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Characterization of myxomycetes in two different soils by TRFLP-analysis of partial 18S rRNA gene sequences

T. Hoppe[1] and M. Schnittler[1]

[1] *Institute of Botany and Landscape Ecology, Ernst Moritz Arndt University of Greifswald, Soldmannstr. 15, D-17487 Greifswald, GERMANY. e-mail: thomas.hoppe@uni-greifswald.de*

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Abstract

Terminal Restriction Fragment Length Polymorphism (TRFLP) as a molecular technique was adapted to compare myxomycete communities based on genomic DNA extracted from soil. The 18S rRNA gene was amplified by universal primers for dark-spored myxomycetes and digested with the restriction enzyme Hha*I* to obtain fragment polymorphisms. To establish a database for the identification of fragments, we analyzed 167 specimens representing 96 myxomycete species. The specific restriction sites for Hha*I* were determined and a data bank was constructed. Expected fragment sizes were verified by digesting a mock sample generated from DNA aliquots of seven different species. TRFLP profiles were generated from two soil samples. Differences in the composition of the respective myxomycete communities can be shown by comparison of the generated fragment length pattern community. The potential of the technique and difficulties in species identification from fragment sizes are discussed.

Keywords – Community analysis – Dark-spored myxomycetes – environmental PCR – Terminal Restriction Fragment Length Polymorphism

Introduction

Myxomycetes or true slime molds are amoebozoan protists, characterized by three life stages and a complex nuclear cycle (Clark & Haskins 2013). Unicellular amoebae form, usually by syngamy, syncytial plasmodia which convert most of its biomass into a fruiting body which releases meiotic spores. Whereas vegetative plasmodia are mainly negatively phototactic, with the onset of fructification they become positively phototactic. Therefore, in contrast to the often hidden plasmodia, the immobile fructifications can be easily collected on decaying wood or leaf litter. Solely based on the minute, yet for most species macroscopic fructifications, a considerable body of data on world-wide occurrence of myxomycetes exists (Stephenson et al. 2008). The current morphospecies concept relies on the morphological characters displayed by the fructifications (Clark 2000, Schnittler & Mitchell 2000), whereas amoebae or plasmodia cannot be determined to species (Feest 1985). In the amoebal or plasmodial stage, only larger groups of myxomycetes can be told apart (Hoppe & Kutschera 2014) which sometimes corresponds with plasmodial types (Alexopoulos 1960).

However, Stephenson & Feest (2012) suggested that in most habitats the amoeboid stage is the prevailing life from, since only under favorable conditions, and perhaps only if a critical density

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Corresponding Author: Thomas Hoppe – e-mail – thomas.hoppe@uni-greifswald.de

in amoebal populations is reached, fructification takes place (see discussion in Clark & Haskins 2013). For a deeper understanding of myxomycete ecology the composition of amoebal communities in soil is a key factor (Stephenson et al. 2011). Therefore, developing molecular techniques to tell apart different myxamoebal communities without the need of cultivation (which will most probably select for members of the Physarales, Clark and Haskins 2012) would be a step forward. Denaturing Gradient Gel Electrophoresis (DGGE) as a molecular technique was successfully applied to distinguish myxamoebal communities (Ko Ko et al. 2009). Terminal Restriction Fragment Length Polymorphism (TRFLP), a technique often used to investigate communities of prokaryotic micro-organisms (Liu et al. 1997) may be a cheaper and more time-efficient solution yielding similar results.

The precondition for this technique is a well-known gene fragment which is sufficiently variable within the group under consideration. For protists, one candidate marker are partial 18S rRNA sequences, which were successfully used a) to construct phylogenies for major groups of myxomycetes (Fiore-Donno et al. 2008, 2012, 2013) b) to ascertain the systematic position of a species (Erastova et al. 2013, Novozhilov et al. 2013) and c) to differentiate between closely related species (Novozhilov et al. 2012). Amplicons for partial 18S rRNA sequences are typically 500–700 bases in length, which simply by chance yields one to several cutting positions for restriction endonucleases. With primers that are specific for larger groups of myxomycetes (like dark- or bright-spored myxomycetes), after PCR and treatment with restriction endonucleases the fragments can be visualized on a sequencer, if one of the primers is labelled with a fluorescent probe. Given a sufficiently variable gene, the position of the cutting position for the restriction enzyme should vary among myxomycete species, producing a fingerprint composed of differently sized fragments characteristic for each amoebal community. At least in theory, some abundant species may be identified if they show fragments of unique length, since the size of the expected fragments can be inferred from partial 18S rRNA sequences obtained from fructifications of morphologically identified specimens. However, bulk DNA isolated from soil represents a mixture of different organisms (not only myxomycetes). Consequently, the success of the method depends highly on primer specificity (Liu et al. 1997, Li et al. 2007, Pasternak et al. 2013). The primer pair used to amplify this marker for dark-spored myxomycetes seems to fulfill these requirements: amplifying virtually all myxomycetes of this group but largely excluding other soil protists. In this study, we provide an assessment of the putative diversity in TRFLP fingerprints for dark-spored myxomycetes and show the fingerprints of two soil communities analyzed with this approach.

Material & Methods

Expected fragment sizes – To assess putative TRFLP patterns, an alignment of 133 partial 18S-rRNA sequences from NCBI was constructed to look for an initial variation in positions of digesting sites, ie. expected fragment lengths. MEGA 6 (Tamura et al. 2013) was used for an automated scanning of the restriction site. In addition, DNA was extracted from sporocarps of 34 species and amplified with the primer pair S1 / SU19Rsp as described in Fiore-Donno et al. (2008). The products were used as template for a second PCR. For this nested PCR we designed a new forward primer that amplifies a shorter sequence which still includes all regions variable for myxomycetes in the first amplicon (S3b F: TCT CTC TGA ATC TGC GW AC, SU19Rsp: TGT CCT CTA ATT GTT ACT CGA). Products were first sequenced (ABI 310, Applied Biotechnologies, USA) and analyzed for putative digesting sites for the restriction enzymes Hha*I*, Mbo*I*, HpyCH4*III*, Mae*II* and Bsh1236*I*, searching for sites in the respective alignment with BioEdit 7.2.0 (Hall 1998).

Verifying fragment sizes – A mock sample of genomic DNA from seven morphological determined species was constructed by creating a roughly equimolar mixture of isolated DNA, pooling 0.5 µl of isolated DNA amplified with the primers S1-HEX / SU19Rsp to a final volume of 20 µl (the primer S1 was labelled with the fluorophore HEX at the 5'-end; Invitrogen, Germany). The PCR-product was digested with Hha*I* and the respective electroferogram was analyzed with

GeneMarker V2.2.0 (SoftGenetics, USA). Included were *Diachea leucopodia* (Bull.) Rostaf. (specimen MYX79; Genbank KM977849), *Diderma globosum* var. *europaeum* Buyck (MYX443; KM977852), *Didymium nigripes* (Link) Fr. (MYX51; KM977859), *Fuligo intermedia* T. Macbr. (MYX175; KM977862), *F. licentii* Buchet (MYX305; KM977864), *F. septica* var. *flava* Pers. (MYX1240; KM977867) and *Physarum polycephalum* Schwein. (MYX15; KP323383).

TRFLP profiles from soil myxomycete communities – To fingerprint myxomycete communities in soil, samples were collected from two different localities near Siegen (Germany, B29, 50°50'23.3"N/ 08°02'11.4"E, and B75, 50°50'23.7"N/ 08°02'16.0"E, see Hoppe 2013), air dried and stored in sterile plastic bags. About 0.7 g of soil was used for the DNA isolation applying a kit designed for samples rich in humid acids (NucleoSpin Soil, Machery & Nagel, Germany). After a PCR using the primers S1/SU19Rsp (Annealing Temp. 57 °C, 30–40 cycles) the product was used for a second PCR with the primers S3b F-HEX/SU19Rsp. Subsequently the PCR product was incubated (2 h, 37 °C) with Hha*I* (Fermentas, Germany). For a preliminary assessment fragments were visualized by polyacrylamide gel electrophoresis. For exact determination of fragment length, the labeled fragments were analyzed with a sequencer (ABI310, Applied Biosystems, USA); the resulting fingerprints were analyzed with GeneMarker V2.2.0 (SoftGenetics, USA).

Results

Expected fragment sizes – A dataset for 167 partial 18S rRNA sequences from 96 species covering 20 genera of dark-spored myxomycetes was generated (126 sequences were obtained from NCBI, 34 sequences added by this study, Table 1) and deposited in a web-based tool for the analysis of microbial communities (Shyu et al. 2007, http://mica.ibest.uidaho.edu/). Fragment lengths of amplicons that can be expected for a digestion with Hha*I* ranged from 124 to 692 bp (Fig. 1), whereas the variation for all other restriction endonucleases was much lower. If the forward primer (S3b F) is labelled, detectable fragments include Hha*I* cutting sites which are mainly situated within the variable part of the 18S rRNA gene (helices E8_1, but especially 10, E10_1 and 11, compare Fig. 4 in Fiore-Donno et al. 2012). Due to a second digesting site near the much conserved 3'-end of the amplicon, putative Hha*I* fragments for SU19Rsp as the labelled primer would be very short and almost identical in length (15 to 20 bases).

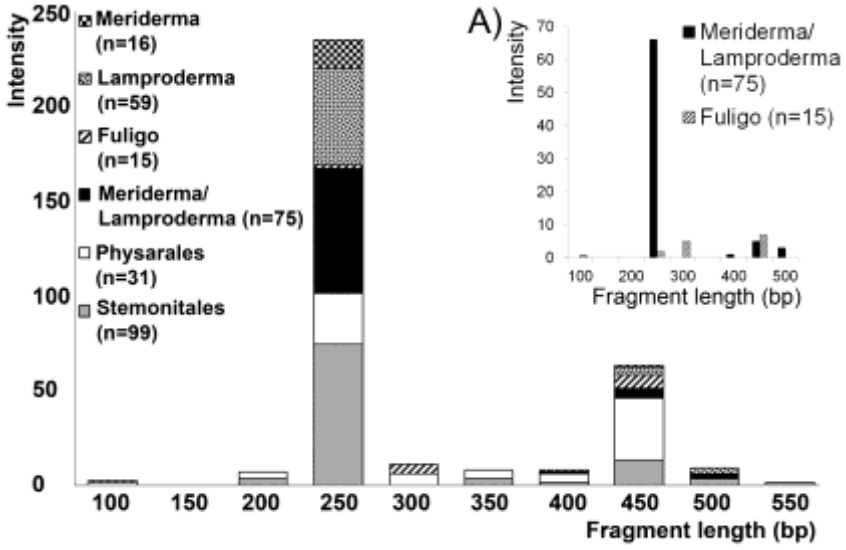

Fig. 1 – Fragment sizes to be expected with a Hha*I* digestion of partial SSU sequences (primers S1-HEX / SU19Rsp) for different myxomycetes, as inferred from an alignment including 167 sequences of 96 species. A) Expected fragments for the genus *Meriderma* (74 specimens from 35 taxa) and *Fuligo* (14 specimens from 7 taxa).

Different genotypes of one morphospecies differ often slightly in the length of the expected fragments, since two kinds of mutations can influence fragment size (Fig. 2): First, length variation in variable loops of the 18S-rDNA sequences alters slightly the fragment length (fairly common). Second, a mutation can extinguish or create a cutting site, which may lead to dramatic changes in fragment length (rare).

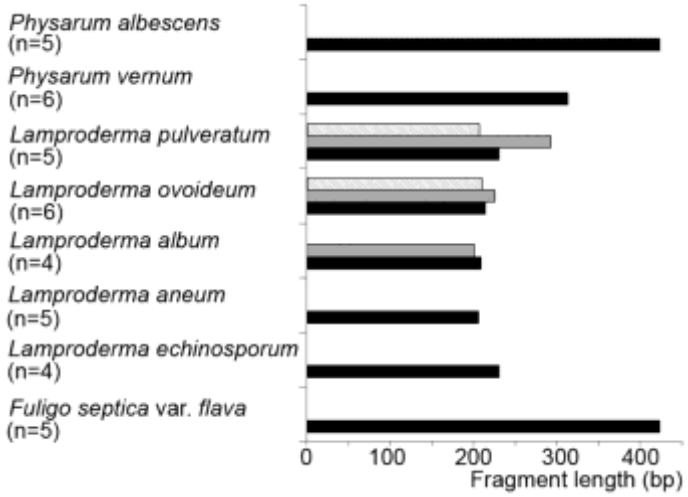

Fig. 2 – Expected intraspecific fragment lengths for 4–6 sequences of eight morphospecies. The digesting sites for Hha*I* were searched in the alignment of partial SSU sequences and fragment length was calculated (±1 bp).

Verifying fragment sizes – Fig. 3 shows the profile generated with the mock sample of seven myxomycete species. All seven species displayed fragments of roughly the expected size. For all but one species, differences between the fragment length calculated from the sequence and the position of the respective peak did not exceed ten bases. Remarkable is the high variation in signal strength for the individual peaks.

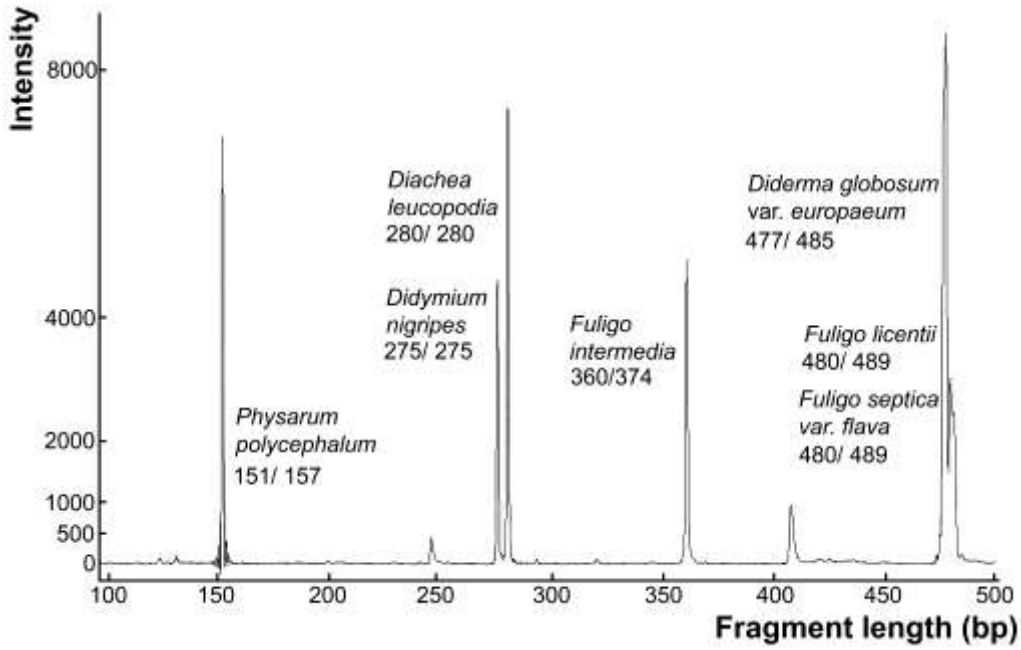

Fig. 3 – TRFLP profile of a mock sample composed from amplicons of partial SSU sequences of seven species of myxomycetes, amplified with the primer pair S1 / SU19Rsp. Numbers indicate fragment lengths calculated from the sequence/ read from the TRFLP profile.

TRFLP profiles from soil myxomycete communities – The TRFLP profiles generated from soil collected in to adjacent sites show peaks reflecting comparable fragment size, but new peaks occur as well (Fig. 4).

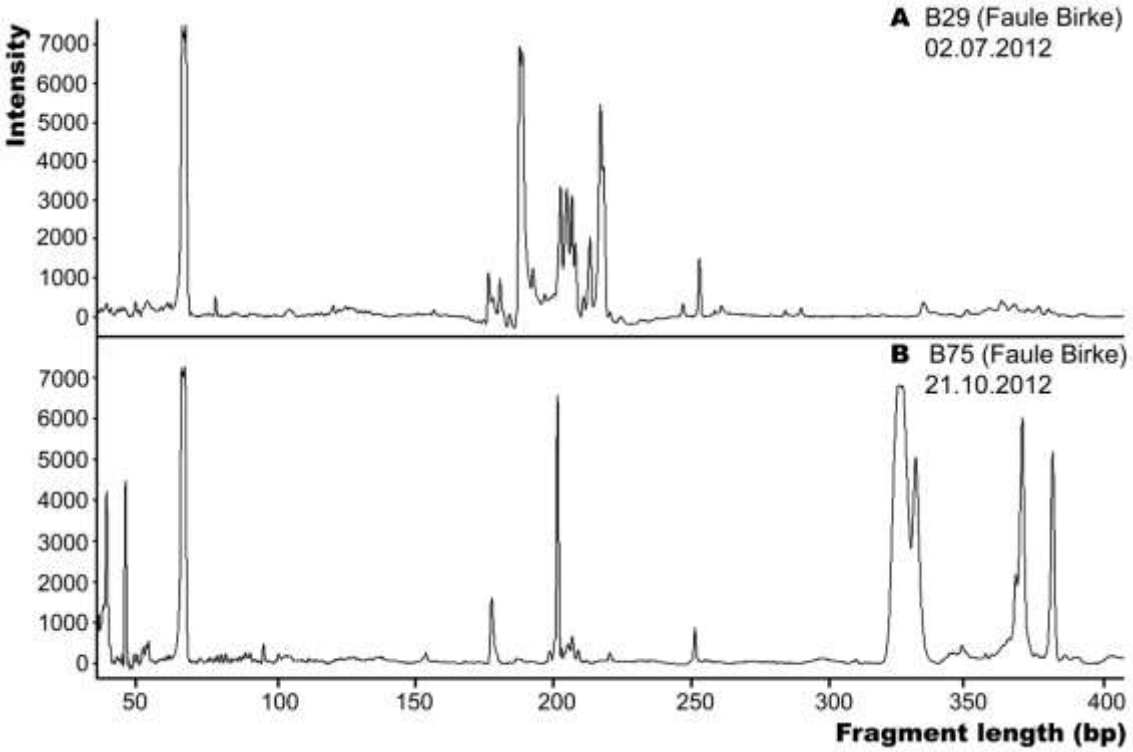

Fig. 4 – Comparison of TRFLP profiles of two soil samples from forest soils with a different sampling date: A) B29 from 02.07.2012 and B) B75 from 21.10.2012 (primer pair: S3b F-HEX/ S19Rsp). Shown is the range between 40 and 400 bp.

In both profiles distinct peaks are visible in the ranges from 150 – 200 and 320 – 400 bp. Non-identical fragments point towards the presence/absence of individual species. For example, sample B29 shows signals between 170 and 250 bp, B75 lacks the most of these signals completely. In contrast, only B75 displays strong signals between 320 and 380 bp.

Discussion

Many species of myxomycetes seem to be widespread, at least at the morphospecies level (Stephenson et al. 2008); others seem to be adapted to particular ecological conditions (Schnittler et al. 2000). A first ePCR study of a community of nivicolous myxomycetes found a large proportion of sequences obviously originating from myxomycetes that cannot be assigned to any known genus or species (Kamono et al. 2012). Together with first results from barcoding attempts which often showed a whole gene pool of slightly deviating partial 18S rRNA sequences for a particular morphospecies (Aguilar et al. 2013; Novozhilov et al. 2013), a significant proportion of hidden molecular diversity can be expected for the group. In addition, the number of morphospecies described as new for science increased steadily at least until the turn of the millennium (Schnittler & Mitchell 2000). Hence, it is important to develop culture-independent methods to analyze naturally occurring amoebal communities of myxomycetes to retrieve the signals of species not fruiting at a given time.

A variety of possible technologies for community analyses of microorganisms exists (Ahmad et al. 2011). Comparable to TRFLP is DGGE/TGGE (Muyzer et al. 1993), the latter technique was successfully applied to compare the molecular diversity of litter-inhabitting myxomycetes (Kamono & Fukui 2006; Ko Ko et al. 2009). In comparison to next-generation sequencing (NGS) techniques, both methods are time- and cost-efficient – but provide only a

community profile. For DGGE/TGGE exact sequences can be achieved by cloning and sequencing of products. However, DGGE/TGGE is more complex and needs more processing time, whereas TRFLP allows the comparison of different myxomycetes in the soils within a couple of hours and would thus be a suitable technique for a rapid characterization of myxomycete communities in soil.

The choice of the marker gene is crucial for the efficiency of this method. Ideally, a suitable target gene should work like a barcode marker: variation should be low within a species, and universal primers recovering DNA from all members of the target community but not for other organisms should exist. Amplicons should include variable sections and produce a sufficient proportion of longer (>50 bp) fragments which stand out in the respective electroferograms. The partial sequence of the 18S rRNA gene sequences chosen for this study, is a good candidate for myxomycete barcoding (Novozhilov et al. 2013), and universal primers for the dark-spored myxomycetes exist (Fiore-Donno et al. 2012), which is the major group of soil myxomycetes.

A suitable endonuclease should produce a maximum variation in fragment length. From the five restriction endonucleases that were screened for 167 sequences from 96 species of dark-spored myxomycetes, Hha*I* yielded the highest interspecific, but not intraspecific variability and was used as well successfully for microbial communities (Pasternak et al. 2013). For myxomycetes, several different fragments of different size can be expected for a given morphospecies (Fig. 2) due to the high intraspecific variation in 18S rRNA sequences (Novozhilov et al. 2013), which makes this gene suitable even for population genetic studies (Aguilar et al. 2013). In accordance, crossing experiments with cultivable members of the Physarales revealed that, most morphospecies comprise several reproductively isolated units (i.e., biospecies, Clark & Haskins 2010). Most likely, complementary mutations in stems of the rRNA loops upstream of the digesting site will add or delete bases, thus producing shorter or longer fragments. Therefore, not the morphospecies, but the (usually unknown) biospecies is the level targeted by molecular methods like TRFLP, it will thus be unlikely that a morphospecies can be identified directly from TRFLP profiles. In addition, the results of the DNA-sequencing and the TRFLP analysis do not completely correspond with each other (Fig. 3). First, the position of a peak in the electroferrogram cannot be determined with the same precision as the position of the digesting site in a sequence. Second, competition effects between fragment cohorts may occur.

Nevertheless, as suggested by the comparison of known sequences (Fig. 1), some peaks in TRFLP profiles seem to be specific for certain orders or genera. As inferred from our alignment of 18S rRNA sequences, signals in the range 200–450 bp (mainly 250 bp) are likely to be generated by members of the Physarales, whereas members of the Stemonitales produce a greater variation in fragment length (200–600 bp). With the settings chosen by us, most fragments can be expected between 70 and 400 bp. However, homoplasies (two different species yield by chance fragments identical in length) cannot be excluded.

The soil samples B29 and B75 (Fig. 4) come from two different locations of one forested, which are similar in vegetation structure (Hoppe 2013). As one may expect, the two TRFLP profiles are similar but not identical to each other, and peaks occurring in one but not the other sample point to differences in species composition. Both amoeba and cysts of myxomycetes can be expected to exist all over the year in the soil, yet in different densities (Feest & Madelin 1988). Comparing forests with similar climatic conditions, soil and vegetation, one must assume a significant overlap in species composition. However, if a new genotype becomes established in a soil patch, new peaks can be expected, making the TRFLP profile distinctive.

Summarizing, TRFLP profiles from soil seem to be a cheap and fast method to compare community structure in soil myxomycetes, but a direct identification of specimens is hampered by several factors. First, small fragments occur more often and in higher concentrations and may represent homoplasies. Second, fragments occurring in high concentrations produce large and broader peaks, which may mask fragments which slightly deviate in size but occur in lower concentration. Third, the rather high intraspecific variation in 18S rRNA genes is likely to produce different fragments for a given morphospecies.

However, a direct comparison of several TRFLP profiles may be a cost- and time-efficient method to screen larger amounts of soil samples for community differences. It can be used as a pre-screening to check the suitability of the isolated DNA for metabarcoding with NGS. This method will be much more informative; but comes at higher expenses in both money and time to be used for the respective bioinformatics pipelines. For direct comparison, TRFLP profiles can be treated like profiles generated by the well-known AFLP procedure (Vos et al. 1995): peaks can be screened and 0/1 matrices of peak positions can be generated.

As such, TRFLP is not an alternative, but a complementary technique to metabarcoding, which currently appears to be the most powerful technique for uncovering the diversity of amoebal myxomycete communities, which, at least for some soils, represent the largest amoebal fraction (Urich et al. 2008). However, even with group-specific primers at hand, NGS is still limited by the short sequences that can be analyzed. This and the high costs of machinery, specially tailored consumables and the time consuming bioinformatics pipeline for analysis may all improve in future. Other limitations, like the implicit danger of creating chimeric amplicons, or the problem that shorter target sequences may outcompete longer ones, are shared by both methods, since they are inherent to environmental PCR (Kircher & Kelso 2014). Even if only exceptionally allowing identification to species. TRFLP may be a cost-efficient alternative to NGS, revealing differences between ecological communities with low effort.

Table 1 – Data for sequences of dark-spored myxomycetes used in this study.

Species/ Specimen	**Collection number**	**Acession number**
Amaurochaete comata		AY842031
Barbeyella minutissima	MM36759	JQ031956
Brefeldia maxima	MM24519	JQ031957
Colloderma robustum	AMFD270	JQ031960
Colloderma robustum	HS2885	JQ031959
Comatrichia anastomosans	Now11379	JQ031962
Comatrichia anastomosans	Now12905	JQ031961
Comatrichia pseudoalpina	MM32556	JQ031963
Comatrichia rubens	MM29181	JQ031958
Diachea leucopodia		JN123462
Diachea leucopodia	MYX79	KM977849
Diachea subsessilis	MM24463	JQ031964
Diacheopsis pauxilla	MM29883	JQ031966
Diacheopsis sp.	AMFD-2011	JQ031965
Diderma alpinum	LE 285209	JQ812622
Diderma alpinum	LE285237	JQ812621
Diderma chondrioderma	MYX439	KM977850
Diderma crustaceum		JQ277927
Diderma deplanatum	MYX440	KM977851
Diderma fallax	LE285162	JQ812629
Diderma fallax	LE285178	JQ812628
Diderma globosum var. *europaeum*	LE285166	JQ812625
Diderma globosum var. *europaeum*	LE285172	JQ812624
Diderma globosum var. *europaeum*	LE285171	JQ812623
Diderma globosum var. *europaeum*	MYX443	KM977852
Diderma hemisphaericum	MYX436	KM977853
Diderma meyerae	LE285226	JQ812659

Species/ Specimen	Collection number	Acession number
Diderma meyerae	LE285165	JQ812626
Diderma niveum	MYX442	KM977854
Diderma radiatum	MYX437	KM977855
Didymium crustaceum	MYX235	KM977856
Didymium dubium	LE285183	JQ812635
Didymium dubium	LE285181	JQ812630
Didymium flexuosum	MYX295	KM977857
Didymium minus	MYX75	KM977858
Didymium nigripes	MYX51	KM977859
Didymium ochroideum	MYX297	KM977860
Didymium sp.	COHH_22c7	GU320584
Didymium sp.	OX13PS	GQ249857
Elaeomyxa cerifera	MM24498	JQ031967
Fuligo cinerea	MYX202	KM977861
Fuligo intermedia	MYX175	KM977862
Fuligo intermedia	MYX352	KM977863
Fuligo licentii	MYX305	KM977864
Fuligo septica var. *candida*	MYX514	KM977865
Fuligo septica var. *flava*	MYX1226	KM977866
Fuligo septica var. *flava*	MYX458	KM977871
Fuligo septica var. *flava*	MYX502	KM977873
Fuligo septica var. *flava*	MYX1519	KM977870
Fuligo septica var. *flava*	MYX1240	KM977867
Fuligo septica var. *rufa*	MYX372	KM977874
Fuligo septica var. *flava*	MYX1412	KM977868
Fuligo septica var. *flava*	MYX1480	KM977869
Fuligo septica var. *flava*	MYX459	KM977872
Fuligo sp.	BX-2002	AY145526
Lamproderma acanthosporum	MM36058	JQ031968
Lamproderma aeneum	LE285901	JQ812663
Lamproderma aeneum	LE285899	JQ812662
Lamproderma aeneum	LE285829	JQ812661
Lamproderma aeneum	MM36255	JQ031969
Lamproderma aeneum	AK06013	JQ031970
Lamproderma album	LE285175	JQ812672
Lamproderma album	LE285285	JQ812660
Lamproderma album	MM37151	JQ031971
Lamproderma album	MM35162	JQ031972
Lamproderma arcyrioides	LE285836	JQ812643
Lamproderma arcyrioides	MM27880	JQ031975
Lamproderma arcyrioides	MM37005	JQ031973
Lamproderma cacographicum	AMFD310	JQ031976
Lamproderma cacographicum	MYX321	KM977875
Lamproderma cf. *arcyrioides*	AMFD338	JQ031974
Lamproderma cristatum	LE285764	JQ812644
Lamproderma cristatum	MM37003	JQ031977
Lamproderma cristatum	MYX399	KM977876

Species/ Specimen	Collection number	Acession number
Lamproderma cucumer	LE285263	JQ812664
Lamproderma disseminatum	AMFD38	JQ031978
Lamproderma echinosporum	LE285911	JQ812676
Lamproderma echinosporum	LE285778	JQ812675
Lamproderma echinosporum	AMFD136	JQ031980
Lamproderma echinosporum	AK06016	JQ031979
Lamproderma fuscatum	MYX328	KM977877
Lamproderma lycopodiicola	AMFD309	JQ031981
Lamproderma maculatum	LE285787	JQ812673
Lamproderma maculatum	MM37059	JQ031982
Lamproderma ovoideoechinulatum	JMF527	JQ031983
Lamproderma ovoideum	LE285772	JQ812670
Lamproderma ovoideum	LE285863	JQ812669
Lamproderma ovoideum	LE285827	JQ812668
Lamproderma ovoideum	LE285910	JQ812666
Lamproderma ovoideum	LE285878	JQ812665
Lamproderma ovoideum	AK06022	JQ031984
Lamproderma pseudomaculatum	AFMD180	JQ031986
Lamproderma pseudomaculatum	MM37354	JQ031985
Lamproderma pseudomaculatum	MYX315	KM977878
Lamproderma pulchellum	LE285222	JQ812667
Lamproderma pulchellum	MM36096	JQ031987
Lamproderma pulveratum	LE285213	JQ812677
Lamproderma pulveratum	LE285766	JQ812642
Lamproderma pulveratum	LE285789	JQ812641
Lamproderma pulveratum	MM37016	JQ031988
Lamproderma pulveratum	MYX342	KM977879
Lamproderma retrugisporum	MM32478	JQ031990
Lamproderma retrugisporum	MM23831	JQ031989
Lamproderma sauteri	LE285199	JQ812678
Lamproderma sauteri var. *atrogriseum*	LE285206	JQ812671
Lamproderma sauteri var. *pulchrum*	AFMD336	JQ031991
Lamproderma scintillans	MA70223	JQ031993
Lamproderma scintillans	JM3204	JQ031992
Lamproderma sp.	LE285191	JQ812674
Lamproderma sp.	AMFD-2011b	JQ031995
Lamproderma sp.	AMFD-2011a	JQ031994
Lamproderma spinulosporum	MM32506	JQ031996
Lamproderma violaceum	MM29783	JQ031997
Lepidoderma alpestroides	AMFD340	JQ031998
Lepidoderma carestianum	LE285229	JQ812618
Lepidoderma chailletii	LE285156	JQ812617
Lepidoderma peyerimhoffii	LE285215	JQ812627
Meriderma sp. MS-2012b	LE285227	JQ812655
Meriderma aggregatum	LE285283	JQ812658
Meriderma carestiae	LE285696	JQ812650
Meriderma carestiae	LE285723	JQ812649

Species/ Specimen	Collection number	Acession number
Meriderma carestiae	LE285668	JQ812645
Meriderma carestiae	MM35985	JQ031999
Meriderma cribrarioides	LE285845	JQ812657
Meriderma cribrarioides	LE285850	JQ812656
Meriderma cribrarioides	LE285847	JQ812651
Meriderma cribrarioides	MM37106	JQ032000
Meriderma echinulatum	LE285815	JQ812654
Meriderma echinulatum	LE285906	JQ812653
Meriderma echinulatum	LE285895	JQ812652
Meriderma echinulatum	LE285711	JQ812646
Meriderma sp. MS-2012a	LE285820	JQ812647
Meriderma sp. MS-2012a	LE285681	JQ812648
Meriderma sp.	MYX327	KM977880
Paradiacheopsis solitaria	DM7368	JQ032001
Physarella oblongata		JQ277932
Physarella sp.	BX-2002	AY145524
Physarum albescens	LE285158	JQ812640
Physarum albescens	9294	JQ812639
Physarum albescens	LE285187	JQ812638
Physarum albescens	LE285271	JQ812637
Physarum albescens	18581	JQ812636
Physarum alpestre	LE285223	JQ812680
Physarum alpinum	LE285134	JQ812679
Physarum bivalve	TNS-M-Y15990	AB259494
Physarum leucophaeum	LE 47431	JX035989
Physarum melleum		JQ277926
Physarum notabile	LE474991	JX035988
Physarum nutans	TNS-M-Y-16082	AB259531
Physarum polycephalum	MYX15	KP323383
Physarum pusillum		JQ277930
Physarum sp.	LE255714	JX035987
Physarum sp.	LE255437	JX035986
Physarum sp.	LE255721	JX035984
Physarum sp.	LE255719	JX035983
Physarum vernum	LE285169	JQ812634
Physarum vernum	LE285186	JQ812633
Physarum vernum	LE285240	JQ812632
Physarum vernum	LE285190	JQ812631
Physarum vernum	LE285155	JQ812620
Physarum vernum	LE285197	JQ812619
Stemonaria irregularis	MYX565	KM977881
Stemonitis axifera		JQ277931
Stemonitis axifera		AY145528
Stemonitis splendens		JN123463
Stemonitopsis hyperopta	MM37295	JQ032002
Stemonitopsis typhina	MM36735	JQ032003

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