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REGULAR PAPER

Unexplored diversity of microscopic myxomycetes: evidence from environmental DNA

Oleg N. Shchepin[1,*], Martin Schnittler[2], Nikki H.A. Dagamac[2], Dmitry V. Leontyev[3,4] & Yuri K. Novozhilov[1]

[1]Komarov Botanical Institute of the Russian Academy of Sciences, Prof. Popov Str. 2, 197376 St. Petersburg, Russia
[2]Institute of Botany and Landscape Ecology, University of Greifswald, Soldmannstr. 15, 17487 Greifswald, Germany
[3]Department of Botany, H.S. Skovoroda Kharkiv National Pedagogical University, Valentynivska Str. 2, 61168 Kharkiv, Ukraine
[4]Department of Biotechnology, Kharkiv State Zooveterinary Academy, Akademichna Str. 1, 62134 Kharkiv, Ukraine
*Corresponding author: ledum_laconicum@mail.ru

Background and aims – Recent studies showed the position of two slime mould species with microscopic sporocarps, *Echinosteliopsis oligospora* and *Echinostelium bisporum*, within the class Myxomycetes. These minute species are seldom seen in studies based on detection of sporocarps and can easily be confused with protosteloid amoebozoans.
Methods – We searched all published ePCR data sets that targeted myxomycete 18S rDNA for the presence of environmental sequences similar to *E. oligospora* and Echinosteliales in traditional circumscription, and performed phylogenetic analyses that included short environmental sequences and full-length 18S rDNA sequences representing all the major groups of myxomycetes.
Key results – We report 19 unique sequences which are closely related to *E. bisporum* or *E. oligospora* based on sequence similarity (73.1–95.2% similarity) and which form well-supported monophyletic clades with these species in phylogenetic analyses. They may represent new species that are not yet described. Our phylogeny based on full-length 18S rDNA sequences further confirms the position of *E. bisporum* and *E. oligospora* within myxomycetes and the paraphyly of the order Echinosteliales in its traditional circumscription.
Conclusions – Our results show that ePCR-based studies can reveal myxomycete taxa that often escape detection by traditional approaches, including potentially new species, and thus provide valuable new data on diversity and ecology of myxomycetes. As such, strategies for studying myxomycetes biodiversity should be revised, focusing also on molecular detection techniques in addition to the sporocarp-based ones.

Keywords – 18S rDNA; Echinosteliales; *Echinosteliopsis*; *Echinostelium*; hidden diversity; slime moulds; SSU.

INTRODUCTION

Myxomycetes (or Myxogastrea in zoological nomenclature), also called plasmodial slime moulds, are a monophyletic group of free-living amoeboid protists (supergroup Amoebozoa, Kang et al. 2017) that have a unique combination of developmental stages: uninuclear amoeboflagellate cells, multinuclear plasmodia and fruiting bodies with internally produced spores (sporocarps). Although they spend the longest part of their life cycle as trophic stages (myxamoebae or myxoflagellates) living in different terrestrial and aquatic habitats, most of the data on diversity, ecology and distribution stem from collections of fruiting bodies (Stephenson et al. 2008; Novozhilov et al. 2017). This is explained by the fact that in spite of a few morphological and behavioral dif-

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ferences (Alexopoulos 1960; Hoppe & Kutschera 2015), trophic cells cannot be identified to species level. In contrast, sporocarps of the majority of known myxomycete species are quite large (1–10 mm for single sporocarps, up to several dm for compound fructifications) and sometimes brightly coloured, which makes them conspicuous enough for an easy detection in the field and in moist chamber cultures. Mature sporocarps can also be preserved as herbarium specimens for a long time.

However, this does not apply to all groups of myxomycetes. One of the five traditionally recognized orders, Echinosteliales, includes species that form extremely minute (microscopic) fruiting bodies. In a recently proposed phylogeny-based classification of myxomycetes this order is split into Echinosteliales and Clastodermatales due to its paraphyly (Leontyev et al. 2019), but for the sake of convenience we will here address the order Echinosteliales in its traditional circumscription (Lado & Eliasson 2017). In the members of Echinosteliales, sporothecae usually do not exceed 50 µm in diam. (up to 300 µm in *Echinostelium novozhilovii* A.Vlasenko, Vlasenko et al. 2018), contain only a limited number of spores (from 2 to c. 250), and have a stalk 10–150 µm long (up to 1500 µm in a few species and absent in *Semimorula liquescens* E.F.Haskins, McGuinn. & C.S.Berry). It is virtually impossible to notice them in the field, therefore almost all records of Echinosteliales come from agar (Haskins & Clark 2016) or moist chamber cultures (Schnittler et al. 2015). But even if a colony is detected, it is difficult to preserve it as a herbarium specimen since the tiny sporotheca can easily detach from the stalk. These circumstances make this group of myxomycetes the most difficult to study by traditional approaches. At least for detection, an approach combining moist chamber and agar cultures prepared from natural substrates, as outlined in Schnittler et al. (2015), seems to be the most promising. Even with these more sophisticated techniques one cannot expect that the diversity of Echinosteliales will be covered as well as that of macroscopic species.

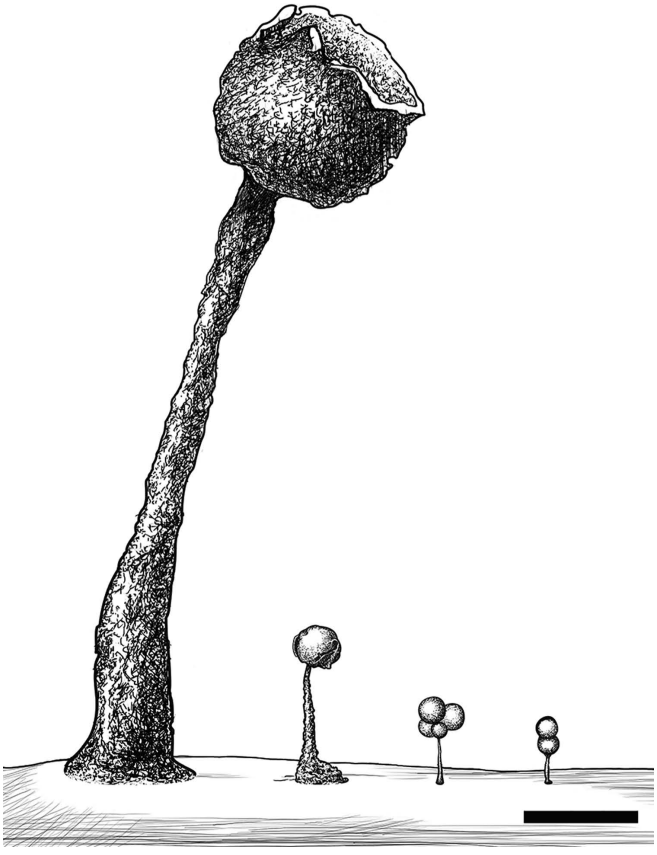

Figure 1 – From left to right: sporocarps of *Licea operculata*, *Echinostelium arboreum*, *Echinosteliopsis oligospora*, *Echinostelium bisporum*. The genus *Licea* comprises the smallest myxomycetes outside the Echinosteliales. Scale bar = 50 µm. Illustration by Elizaveta N. Shchepina.

At the moment, Echinosteliales include only 20 species that are accepted in the nomenclatural database of Lado (2005–2019). One of the species, *Echinostelium bisporum* (L.S.Olive & Stoian.) K.D.Whitney & L.S.Olive, was first described as a protosteloid slime mould *Cavostelium bisporum* (Olive & Stoianovitch 1966) but later was transferred to the genus *Echinostelium* (Whitney et al. 1982) based on ultrastructural traits. Proposing the new combination, these authors wrote: 'With fruiting bodies less than 30 µm high, *E. bisporum* is the smallest member of the myxomycetes. It is difficult to conceive of a smaller one awaiting discovery'. Indeed, this species with sporocarps containing only two spores still retains the position of the smallest one in the class (fig. 1). Its inclusion into Echinosteliales was recently confirmed in two molecular studies based on the analysis of nucleotide sequences derived from the same single isolate (Kang et al. 2017; Fiore-Donno et al. 2018). In addition, in these publications *Echinosteliopsis oligospora* D.J.Reinh. & L.S.Olive, a slime mould species with unknown affinity characterized by the absence of a flagellated stage, was assigned to myxomycetes. It is as well one of the smallest species of myxomycetes forming less than 10 spores per sporocarp. In phylogenies of Fiore-Donno et al. (2018) the single accession of *E. oligospora* appears either as a basal member of the dark-spored clade sister to its remaining species or as a member of Echinosteliales, and the authors conclude that it occupies an unresolved position within the dark-spored clade.

Considering the difficulties in detection of fruiting bodies of these microscopic myxomycetes, we screened the data sets of the few available studies that employed environmental PCR (ePCR) to explore myxomycete diversity. In this study we report and discuss environmental sequences belonging to the basal clade of the dark-spored myxomycetes clustering closely with *E. bisporum* and *E. oligospora*.

MATERIALS AND METHODS

Data mining

To search for environmental sequences closely related to the members of the order Echinosteliales in its traditional circumscription and to *Echinosteliopsis oligospora*, all sequences resulting from ePCR-based studies targeting myxomycete

Table 1 – Studies that employed environmental PCR to investigate myxomycete diversity.

Target group	Primers	Region	Habitat	Method	Threshold (%)	OTUs found	Reference
Didymiaceae, Physaraceae	phf1b/phr2b	Japan	Air	sequencing of DGGE bands	no	9	Kamono et al. 2009a
Didymiaceae, Physaraceae	f1b/phr2b	Japan	Soils of city parks	RT PCR, sequencing of DGGE bands	no	15	Kamono et al. 2009b
Didymiaceae, Physaraceae	phf1b/phr2b	Thailand	Deadwood and ground litter in tropical forest	sequencing of DGGE bands	no	13	Ko et al. 2009
Dark-spored myxomycetes	718RL, S2/ SP03r	French Alps, Scotland, Japan	Alpine soils	RT PCR, cloning	98.0	74	Kamono et al. 2013
Bright-spored myxomycetes	6 primer combinations	Central Germany	Deadwood in temperate beech forest	cloning	98.0	29	Clissmann et al. 2015
Dark-spored myxomycetes	S1/SR19Dark, S1/SF2Dark	Germany	Grassland soils	pyrosequencing	97.0	338	Fiore-Donno et al. 2016
Dark-spored myxomycetes	S1/SU19R, S3bF/S31R	Northern Caucasus	Alpine soils	Illumina MiSeq, cloning	99.1	27	Borg Dahl et al. 2018a
Dark-spored myxomycetes	S1/SU19R, S3bF/S31R	German Alps	Alpine soils	Illumina MiSeq	99.1	208	Borg Dahl et al. 2018b
Dark-spored myxomycetes	S3bF/S31R	Northwestern Russia	Ground litter and soil in boreal coniferous forest	Illumina MiSeq	99.1/98.0	187/101	Shchepin et al. 2019

18S rDNA were obtained from GenBank (table 1). Since filtering steps performed in the original analyses of Next Generation Sequencing (NGS) data could have removed sequences interesting for this study, we have re-analyzed three data sets where the raw sequencing data was available (Borg Dahl et al. 2018a, 2018b; Shchepin et al. 2019). The script used for the analyses is available as supplementary file 1. After quality filtering and *de novo* chimera detection steps these data sets resulted in 64, 396 and 2459 OTUs (operational taxonomic units), respectively, clustered with 98% similarity threshold, as substantiated in Shchepin et al. (2019). Together with the other environmental sequences from table 1, this summed up to 3297 environmental sequences.

The reference data set consisted of 48 full-length 18S rDNA sequences representing all major groups of bright- and dark-spored myxomycetes, including all available sequences belonging to Echinosteliales (nine sequences of eight species) and *E. oligospora* (one sequence). Reference sequences were compared with environmental sequences using 'usearch_global' command in VSEARCH version 2.6.2 (Rognes et al. 2016) with 70% similarity threshold for matches. Nineteen environmental sequences that had the best match to the members of Echinosteliales or to *E. oligospora* were included in the further analyses. These selected environmental sequences together with ten reference sequences were searched with BLASTn across the GenBank Nucleotide collection, resulting in one additional environmental sequence with a close match to *E. bisporum* (query cover 100%, identity 95%). The detailed information on the environmental sequences (similarity to references, region and substrate of origin etc.) is given in table 2.

Phylogenetic analysis

The same set of 48 full-length 18S rDNA reference sequences was aligned with MAFFT 7 online service (Katoh et al. 2017) using the E-INS-i option (Katoh et al. 2005) and default gap penalties. From the total of 14637 positions 1233 well-aligned positions were chosen using GBlocks version 0.91b (Talavera & Castresana 2007) with parameters set as follows: 'Allowed Gap Positions' = 'half', 'Minimum Number of Sequences for a Flank Position' = 65%. Twenty environmental sequences with truncated primer regions were added to the reference alignment with MAFFT online service using options 'addfragments' and 'keeplength'. Since one of the OTUs had a long insertion in a conservative region of the alignment, it was excluded from the further analysis. The resulting alignment (supplementary file 2) was truncated according to the mask obtained with GBlocks.

Phylogenetic analysis was carried out with Maximum Likelihood (ML) and Bayesian inference (BI). ML was run on IQ-Tree version 1.6.8 web server (Trifinopoulos et al. 2016) with 1000 replicates of ultrafast bootstrap (Minh et al. 2013) and with the optimal substitution model (SYM+R4) chosen with ModelFinder (Kalyaanamoorthy et al. 2017) according to BIC tests. BI was computed with MrBayes version 3.2.1 (Huelsenbeck & Ronquist 2001) using one cold and three heated Monte Carlo Markov chains in four simultaneous runs with the evolutionary model set to GTR+G4+I. The number of generations, sample frequencies and burn-in ratio were set to 20 million, 1000 and 0.25, respectively. Clade confidence scores resulting from BI analysis were transferred to the ML tree using IQ-Tree. Alignment and tree were submitted to TreeBase (S23604).

Table 2 – List of environmental 18S rDNA sequences closely related to the members of Echinosteliales or to *Echinosteliopsis oligospora*.

Env. sequence	GenBank accession	Best match	Similarity (%)	Data set	Region	Samples (amount and type)	Altitude (m a.s.l.)
OTU370	MK178532	Echinosteliopsis oligospora MH809394	81.6	Borg Dahl et al. 2018b (re-analyzed at 98% similarity)	Germany, German Alps, below the Alpspitz summit	1 meadow soil	1400
OTU709	MK111082	Echinosteliopsis oligospora MH809394	92.4	Shchepin et al. 2019 (98% similarity OTUs)	Russia, Leningrad region, Nizhne-Svirskiy Reserve	1 forest ground litter	25
OTU762	MK111083	Echinosteliopsis oligospora MH809394	92.9	Shchepin et al. 2019 (98% similarity OTUs)	Russia, Leningrad region, Nizhne-Svirskiy Reserve	3 forest ground litter	25
Uncultured eukaryote UD_67	JQ900843	Echinosteliopsis oligospora MH809394	95.2	Kamono et al. 2013 (original data)	Japan, Hokkaido, Uryu experimental forest	forest soil	595
Uncultured eukaryote e4_1_15	GQ462942	Echinostelium bisporum MH809395	95.0	Suutari et al. 2010 (original data)	Panama, Barro Colorado Island	1 bark of a living tree	25–145
OTU365	MK178538	Echinostelium bisporum MH809395	79.9	Shchepin et al. 2019 (98% similarity OTUs)	Russia, Leningrad region, Nizhne-Svirskiy Reserve	3 forest ground litter	25
OTU255	MK178533	Echinostelium bisporum MH809395	82.4	Shchepin et al. 2019 (98% similarity OTUs)	Russia, Leningrad region, Nizhne-Svirskiy Reserve	6 forest ground litter	25
OTU104	MK178534	Echinostelium bisporum MH809395	83.5	Shchepin et al. 2019 (98% similarity OTUs)	Russia, Leningrad region, Nizhne-Svirskiy Reserve	5 forest ground litter	25
OTU81	MK178541	Echinostelium bisporum MH809395	83.8	Borg Dahl et al. 2018b (re-analyzed at 98% similarity)	Germany, German Alps, below the Alpspitz summit	1 meadow soil	2050
OTU1123	MK178527	Echinostelium bisporum MH809395	84.1	Shchepin et al. 2019 (98% similarity OTUs)	Russia, Leningrad region, Nizhne-Svirskiy Reserve	1 forest ground litter	25
OTU36	MK178535	Echinostelium bisporum MH809395	84.8	Shchepin et al. 2019 (98% similarity OTUs)	Russia, Leningrad region, Nizhne-Svirskiy Reserve	5 forest ground litter	25
OTU182	MK178528	Echinostelium bisporum MH809395	84.9	Shchepin et al. 2019 (98% similarity OTUs)	Russia, Leningrad region, Nizhne-Svirskiy Reserve	3 forest ground litter	25
OTU51	MK178536	Echinostelium bisporum MH809395	84.9	Shchepin et al. 2019 (98% similarity OTUs)	Russia, Leningrad region, Nizhne-Svirskiy Reserve	8 forest ground litter, 2 forest soil	25
OTU917	MK178539	Echinostelium bisporum MH809395	85.8	Shchepin et al. 2019 (98% similarity OTUs)	Russia, Leningrad region, Nizhne-Svirskiy Reserve	3 forest ground litter	25
OTU1598	MK178529	Echinostelium bisporum MH809395	86.2	Shchepin et al. 2019 (98% similarity OTUs)	Russia, Leningrad region, Nizhne-Svirskiy Reserve	1 forest ground litter	25
OTU160	MK178537	Echinostelium bisporum MH809395	87.3	Shchepin et al. 2019 (98% similarity OTUs)	Russia, Leningrad region, Nizhne-Svirskiy Reserve	1 forest ground litter	25
OTU137	MK178530	Echinostelium bisporum MH809395	87.9	Shchepin et al. 2019 (98% similarity OTUs)	Russia, Leningrad region, Nizhne-Svirskiy Reserve	4 forest ground litter	25
OTU228	MK178531	Echinostelium bisporum MH809395	88.7	Shchepin et al. 2019 (98% similarity OTUs)	Russia, Leningrad region, Nizhne-Svirskiy Reserve	6 forest ground litter	25
OTU1082	MK178540	Echinostelium bisporum MH809395	88.8	Shchepin et al. 2019 (98% similarity OTUs)	Russia, Leningrad region, Nizhne-Svirskiy Reserve	3 forest ground litter	25

RESULTS

Investigating the available sequence data from nine ePCR-based studies targeting the diversity of the trophic stages of myxomycetes (table 1), in three of these we found a total of 14 environmental sequences that had best matches to *Echinostelium bisporum* (73.1–88.8% similarity) and 4 to *Echinosteliopsis oligospora* (81.6–95.2% similarity). In addition, a BLASTn search in GenBank Nucleotide collection retrieved another sequence with 95% similarity to *E. bisporum*, labeled as an uncultured eukaryote from a tree bark in Barro Colorado Island, Panama (table 2). Phylogenetic analysis of the full-length 18S rDNA reference sequences and short environmental sequences resulted in a well-resolved phylogeny

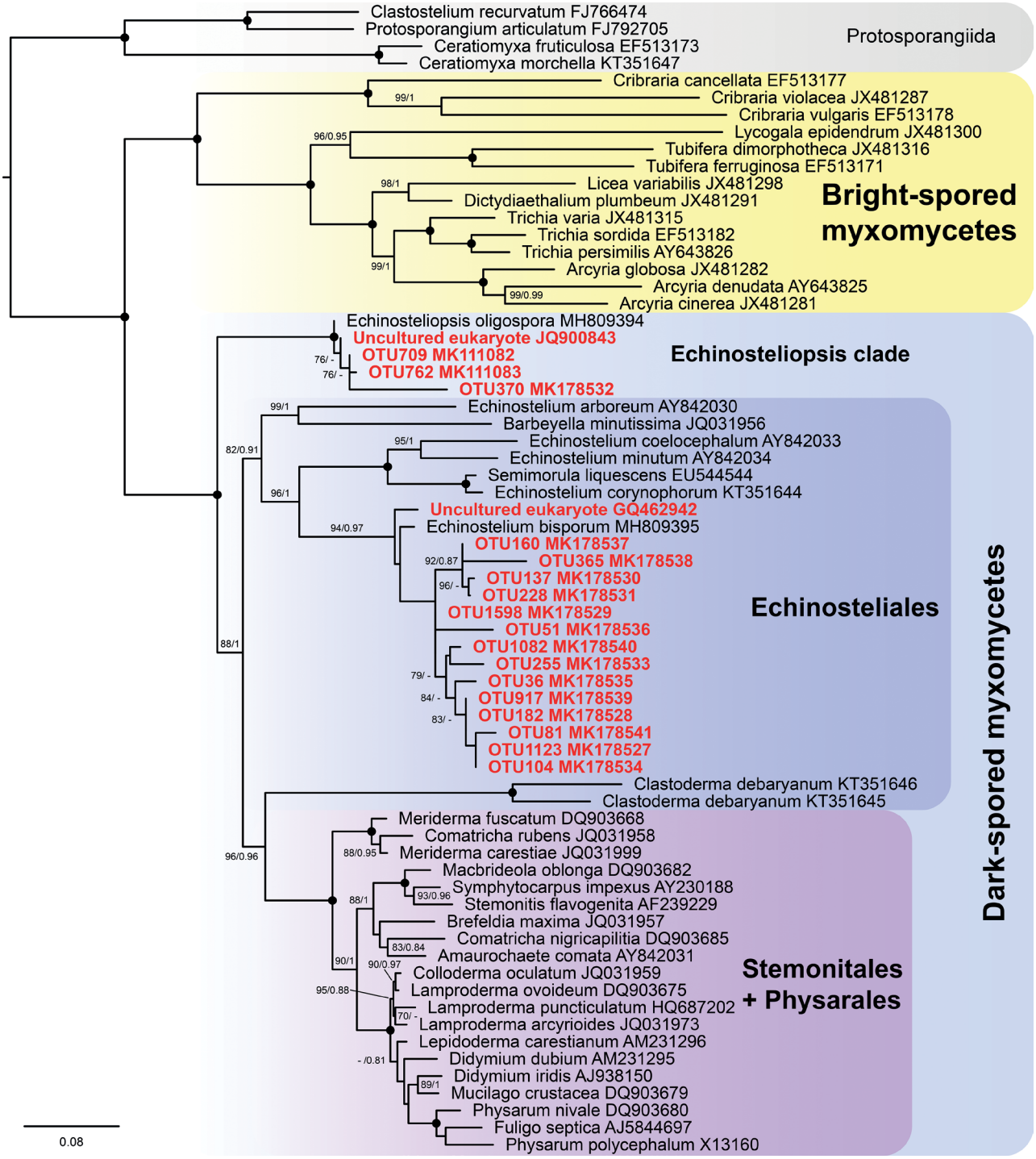

Figure 2 – Maximum Likelihood phylogenetic tree based on full-length 18S rDNA sequences showing the position of environmental sequences (marked in red) within myxomycetes. Ultrafast bootstrap/posterior probability support values ≥ 70/0.7 are indicated near the branches. Fully supported branches (100/1.00) are marked with solid circles.

of myxomycetes with high values of ultrafast bootstrap support and posterior probabilities for most of the major branches (fig. 2). The position of the environmental sequences in the phylogeny is also well-supported and confirms that they are closely related to *E. bisporum* and *E. oligospora*. The *Echinosteliopsis* clade, which now contains one isolate-derived and four environmental sequences, represents a fully supported basal clade of the dark-spored myxomycetes sister to the remaining dark-spored species. The order Echinosteliales in its traditional circumscription appears paraphyletic, as *Clastoderma debaryanum* branches together with the other dark-spored myxomycetes, but not with the members of the order.

Environmental sequences from NGS-based studies that are related to *E. oligospora* have a length 358–366 bp, whereas those related to *E. bisporum* span 269–275 bp. This corresponds well to the fragment size covered by the primers S3bF/S31R in reference sequences of these two species (fig. 3). None of the environmental sequences show any obviously erroneous positions in highly conservative regions, except for the one with long insertions that was excluded from the analysis. Surprisingly, no sequences related to the genus *Clastoderma* were retrieved in the analyzed ePCR data, although reference sequences of *Clastoderma debaryanum* have the lowest number of mismatches to this primer pair among the members of Echinosteliales in its traditional circumscription.

DISCUSSION

Recent studies showed that two slime mould species with extremely minute sporocarps, *Echinosteliopsis oligospora* and *Echinostelium bisporum* (initially described as a protosteloid amoebae), belong to myxomycetes. *Echinosteliopsis oligospora*, the only described member of its genus, differs from all other known myxomycete species in that it does not possess a flagellated stage. The topology of our tree based on full-length 18S rDNA sequences is in agreement with the two-gene ML phylogeny of Fiore-Donno et al. (2018) and shows the position of *E. oligospora* as a sister clade to all the remaining dark-spored myxomycetes. Together with four environmental sequences that are not more than 95.2% similar to it, *E. oligospora* forms a fully supported and well-separated clade that probably deserves an erection of a higher-rank taxon of its own if its position will be corroborated with additional molecular markers. The presence of environmental sequences that cluster closely with the reference sequence but show genetic differences much bigger than 98–99.1% (the level reported as an average intraspecific variation for the studied fragment of 18S rDNA in dark-spored myxomycetes, see Borg Dahl et al. 2018a) suggests that there is a number of species closely related to *E. oligospora* that are not yet described.

Echinostelium bisporum, the tiniest known myxomycete species and the only one lacking a multinucleate plasmodial stage, occupies in our phylogeny the same position within Echinosteliales as in Fiore-Donno et al. (2018). Together with 15 environmental sequences it forms a well-supported clade within the group *Echinostelium-Barbeyella-Semimorula*, while *Clastoderma* branches separately. This topology reproduced in an independent analysis strengthens the conclusion of Fiore-Donno et al. (2018) about the paraphyly of Echinosteliales and supports the description of a separate higher-order taxon for *Clastoderma* that was done by Leontyev et al. (2019). However, so far this concerns *C. debaryanum* only, whereas no sequence data are available for the two other species described in the genus. The diverse environmental sequences closely related to *E. bisporum* might represent either new species with minute sporocarps waiting to be described in the future or some of the 11 species of Echinosteliales that were described but not yet sequenced.

The four environmental sequences related to *E. oligospora* and 14 related to *E. bisporum* come from soil and plant litter samples from different regions: high-altitude meadows of the German Alps, lowland taiga in northwestern Russia, and a low mountain forest in northern Japan (table 2). One additional sequence from the *E. bisporum* group was derived from a bark of a living tree in Panama. All these substrates are typical for *E. bisporum*. While the only known substrate for isolation of *E. oligospora* is a dead plant material, our study is also the first to show that it can occur in soil as well. According to GBIF.org (2019a), *E. oligospora* occurs in very wide geographical ranges, reaching from the North America over Europe and Africa to Asia and Australia, and in nearly all vegetation zones, from tropical to boreal forests. For *E. bisporum* GBIF shows similarly wide geographical ranges (GBIF.org 2019b). However, we expect that a more thorough investigation may show that the organisms morphologically identified as *E. oligospora* and *E. bisporum* are two complexes of cryptic species with their members occupying different ecological niches and showing narrower areas of distribution, as it is the case for a number of other species of

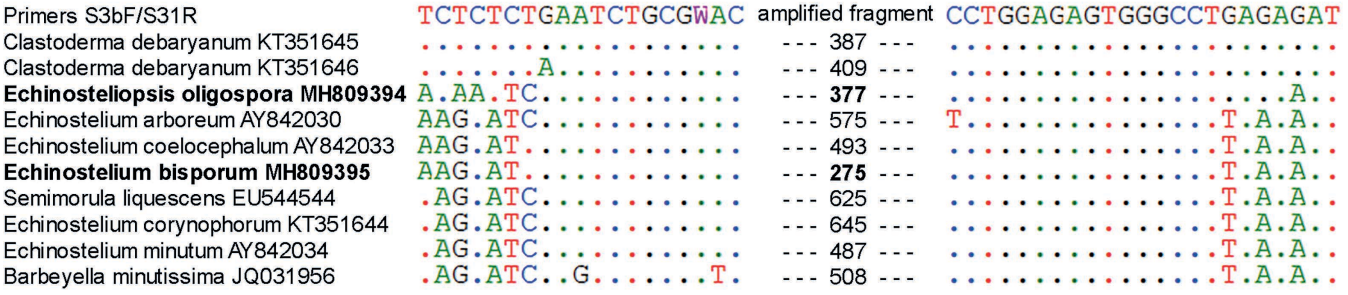

Figure 3 – Comparison of the regions of 18S rDNA sequences of Echinosteliales in traditional circumscription and *E. oligospora* covered by the primer pair S3bF/S31R that produced most of the environmental sequences considered in this study.

myxomycetes (Aguilar et al. 2013; Novozhilov et al. 2013; Feng & Schnittler 2015; Feng et al. 2016; Shchepin et al. 2016; Dagamac et al. 2017).

In comparison to virtually all other myxomycetes, all the species mentioned above are an order of magnitude smaller (fig. 1). These microscopic species are seldom seen and can easily be confused with protosteloid amoebozoans. If there are more forms of Echinosteliales that have lost the ability to form a stalk (like it happened in *Semimorula liquescens*, see Fiore-Donno et al. 2009), this makes the detection of their fructifications even more difficult. Considering this, we think that the diversity of Echinosteliales and *Echinosteliopsis* is now underestimated. As such, strategies for studying myxomycetes biodiversity should be revised, focusing also on molecular detection techniques in addition to the sporocarp-based ones.

Surprisingly, our data mining did not yield any environmental sequences related to any other members of Echinosteliales represented in the reference data set except for *E. bisporum*. This is especially strange for *Clastoderma debaryanum*: in contrast to other Echinosteliales, the primers S3bF/S31R have no or almost no mismatches to its available sequences and cover a fragment 389–407 bp in length which is not overly long for Illumina sequencing (fig. 3). A possible explanation may be that *C. debaryanum* is more specialized in substrate preferences and rarely occurs in soil and forest floor litter.

SUPPLEMENTARY FILES

Two supplementary files are associated to this paper:
(1) The script used for the analyses of NGS-based data sets (pdf)
https://doi.org/10.5091/plecevo.2019.1621.1897
(2) The alignment of reference 18S rDNA sequences of myxomycetes together with environmental sequences discussed in this study (FASTA). The mask produced by GBlocks is included as the first line.
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