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Quick n' Cheap – a simplified workflow to barcode plasmodial slime molds (*Myxomycetes*)

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Abstract

We present a workflow for efficient barcoding of myxomycete fructifications, which (i) requires less than 1000 spores, (ii) allows to collect spores with only a needle, (iii) works without any commercial kits, and (iv) is optimized for the use of 96-well PCR plates throughout the process. Specimens of 291 dark-spored nivicolous myxomycetes and 121 bright-spored members of the Trichiaceae were sequenced for the barcode marker 18S rDNA (SSU) with a low rate of failure and no detectable cross-contamination. Crude DNA extracts can be stored for further analyses: the elongation factor 1 alpha gene (EF1A), a single-copy marker, was successfully amplified after four weeks of storage. As such our procedure will allow a time- and cost-efficient barcoding of large series of specimens.

Introduction

For many groups of organisms, DNA barcoding (Hebert et al. 2002) is the method of choice for safe identification of specimens (Casiraghi et al. 2010), widely used in taxonomic (Hebert & Gregory 2005) and ecological research (Valentini et al. 2009). For myxomycetes, with the first molecular studies (Fiore-Donno et al. 2005, 2008) a natural system of the group started to emerge (Fiore-Donno et al. 2012, 2013), which was recently used for a new classification (Leontyev et al. 2019b). As a side effect, markers for barcoding became available (Schnittler et al. 2017; Borg Dahl et al. 2018), with the first part of the 18S rRNA gene (small ribosomal subunit, SSU; Johansen et al. 1988; Fiore-Donno et al. 2012) used most often, as in other groups of protists (Adl et al. 2014). The short sequences (320–550 bp, according to the primer pairs used) can reliably differentiate species at a similarity threshold of 99.1% (Leontyev et al. 2015; Borg Dahl et al. 2018).

For DNA barcoding, commercial extraction kits are typically used, which require a noticeable amount of material (3–5 sporocarps of ca. 0.5 mm diameter, Borg Dahl et al. 2018). This often limits barcoding for scanty material, old sporocarps with most of the spore mass blown away, species with minute sporocarps, and valuable collections from rare species or type material. Here we describe a workflow to barcode series of myxomycete collections with rather simple equipment and minimum effort, omitting any cost-intensive commercial kits.

Materials & Methods

Spore sampling

To minimize cross-contamination, specimens were collected in separate boxes already in the field and wrapped with paper or put into paper bags (plastic bags are discouraged since the specimens cannot dry out and become moldy). Spores were later collected into 200 µl PCR tubes arranged in stripes of eight with individually bound lids (NEST Biotechnology), using a rather thick and blunt preparation needle (see Supplement 1, YouTube video). Between 300 and 1000 mature spores were carried over to the tube by touching a sporocarp with the needle and detaching the spores by twisting the needle at the inner side of the PCR tube. For species with minute fructifications, like *Perichaena pedata* or *Hemitrichia pardina*, whole sporocarps were collected. Spore numbers were estimated by counting attached spores in small areas of a tube wall under a dissecting microscope, and extrapolating this to all visible spores. After each transfer, the needle was thoroughly wiped with a clean paper towel. From time to time it was visually inspected to be free of spores under a dissecting microscope. After spore sampling one steel ball of 1.8 mm diameter (Kugel Winnie) was added to each tube. After this step, the tubes can be stored dry at room temperature.

DNA extraction

PCR tubes filled with spores were placed in a 96-well PCR rack, and the position (A1 to H12) of each tube was recorded in an Excel spreadsheet (compiling as well unique name strings for the later sequences). The plate was centrifuged for a few seconds at 795 rcf (2500 rpm) in a plate centrifuge, to ensure that all spores assemble at the bottom of the tube, thus generating the largest area of attack for the steel ball. After centrifugation, the samples were pre-cooled to 4–8°C for 30 minutes, and fitted into a custom-made adaptor for a Retsch MM301 ball mill. To break the spores, the rack was shaken at 30 Hz for 70 seconds. If no mill is available, the rack can as well be vortexed 3 times for 1 min at maximum speed.

After a brief centrifugation (795 rcf) in a plate centrifuge, the rack was immediately put on ice. Using an 8-channel pipette, 20 µl of Lysis buffer (0.05 M KCl, 0.05 M TRIS pH 8, 2.5 mM EDTA, 0.9% Triton X) was added to each tube, followed by 0.5 µl of Proteinase K (Roth, 20 mg/ml). After vortexing for 10 seconds, the tubes were incubated for 25 minutes at 55°C and finally for 7 minutes at 97°C (for protein denaturation). In order to achieve a clear supernatant free of spores and debris, the tubes were centrifuged for a minimum of 3 minutes at 2240 rcf (3700 rpm) in a plate centrifuge. Without any filtering or elution, the supernatant was used as a template for PCR immediately after DNA extraction or after storage at 5°C over night.

PCR amplification

For the PCR, 4 µl of the clear supernatant was transferred to a fresh 200 µl tube with 21 µl of a self-made PCR mixture consisting of 1x Molzym PCR buffer, 3.4 mM $MgCl_2$, 0.2 mM dNTPs, 0.25 µM of each primer as final concentrations, and 1 U Moltaq polymerase; concentrations were adjusted with water for molecular biology (ddH_2O, Applichem).

The following primers were used for amplification of a fragment of 18S rDNA (see Table S1): for dark-spored myxomycetes (see Leontyev et al. 2019b for circumscription), S3bF / S31R (short fragment, annealing temperature T_a 52°C), and for bright-spored myxomycetes, SFATri / SR4Bright (T_a 59°C). Alternatively, the primers S2 / S31R (short fragment, T_a 56.5°C), or S2 / SU19R (longer fragment, T_a 58.5°C) were used for some replicate sequences (data not shown). To test the suitability of the method for amplification of single-copy genes, a fragment of EF1A was amplified with the primers EF1a_DS_F1 / EMerR1b (T_a 55.5°C) after four weeks of storage at 5°C. PCR conditions were as follows: initial denaturation 95°C / 2 min; 40 cycles with denaturation 95°C / 30 sec, annealing T_a / 30 sec, and elongation 72°C / 60 sec; plus a final elongation at 72°C / 5 min. After the PCR was finished, the PCR products were vortexed, and droplets and condensed water were centrifuged down. PCR success was verified by loading 4.5 µl of the product on a 1.8 % agarose gel with 6 µl of the marker PBR328 (1 µg) as DNA ladder. The electrophoresis was run for 1 hr on 100 V and bands were stained with ethidium bromide for 40 min.

Sequencing

PCR products were purified with a mix of 1µl 2 U Exonuclease I and 0.2 U FastAP Thermosensitive Alkaline Phosphatase for 5 µl PCR product (fivefold diluted compared to the manufacturer's protocol, ThermoScientific). In order to compensate the lower enzyme concentration, the incubation at 37°C was extended to 1 hr, followed by heating the mixture to 85°C for 15 min for enzyme deactivation. Based on photometric concentration measurements, the purified PCR products were diluted to 20 ng/µl with ddH_2O. The product can be stored at 5°C (for use within several weeks) or at –20°C (long-term storage) for a later investigation.

For cycle sequencing, 2.5 µl of the purified PCR product was used with 1.5 µl 5x sequencing buffer (Applied Biosystems), 0.25 µl Half Big Dye (Sigma), 0.25 µl Big Dye 3.1 Terminator Ready Reaction Mix (Applied Biosystems) and 0.64 µl 10 µM reverse primer. After adjustment with ddH_2O, a total volume of 10 µl was reached. The sequencing PCR was done with initial denaturation 96°C / 1 min followed by 25 cycles with denaturation 96°C / 10 sec, T_a / 5 sec, and elongation 60°C / 4 min (T_a = 52°C for sequencing with S31R, 56°C for the other primers).

Products of the sequencing PCR were purified by ethanol precipitation: 30 µl 96% EtOH was added, vortexed, and mixtures were centrifuged at 2240 rcf / 25 min in a plate centrifuge. After discarding the supernatant, the pellets were washed with 100 µl 70% ethanol and centrifuged again (2240 rcf / 25 min). EtOH was discarded and the pellets were drained for 1h at 37°C and finally dissolved in 20 µl ddH_2O. After vortexing and spinning down, the product was sequenced in an ABI 3100 16-capillary Genetic Analyzer (Applied Biosystems).

All obtained chromatograms were inspected using BioEdit v. 7.2.5 and rare base-calling errors were corrected manually. The corrected 18S rDNA sequences were searched against NCBI GenBank using BLAST, which contains myxomycete sequences mostly obtained with DNA extracted by standard laboratory methods (see Borg Dahl et al. 2018 for exemplary details). A detailed list of all steps, required equipment, chemicals, and time is provided in Supplement 2.

Results

The method was first tested on 291 specimens of nivicolous myxomycetes collected at two surveys in the French Alps (Albertville region, 2019 and Hautes Alps, Grenoble region, 2014). Spores of all specimens, including scanty, malformed, or slightly immature colonies, were sampled in four 96-well plates and sequenced for the 18S rDNA barcode (together with some primer trials for different material). Ta-

Table 1. Results of the simplified barcoding procedure presented in this study, tested on 291 specimens of nivicolous myxomycetes from two regions of the French Alps (Fr19: around Albertville; HAlps14: Hautes Alpes, Grenoble), 121 specimens of Trichiaceae from moist-chamber cultures obtained from the Tropics (CR20: Costa Rica, around Turrialba; Et12: Ethiopian highlands), and 29 specimens that were sequenced for the EF1A gene.

Survey	**Fr19**	**HAlps14**	**CR20**	**Eth12**	**HAlps14**
Marker	18S rDNA	18S rDNA	18S rDNA	18S rDNA	EF1A
Primer F	S3bF	S3bF	SFATri	SFATri	EF1a_DS_F1
Primer R	S31R	S31R	SR4Bright	SR4Bright	EMerR1b
Group	dark	dark	bright	bright	dark
Specimens investigated	195	96	67	54	29
Sequences obtained	189	84	46	43	26
% successful	96.9	87.5	68.7	79.6	89.7
Matches in GenBank					
100% identical	149	55	0	2	n.d.
>=99.1% similarity	21	22	0	16	n.d.
>=85.0% similarity	19	7	46	23	n.d.

ble 1 gives a statistic for sequencing success and the matches with sequences in GenBank; Supplement 3 shows the detailed results.

Most accessions of nivicolous myxomycetes were successfully barcoded (97 and 88% for the surveys conducted in 2019 and 2014, respectively). The BLAST search in NCBI GenBank revealed that 78 and 57% of these sequences were 100% identical to ribotypes already stored in GenBank, another 11 and 23% were new, but within the 99.1% threshold proposed by Borg Dahl et al. (2018) for species identification. All sequences were found to belong to myxomycetes. We analyzed the 18 specimens for which the amplification failed: for seven, we found no obvious reason for the failure. Another seven were damaged by insects. From the remaining four specimens, three were immature (spores baked together into a dense mass) and the last one was the bright-spored *Trichia alpina*, accidentally processed together with the dark-spored specimens.

The second test included 121 specimens of bright-spored Trichiaceae obtained from moist-chamber cultures (mostly the genus *Perichaena*) from two surveys (Costa Rica, Turrialba region, 2020; Ethiopian highlands and Simien Mountains, 2012, see Dagamac et al. 2017a). Here, the success rate was much lower (69 and 80% for the surveys conducted in 2020 and 2012, respectively). The most prominent reason for failure were the conditions in the moist chambers, especially specimens from very moist habitats in Costa Rica did not mature properly. Sequence identity with sequences found in Genbank was much lower (average sequence identity of 90.3 and 94.8% for Costa Rica and Ethiopia, respectively). All sequences retrieved by the BLAST search belonged to myxomycetes.

The third test involved 29 specimens of nivicolous myxomycetes from the French Alps survey (HAlps14). DNA extracted using the simplified method presented herein was used to amplify the single-copy gene EF1A. In total, 26 specimens gave readable sequences. Of these, 22 were homozygous and 4 were heterozygous showing definite peak overlays in chromatograms due to SNPs and/or indels. One se-

quence of *Meriderma aggregatum* ad. int. was readable only in the first/last part (sequenced forward/reverse) due to the spliceosomal intron described in Feng et al. (2016), consisting of a long C-homopolymer followed by a $(CA)_n$ microsatellite motif.

Discussion

For a group like myxomycetes, DNA barcoding is of crucial importance for both taxonomy and ecological research due to several reasons. First, many morphospecies, like in the genera *Licea* and *Perichaena* (Eliasson 2017), show reductive evolution (Leontyev & Schnittler 2017) and thus have only a limited display of morphological characters. Second, morphological similarity can arise by convergence. Both cases complicate species identification (Leontyev et al. 2019a). Third, to describe ecological niches of species, we need surveys considering all fructifications (as described in Novozhilov et al. 2013; Schnittler et al. 2015a). Specimens from marginal habitats are often maldeveloped and do not show typical characters. Fourth, cryptic speciation, as described in Feng & Schnittler (2015, *Trichia varia*), Leontyev et al. (2015, *Tubifera ferruginosa*), Feng et al. (2016, *Meriderma* spp.), Shchepin et al. (2016, *Lepidoderma chailettii*), and Dagamac et al. (2017b, *Hemitrichia serpula*), seems to be common in myxomycetes. Once the respective biological species are molecularly characterized, barcoding will allow a safe taxonomic assignment (see discussion in Feng et al. 2016 and Walker & Stephenson 2016).

Our tests showed that a simple, non-sterile method reliably gives barcode sequences. Of the 291 samples of nivicolous myxomycetes, 47% were "white" (i.e. Physarales with a calcareous peridium) and 53% "black" (Stemonitidales and Meridermatales with a non-calcified peridium). We did not encounter any cross-contamination between the two groups. As explained in Novozhilov et al. (2013) and Janik et al. (2020), cross-contamination between samples can be a serious problem. From this reason, the latter authors use disposable sterile syringe needles. However, with primers specific for myxomycetes, prokaryotes are not a problem and sterile conditions are not necessary. We thus relied on a simpler procedure, taking up spores with a blunt preparation needle which was wiped clean with a paper towel, instead of sterilization by EtOH. Visual inspection under a dissecting microscope revealed this to be sufficient to clean a smooth needle from spores. We think that cross-contamination during field collecting (storing many specimens together in styrofoam-lined plastic boxes) is a more serious problem.

The method worked as well for older material (collected in 2014 and 2012) and thus might be applicable to type material. The DNA extracts can be stored at 5°C for at least four weeks and gave readable sequences for the single-copy gene EF1A (for long-term storage, –20°C is recommended). As such, the procedure described herein fulfills several requirements needed for a routine barcoding.

(1) EASY COLLECTION OF SPORES: Spores can be collected with a needle wiped clean with a paper towel as laid out in a YouTube video (Supplement 1). This makes it easy to collect spores immediately after a survey before the specimens are fully dried out and eventually shed spores, reducing the risk of cross-contamination between specimens. With some modifications, this protocol should as well work for plasmodia emerging from moist chamber cultures (Shchepin et al. 2017).

(2) CHEAP: In contrast to Janik et al. (2020), we omitted a commercial direct PCR kit (these authors used the Phire Plant Direct PCR Master Mix, Thermo Scientific, Waltham, Massachusetts, US) and rely entirely on crude DNA extraction followed by a regular PCR. With this workflow, barcoding can be done in a lab with rather basic equipment (see supplement 2): essentials are a homogenizer (ball mill or vortexer), a pipette, a centrifuge (best a plate centrifuge to allow for the use of 96-well plates), and a thermal cycler; sequencing itself is easy to outsource to commercial labs. An additional test with 16 specimens sampled with two aliquots, treated with a ball mill and a vortexer, gave in all cases high-quality sequences.

(3) TIME-EFFICIENT: This is important for ecological questions, where large numbers of specimens are processed. All procedures are laid out for use with 96-well plates, which enables the use of multichannel pipettes and pipetting robots. For large-scale barcoding, next generation sequencing

methods (Shokralla et al. 2014, Wang et al. 2018) may replace in future Sanger sequencing (Sanger et al. 1977), which is still the gold standard in barcoding.

(4) LOW INPUT: Taxonomically critical material is often very scanty, especially specimens from moist-chamber cultures which are crucial for surveys in arid (Schnittler et al. 2015b) and tropical regions (Novozhilov et al. 2017, 2019). Therefore, one needs to obtain material for sequencing without destroying a sporocarp. As recently shown by Janik et al. (2020), a few hundred spores are usually sufficient for gene amplification, and even PCR from a single spore can be successful (Feng & Schnittler 2015). The method presented herein works best with 300–1000 spores, while more spores (like a complete sporocarp) seem to inhibit the PCR reaction.

(5) RELIABILITY: The reliability of this procedure is comparable with that of Janik et al. (2020), who reported 95% successful amplifications with the Phire Plant Direct PCR Master Mix. We had 97–69% successful amplifications, with most of the failed samples explained by specimen condition (immature specimens and insect damage). For the specimens damaged by insects, we tried to amplify the barcode from the spore remainders left by the insects to see if there is DNA left, but the result was negative. Sampling intact spores from within sporocarps would most probably have worked.

Currently, barcoding works much better for dark-spored than for bright-spored myxomycetes. There are three reasons. First, bright-spored myxomycetes show a much higher overall variability of 18S rDNA partial sequences (Fiore-Donno et al. 2012, 2013), sometimes to the extent that sequences from one taxonomic group are hardly alignable (e.g. *Tubifera* spp. in Leontyev et al. 2015). Second, for dark-spored myxomycetes there are nearly universal primers available, only some *Comatricha* spp. and *Stemonitopsis* spp. cause problems. For bright-spored myxomycetes, members of Cribrariales, Reticulariales and the Liceales require special primers (Leontyev et al. 2019a), and this is as well the case for basal groups of the dark-spored clade, like Echinosteliales. Third, the number of available sequences is much larger for the dark-spored, especially nivicolous, myxomycetes: a bulk search in GenBank for non-environmental 18S rDNA sequences resulted in 3906 for dark-spored, but only 470 for bright-spored myxomycetes.

Thus, an even more difficult task remains: to build up a quality-checked comparison data base for myxomycetes – the more barcodes we have, the better. This spurred the development of MyxoSeq, a new curated database (https://dna.myxomycetes.org). It is devoted specifically to reference nucleotide sequences of myxomycetes. All sequences there are connected to herbarium specimens with confirmed taxonomic annotation and extensive specimen and sequence metadata.

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Supplementary material

Supplement 1: Collecting procedure for spores (Video at https://youtu.be/0A4kTLNt9L8).

Supplement 2: Documentation of preparation steps, necessary equipment and time for barcoding a 96 well plate of myxomycetes (Microsoft Excel).

Supplement 3A–C: List of specimens sequenced and BLAST statistics (Microsoft Excel).