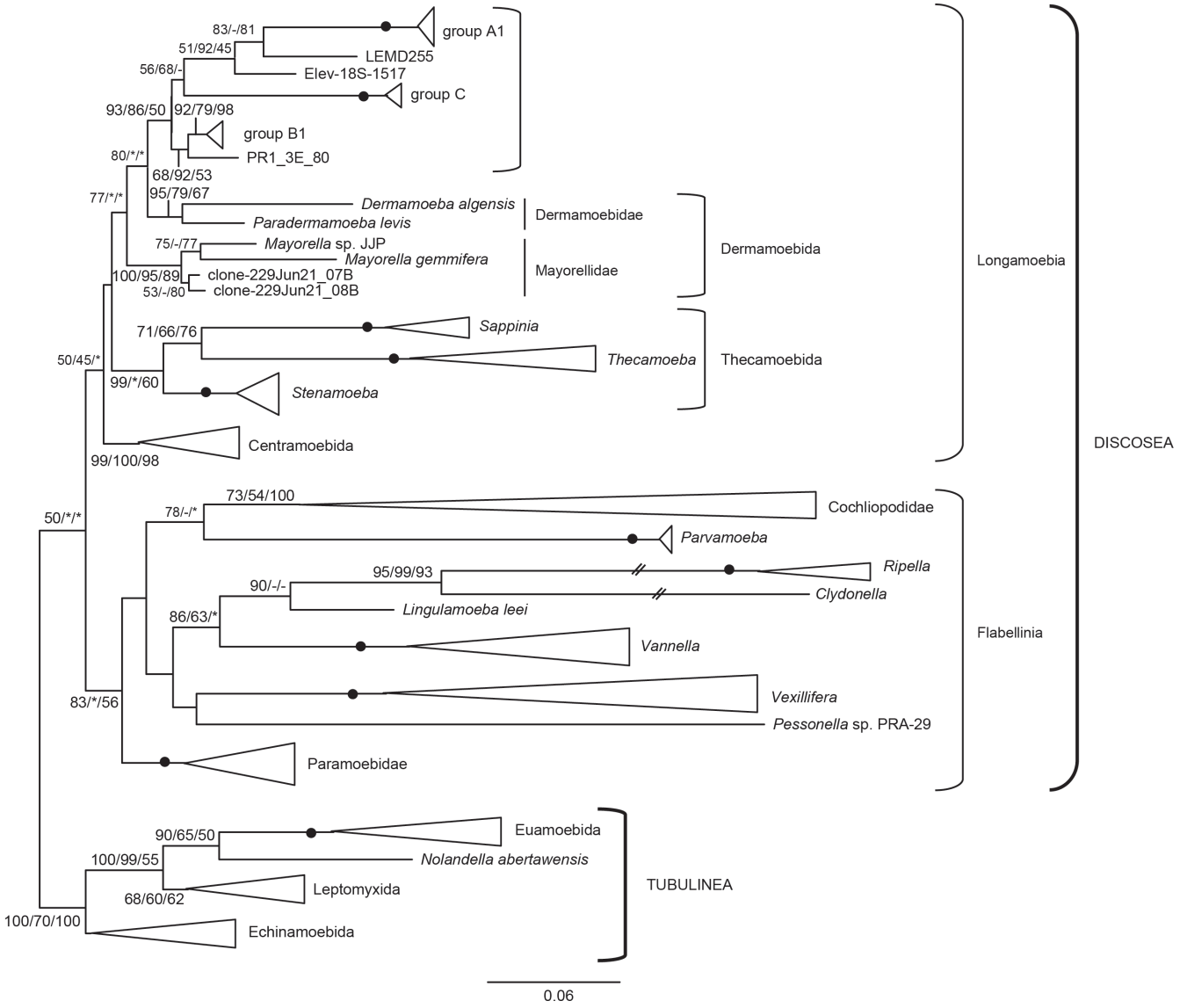

Fig. 2. Maximum-Likelihood SSU tree of subphylum Lobosa, with emphasis on major representatives of the class Discosea. The monophyletic resolution of Flabellinia and Longamoebia was obtained after omitting unstable taxa *Stygamoeba* and *Vermistella* (see Fig. 1). Members of the class Tubulinea were used as outgroup. Bootstrap values (BV) for ML/NJ/MP were presented at nodes; filled circles – 100% BV with all methods; * – node supported but BV < 40%. For acc. nos. – see Fig. 1.

sequences, originating from European trembling aspen rhizosphere under 'ambient' and 'elevated' CO_2 condition, respectively (Lesaulnier *et al.* 2008), and the clone LEMD255, originating from the anoxic sediment of lake Lemon, USA (Dawson and Pace 2002), form four distinct lineages, called here for convenience A1–A3 and C (Fig. 1). It should be noted that many of the 'Amb-' and 'Elev-' sequences have been misassigned to uncultured alveolates, as showed by us (this study) and by others (e.g., Smirnov *et al.* 2009). Our strains emerge in a highly supported (98/99/91% BV for ML/NJ/MP) holophyletic B lineage. The clone

PR1_3E_80, derived from an European pristine peat bog (Lara *et al.* 2011), is basal (subgroup B2), whereas uncultured picoeukaryotes from North American (LG11-03, LG30-03) and European (LKM74) lakes (van Hannen *et al.* 1999, Richards *et al.* 2005) form a more inclusive and supported subgroup B1 with our strains (Fig. 1). All members of the subgroup B1 share similarity values > 98%, and 90.6–91.0% with PR1_3E_80 (Table 1).

Through BLAST search, several additional partial (about 550-660-bp) SSU sequences were retrieved and/or identified herein as belonging to the group B. Two 660-bp sequences, corresponding to the SSU Ami portion (primer set 6F/9R), were distinct representatives of this clade (Fig. 3A) recorded in large number in a recent clone library study on drinking water system in USA (Buse *et al*. 2013). Other sequences corresponded to the 5' portion, just anterior to Ami fragment (Fig. 3B). One sequence, UF-75, was obtained by 18S rDNA clone library from urban fringe aerosols in Phoenix, Arizona, with particulate matter of diameter between 2.5 and 10 µm (PM10), and was misassigned to glomeromycotan fungi in the original report (Boreson *et al.* 2005). The remaining six sequences originated from an European sandy soil *Pinus* forest. These sequences are closely related to either the peat bog PR1_3E_80 or to our amoebae, sharing with them 93.5% and up to 99% sequence similarities, respectively, and as confirmed by phylogenetic analysis (Fig. 3).

As a whole, our strains and these uncultured eukaryotes emerge as a novel lineage within the subclass Longamoebia, closely related to the order Dermamoebida, either as sisterhood or as their new suborder or family.

This group is present in Europe and North America in both terrestrial and lacustrine environments, and probably shows a wider distribution. Filter-based sampling (Boreson *et al.* 2005, Richards *et al.* 2005) and direct observation of our strains (this study) indicate that small size, < 10 µm, could be typical, at least for the group B. Various studies showed that dispersal over long distances is inversely related to size (e.g., Heger *et al.* 2009, Yang *et al.* 2010, Wilkinson *et al.* 2012). Thus, such a small size might contribute to the wide distribution/cosmopolitanism of the group. Further efforts are needed to reisolate members in order to provide morphological description for full characterization of this lineage, for which we propose the informal name "Microdermamoebida".

Table 1. Pair-wise 18S rDNA sequence similarity values.

	Taxa/phylotypes	Novel lineage						Dermamoebida			Stygamoebida		
		A1	A2	A3	B1	B2	C						
		1	2	3	4	5	6	7	8	9	10	11	12
1.	A1 Amb clones[a]	99.1	84.8	86.1	84.1	83.6	85.5	74.1	73.1	69.6	73.7	79.2	76.8
2.	A2 LEMD255		100	84.0	82.4	81.6	84.4	73.5	73.8	73.0	78.0	78.2	76.7
3.	A3 Elev-18S-1517			100	88.0	87.3	85.5	76.8	76.6	73.2	77.8	82.4	80.5
4.	B1 LG/LKM[a]				98.8	90.7	81.9	76.1	78.2	73.1	77.7	82.8	81.0
5.	B2 PR1-3E-80					100	81.2	74.9	74.6	73.5	79.5	80.9	78.4
6.	C Elev clones[a]						99.6	73.8	73.2	71.6	75.5	79.4	77.6
7.	*Dermamoeba algensis*							100	75.6	71.6	73.2	75.0	76.3
8.	*Paradermamoeba levis*								100	72.0	73.2	77.9	78.1
9.	*Mayorella gemmifera*									100	76.7	74.2	74.6
10.	*Mayorella* sp. JJP										100	76.9	76.8
11.	*Vermistella antarctica*											100	82.2
12.	*Stygamoeba regulata*												100

[a] cluster of multiple phylotypes (see Fig. 1); mean sequence similarity values were used.

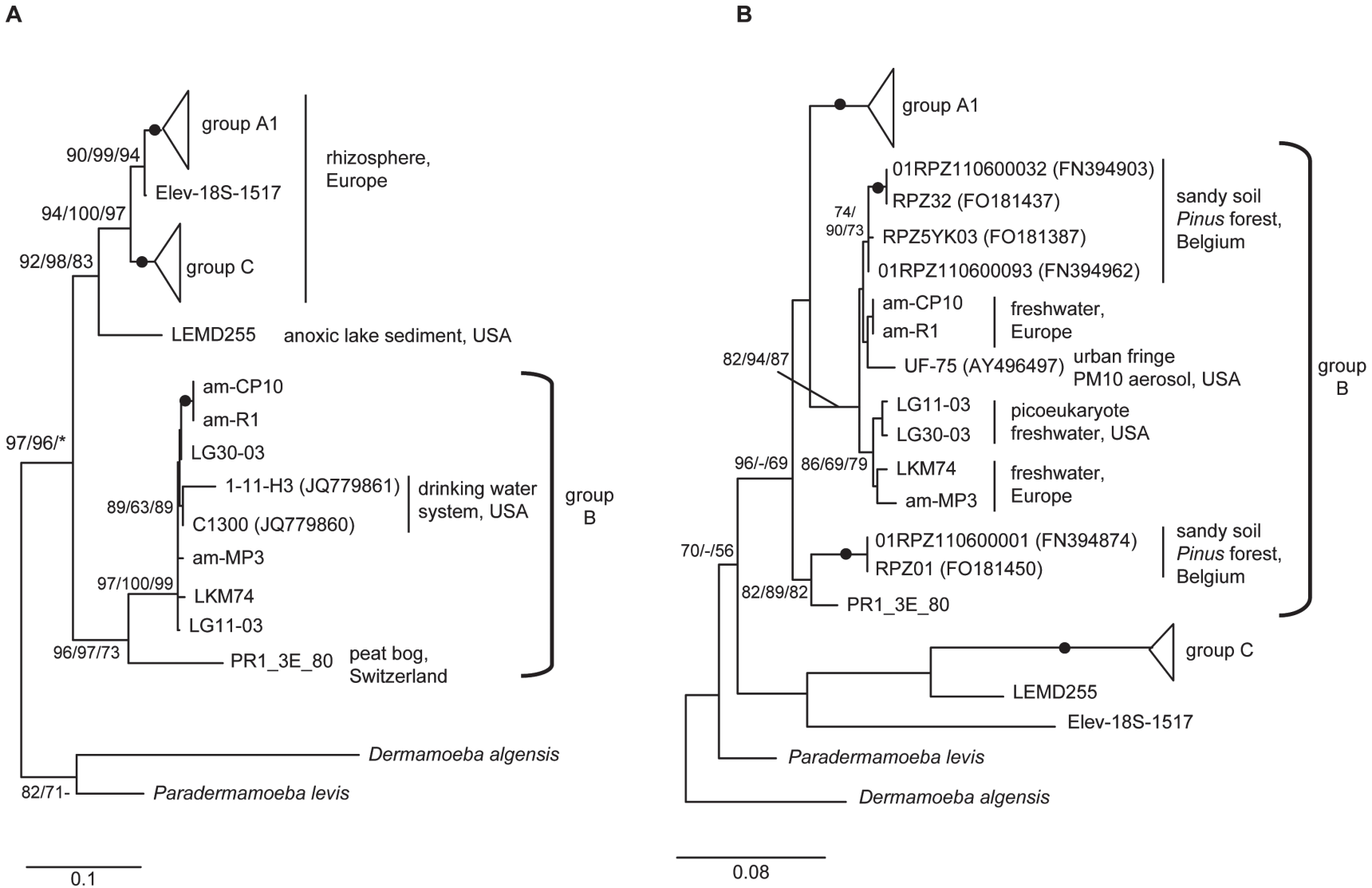

Fig. 3. Maximum-Likelihood trees including partial sequences retrieved from GenBank, based on the 660-bp Ami fragment (**A** – LG30-03 positions: 566-1228), and on the first 5' 550-bp portion (**B** – LG30-03 positions: 1-547). Trees were rooted on the Dermamoebidae. At nodes, BV for ML/NJ/MP (filled circles – 100% BV with all methods; * – node supported but BV < 40%). See Fig. 1 for acc. nos.

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