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Fine-tuning a method for DNA-extraction of myxomycetes

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Abstract: In this paper we describe in detail the procedure for a genomic DNA extraction method from spores of myxomycetes that was inspired by the direct PCR technique. We include results of small-scale tests that were set up for the optimization of this extraction method. These tests were performed to select the grinding breads, assess the result of different estimated spore quantities, compare the extraction medium, evaluate the temperature to weaken the cell walls of amoeboflagellates, and to ascertain whether the storage method of the extract is appropriate. The results are presented as agarose gel images of either crude genomic DNA extracts or PCR products of partial SSU and are discussed in detail. From the tests, the combination of features determining the best method were an extraction solution TE pH: 8, grinding with 1 mm Zirconia/Silica beads during 60 sec at 30 Hz, cell weakening at 90°C and storage at -20°C. The specimens that were used for the tests are listed in this paper along with collection data and the GenBank accession numbers for the submitted SSU sequences. This simple approach can serve as a fast, cheap, and cleaner alternative to other extraction protocols or it can be used as a preparation phase for such established methods.

Keywords: genomic DNA, myxogastria, molecular analysis, SSU

Introduction

In March 2016 the first author had the opportunity to receive a course focused on the particularities of molecular laboratory work on myxomycetes, excellently taught by Dr. Anna Maria Fiore-Donno of the Terrestrial Ecology Group (University of Cologne, Germany). A very intense week saturated with valuable information on what can go wrong and how to avoid disaster resulted in a few beautiful partial SSU sequences.

Extraction of DNA can be achieved, in general, by means of various methodologies that range in both complexity and price tags. For example, in Cologne we used a fairly fast but costly DNeasy Plant Kit (Qiagen Manchester, United Kingdom). The ideal method, in addition to being effortless, should be fast, economical, and use up a minimum quantity of the evaluated specimen. In the case of myxomycetes, the latter is mostly an absolute requirement due to the scarcity of available material, mostly fruiting bodies containing spores that are the only source of DNA. The method should also avoid obtaining spores from

more than one sporocarp to reduce the risk of double bands and chimera sequences, resulting in additional stress for the unfortunate researcher.

In the molecular laboratory of the Meise Botanic Garden the most common methods used for the extraction of genomic DNA are either the relatively inexpensive but laborious protocol using Cetyltrimethylammonium bromide (CTAB) in combination with phase extraction by chloroform/isoamyl alcohol (Amalfi and Baert 2016) or the commercially available but costly enzymatic and/or spin-column kits. Our resident lichenologist, Dr. Damien Ertz, frequently utilizes an adapted version of the direct PCR method (Wolinski et al. 1999; Ertz et al. 2015), in which small portions of a lichen are directly used as a template for the polymerase chain reaction (PCR), thus skipping the DNA isolation process entirely. By doing so, the laboratory time decreases substantially from hours to just the time necessary for cutting out the selected portion of the lichen, and the cost is reduced to zero for the extraction itself.

A disadvantage of the direct PCR procedure is that the material can only be used for only one run, leading to the use of more material if more than one molecular marker is to be tested. We, therefore, aimed to adapt this technique in order to have an extract that can be used multiple times and even stored for long term. We present here a detailed explanation of fine-tuning the protocol for DNA extraction from myxomycetes spores, used in the molecular laboratory of the Meise Botanic Garden since 2018. This approach can be used as an alternative for the methods already published (Janik et al. 2020; Schnittler et al. 2020) and could even serve as a preparation protocol before these and other extraction methods. Every technique has its pros and cons, and having multiple options is therefore advantageous.

Materials and methods

Herbarium specimens used in this study

The specimen of *Badhamia utricularis* was chosen because it consists of a suitably large colony of about 30 cm², with many closed fruiting bodies that would provide enough spores for multiple tests. Also, a plasmodium was revived from a sclerotium of *Physarum polycephalum* from Carolina Science GmbH, and the fruiting bodies formed after 4 weeks were used as the source of spores for one of the tests cited in this paper. These and the other herbarium specimens cited in this study are listed in Table 1. More information and images can be found on the website MYXO-BE (2023).

Tests for optimization of the genomic DNA extraction protocol

Selecting beads

Spores of myxomycetes should be opened to make the genomic DNA available for further analysis. In many cases myxomycete spores are “tough eggs to crack”, and because mechanical crushing has proven to be a good option in extractions of other organisms, we chose this option. More specifically we tested grinding with commercially available beads (e.g., metal, silica, zirconium, etc.).

The concept is that a sample (spores) is placed in a sealable tube containing beads, and subsequently ground in a bead beater (MM200 TissueLyser, Retsch, Germany). A disadvantage of mechanical crushing is the shearing of the DNA, and a damaged template is not ideal for further analysis. The amount of shearing can be influenced by the duration and strength of the grinding process, and it can also be influenced by the type and the diameter of the beads. A small optimization test was set up with different types and dimensions of beads as shown in Table 2.

Table 1. Information of specimens from which spores were used to extract genomic DNA in this study.

Species	Herbarium number	GenBank accession number	Collection Date	Location	Coordinates
Badhamia utricularis (Bull.) Berk.	MdH1510013	OQ727553	16/10/2015	Ronde Put, Postel, Mol, Belgium	51.287493, 5.153968
Craterium minutum (Leers) Fr.	MdH1709004	OQ727554	23/09/2017	Ralisbroek, Walenbos, Tielt-Winge, Belgium	50.940780, 4.917275
Paradiacheopsis solitaria (Nann.-Bremek.) Nann.-Bremek.	MIRWART-MC01C003	OQ469816	14/07/2022	Domaine Provincial de Mirwart, Saint-Hubert, Belgium	50.027019, 5.255121
Perichaena liceoides Rostaf.	MAIZE40-MC01C001	OQ692057	14/12/2020	Maize field BE05, Wolvertem, Meise, Belgium	50.988375, 4.319733
Physarum didermoides (Pers.) Rostaf.	BW2348c	OQ690718	12/11/2016	Botanic Gardens of Strasbourg University, Strasbourg, France	48.584054, 7.766122
Physarum polycephalum Schwein.	Ppoly01	Not submitted	9/03/2018	Molecular Laboratory, Meise Botanic Garden, Meise, Belgium	N.A.

Table 2. Information on the tissue grinding beads used in the grinding optimization test.

Type	Diameter (mm)	Brand
Zirconia/Silica	1	BioSpec Products, USA
Stainless steel	2.8	Ops Diagnostics, Lebanon
Stainless steel	5	Ops Diagnostics, Lebanon

The beads were deposited in sterile tubes (2 mL Protein LoBind, Eppendorf, Belgium). This tube model is generally used in our laboratory for the extraction of plant or fungal material, it has a rounded conical bottom to ensure optimal free, and thus efficient, movement of the beads. Eight sporocarps of approximately the same size were selected, each sporocarp was opened with sterile tweezers on a sterile microscopy slide, the peridium was removed, and 50 µL of TE buffer was pipetted on the spores, mixing everything by pipetting up and down. The spore/TE buffer mix of each sporocarp was pipetted in a tube containing a different type, number, or dimension of beads. The tubes were placed in a bead beater at

maximum frequency of 30 Hz, for 30 or 60 sec. To visualize the genomic DNA in the extracts, 2 µL of each sample were pipetted in the wells of 1% agarose gels, prepared as described in Lee et al. (2012). Color inverted images of the 1% agarose gels stained with 0.005% Ethidium bromide (EthBr) on a UV-transilluminator were obtained (Fig. 2).

Testing the spore quantity

A quick test was conceived to find out if enough genomic DNA could be extracted and still be useful for PCR by using the quantity of spores from one fruiting body, from half, from a quatre or from one-eighth of a sporocarp. A color inverted photograph of a 1% agarose gel stained with 0.005% EthBr on a UV-transilluminator was obtained (Fig. 3).

Choosing an extraction medium and temperature

The direct PCR preparation of the lichens is normally done in sterile water because the complete content of each tube/extract is used as the template for the PCR run. Crude CTAB extracts of genomic DNA are generally stored in sterile TE buffer pH 8.0 in our laboratory. We therefore chose to use the same buffer as extraction solution. In the direct PCR protocol, a step of 10 min at 95°C is added just before the PCR program begins to weaken the cell walls of the lichen. To verify if this step makes a difference in gain and/or if this had an effect on the PCR outcome, a comparison test was performed. Equal amounts of spore mass from two species, *Badhamia utricularis* and *Physarum polycephalum*, were extracted with Zirconia/Silica (60 sec at 30 Hz) in water and in TE buffer, each incubated for 10 min at three different temperatures: 90, 95 or 99 °C. A color inverted photograph of a homemade 1% agarose gel stained with 0.005% EthBr on a UV-transilluminator was obtained (Fig. 4).

Testing the preservation condition of the extracts

All extracts are preserved in the original extraction liquid, TE buffer pH 8.0, and are kept in a freezer at -20 °C. To test if this is an adequate storage method, a PCR of a selection of the extracts was run again after 5 years. This being the maximum age of the extracts up to now, this test can be repeated after 5 years. The results are presented in Fig. 5 as a color inverted image of a precast E-Gel EX 1% agarose gel pre-stained with SYBR Gold II DNA gel stain run in a E-Gel Power Snap Electrophoresis Device with integrated blue-light transilluminator for imagining (Thermo Fisher Scientific, Merelbeke, Belgium).

Miscellaneous testing

Bright spored taxa such as *Perichaena liceoides* (Mironova et al. 2023) were also subjected to DNA extraction and sequencing. This species and *Paradiacheopsis solitaria* (Table 1) also served as examples of extracting small fruiting bodies. New sequences generated from the PCRs in this paper have been deposited in the GenBank/EMBL database and can be found in that repository under the Accession Numbers listed in Table 1.

Protocol for DNA extraction from myxomycetes spores

Preparations

Use clean, powderless latex gloves throughout the entire procedure and regularly use new ones or wipe them with a paper towel and 70% ethanol. The work bench and stereomicroscope should be wiped cleaned with 70% ethanol. The base of the stereomicroscope should be covered with a clean paper sheet or paper towel. Microscopy slides must be cleaned with 70% ethanol using paper lens wipes. This can be done in advance, keeping the slides ready for use in a clean container or a plastic Ziplock bag.

Two clean beakers are necessary (volume 20 mL), one filled with 15 mL of sterilized demineralized water and one with the same volume of 100% ethanol. A spirits burner (alcohol burner) with a cotton wick should be lit and placed nearby at a safe distance from the stereomicroscope. The sterilization of tweezers (or needles) is done in this order: dip in water, dip in ethanol and hold in flame until red-hot. Before utilizing let the metal cool down on a sterile surface (e.g., the rim of a glass petri dish).

PCR tubes (0.2 mL, BIOzym TC, Landgraaf, The Netherlands), in our case, were prepared by inserting 10 to 15 Zirconia/silica beads in each tube with sterile tweezers with broad ends (KNIPEX Universal Tweezers, ref. 92 61 02, Wuppertal, Germany), subsequently closing the tube lid. Filling the tubes with beads can also be done well in advance by keeping them ready for use in a clean, dry container. When utilized for extraction, a serial number or reference code referring to each specimen was written with a permanent marker on the side of respective tube. An Excel spreadsheet was filled out with all data concerning the specimen and all subsequent handlings leading up to the sequencing.

The sampled spores were then preserved in TE buffer (= TRIS-EDTA buffer solution pH 8.0). This can be purchased (SIGMA-ALDRICH, Overijse, Belgium) or prepared following this recipe:

> In a 100 mL flask pipet, add 0.2 mL of 0.5M EDTA (pH 8.0) and 1 mL of 1M TRIS-HCl (pH 8.0). Then add H_20 to reach the mark of 100 mL, and sterilize by autoclaving 20 min at 121 °C.

The extraction protocol

Precision Dumostar tweezers (DUMONT, Montignez, Switzerland) with extra fine tips of 0.01 mm were used to sample the spores. Ideally two tweezers or one set of tweezers in combination with a fine needle should be available. The tips of the tweezers and needles should be sterilized (water, ethanol, flame) between samples, and always placed on a sterile surface (e.g., on the rim of an open glass petri dish) to cool down before use.

A (herbarium or fresh) specimen should be placed under the stereomicroscope (Fig. 1A) and one sporocarp selected. Ideally the sporocarp should be closed and devoid of all traces of other organisms (e.g., hyphae, bacteria, other amoebae, nematodes, etc.). Since this is usually tricky, it is recommended to isolate only a small but "clean" portion of the spores, separating the stalk and the hypothallus from the peridium as much as possible.

The selected sporocarp should be placed on a microscope slide (Fig. 1A), all non-spore material (e.g., stalk, peridium, capillitium) removed. The capillitium is mostly impossible to remove completely, but residual elements will not interfere with the analyses. The remaining spores should be kept together as in Fig. 1B.

Using a 20 µL manual single channel pipette (Pipet-Lite LTS Pipette L-20XLS, RAININ, Mettler-Toledo, Zaventem, Belgium) equipped with a filter tip (GP LTS 20µL F 960A/10, RAININ, Mettler-Toledo, Belgium) two large droplets of 15 µL TE buffer each, should be pipetted on the microscope slide in the vicinity of the spores (Fig. 1B).

The first droplet of buffer must be sucked up, carefully pipetted onto the spores and mixed with these structures by pipetting up and down (Fig. 1C). As much as possible of the buffer/spore mix should be pipetted to a PCR tube ready with 10-15 Zirconia/Silica 1 mm beads (Fig. 1D). The second droplet of TE buffer should be sucked up, pipetted carefully on the remaining spores, and mixed again by pipetting up and down (Fig. 1E). The remaining buffer/spores mix should be pipetted into the PCR tube containing the first buffer/spore mix (Fig. 1D).

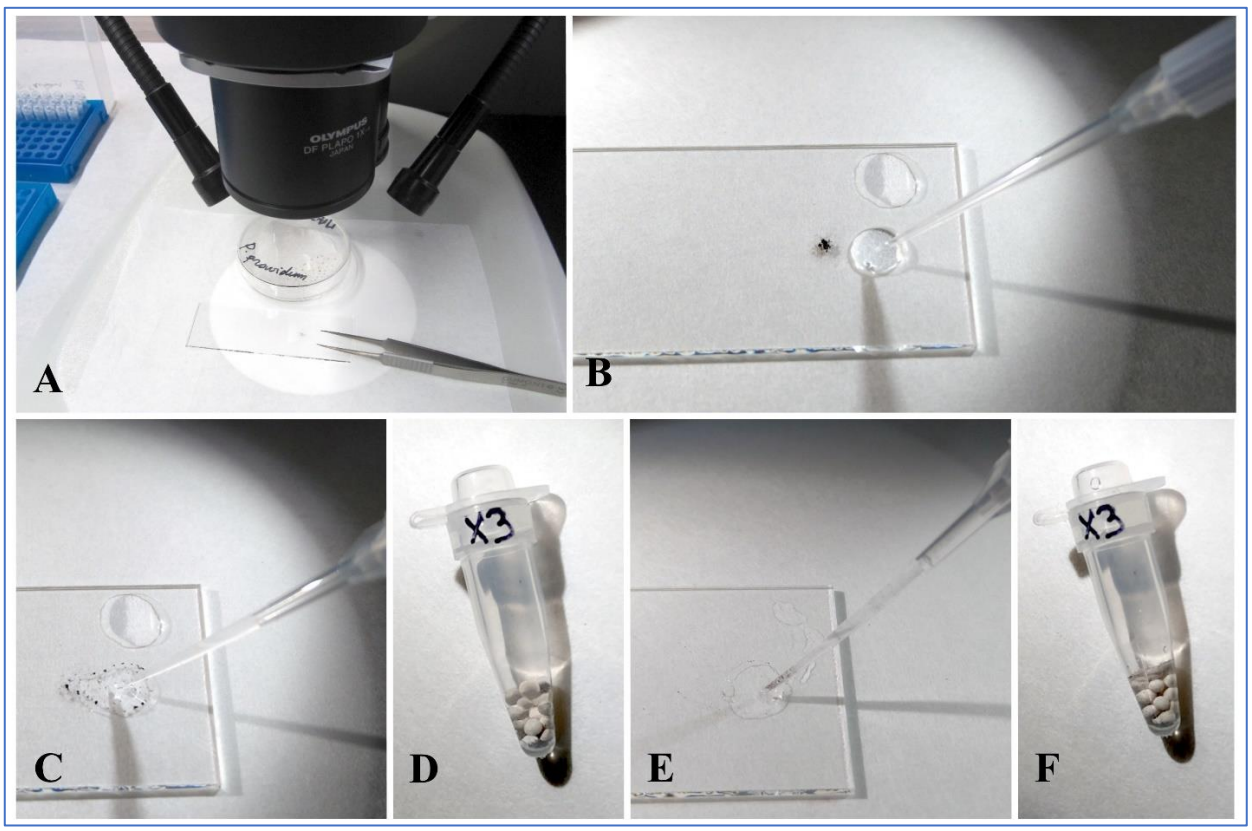

Figure 1. Image of the set up for spore harvesting from sporocarps of myxomycetes. A. Specimen and microscopy slide with harvested spores under stereomicroscope. B. Pipetting two drops of 15 µL TE buffer next to spores. C. Mixing the first drop of TE buffer with the spores. D. Transferring the first drop to a PCR tube marked with a unique extraction number, containing Zirconia/Silica beads. E. Mixing the second drop of TE buffer with remaining spores. F. Transferring the second drop to the PCR tube.

When harvesting spores from the same specimen a clean area of the same microscopy slide can be used, but tweezers and needles need to be cleaned and sterilized again. If a new specimen is sampled, a new microscopy slide should always be used.

All tubes must be inserted in the tube holde and placed in a bead beater for the material to be subjected to grinding for 60 sec at 30 Hz. After this, the tubes should be spun down and kept at -20 °C until the PCR can be run. Before performing the PCR run the tubes are thawed to room temperature and placed in a thermocycler for 10 minutes at 90 °C, then spun down and kept on ice while preparing the PCR run.

The PCR and sequencing protocol used in this study

The nearly complete small-subunit ribosomal RNA gene (SSU) is obtained by using the following primers:

AmyxF or S3: GATCCTGCCAGTAGTGTATGC (Forward)
Rib2: GCAGGTTCACCTACGATTACC (Reverse)

The PCR mix should be prepared in a total volume of 25 µL, using the PCR reagents, containing 1.25x DreamTaq^{TM} buffer (including 2.5 mM $MgCL_2$), 0.25 µg/µl Bovine Serum Albumine, 1 mM of each of the deoxynucleotide triphosphates and 1 U DreamTaq^{TM} DNA Polymerase (Thermo Fisher Scientific, Merelbeke, Belgium).

The PCR cycler should be run using the following program: 95°C for 2 min; 95°C for 30 sec, 55°C for 30 s and 72° for 2 min. The last three steps are repeated 34 times and the last cycle is followed with a final elongation step at 72° for 10 min. When the program is finished the PCR tubes should be kept at 4°C.

The PCR products are enzymatically purified by adding 1 unit of Exonuclease I and 0.5 unit of Fast Alkaline Phosphatase (Thermo Fisher Scientific, Merelbeke, Belgium). Sequencing of the purified samples (for the purposes of developing this protocol) was carried out by Macrogen Europe B.V. (Amsterdam, The Netherlands)

Results and Discussion

Selecting beads

In Fig. 2 the results of the PCR related to the grinding test are shown. The grinding speed of 30 Hz and time of 60 sec (lane 2) gave the best result in combination with 30 Zirconia/Silica beads (1 mm diam.). A narrow clearly visible band was an indication that there was no shearing of the extracted DNA. The band in lane 1 (30 sec, Zirconia/Silica beads) was narrower, which could be attributed to the shorter grinding time. In lane 3 (30 sec, one bead, 5 mm diam.) there appeared to be no visual DNA, suggesting that the spores were not ground enough. In lane 4 (60 sec, one bead, 5 mm diam.) tailing is visible below the band, meaning smaller fragments because of shearing. Also, Zirconia/Silica beads (1 mm diam.) in lane 5 also gave better result in comparison to lanes 6 (four metal beads, 2 mm diam.), 7 (one metal bead,

5 mm diam.) and 8 (two metal beads, 5 mm diam.), because all these showed smears instead of bands. Lane 5 shows the fronting of the DNA indicating an overload of the gel's capacity. The number of Zirconia/Silica beads was reduced from 30 to 10-15 fitting the smaller dimensions of the 200 µL PCR tubes.

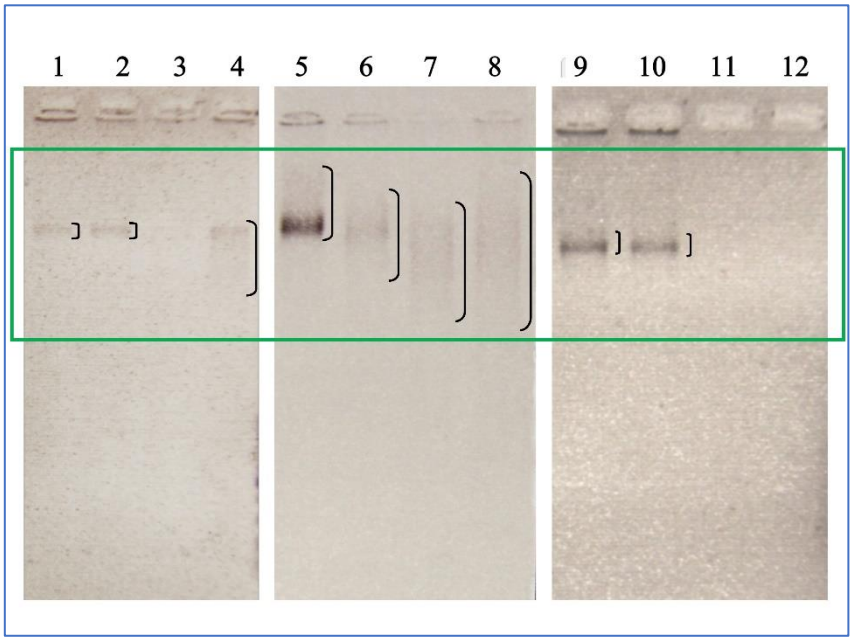

Figure 2. Color inverted image of three 1% Agarose gels (the numbers indicate the gel lanes in which 2 µL crude genomic DNA extract was run). The green frame indicates the area of interest. 1. Zirconia/Silica 1 mm beads (1 mm diam.). 2. Zirconia/Silica beads (1 mm diam.). 3. One metal bead (5 mm diam.). 4. One metal bead (5 mm diam.). 5. Zirconia/Silica beads (1 mm diam.). 6. Four beads (2.8 mm diam.). 7. One metal bead (1 mm diam.). 8. Two metal beads (1 mm diam.). 9. Whole sporocarp. 10. Half of a sporocarp. 11. Quarter of a sporocarp. 12. One eighth of a sporocarp. Samples 1 and 3 were ground for 30 seconds; all other samples were ground for 60 s, and all samples were ground at 30 Hz.

Testing the spore quantity

The spore quantity test showed a good visual result (Fig. 3) at the expected height of approximately 2000 bp in all lanes and the blank sample (BL). When the sample was replaced with TE buffer, the result was negative. The bands in lanes 1 and 2, respectively, with the PCR product of the spore extract from a complete sporocarp and from half of a sporocarp, seemed equally pronounced. Once the amount of spore was reduced to a quarter or one eight of a sporocarp, the crude DNA bands were not visible on agarose gel. The estimated concentration of the band in lane 1 was 150 ng/µL, and even the narrowest band (lane 4) corresponded to an estimated concentration of 90 ng/µL PCR product. For sequencing a fragment of >700 bp only 50 ng/µL is needed as advised by Macrogen Europe BV (2023).

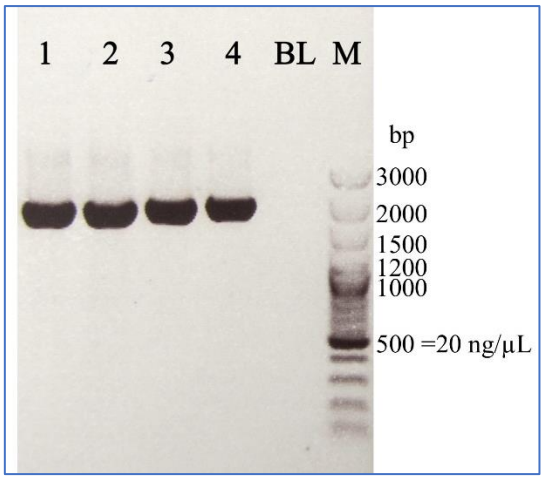

Figure 3. Color inverted image of 1% agarose gel stained with EthBr of 2 µL of the PCR products obtained from the genomic DNA extraction test with different amount of spores from *Badhamia utricularis*. 1. A whole sporocarp. 2. Half of a sporocarp. 3. A quarter of a sporocarp. 4. One eighth of a sporocarp. BL. Blank. M. DNA Marker or standard 100 bp DNA ladder with a range of 100 to 3000 pb, with known DNA quantity of 20 ng/µL of the 500 bp band for an estimation of the amount of PCR product in the samples.

Choosing an extraction medium and temperature

The gel image of a SSU PCR (AmyxF/Rib2, Fig. 4) of two species *Badhamia utricularis* (Bu) and *Physarum polycephalum* (Pp) shows the comparison of results between H_2O (sterile demineralized water) and TE (Tris-EDTA buffer pH 8.0) as extraction liquids. In all cases, TE (lanes 4-6, 12-14, 20-22) was much better as an extraction medium than water (lanes 1-3, 9-11, 17-19). Only in lane 3 a faint band of the spore extract from *Physarum polycephalum* was visible. There were no bands visible in any lanes were water was used to extract the spores.

Regarding the extraction temperature, the best option out of the three used herein was 90°C, as demonstrated by the most prominent bands in lanes 4-6. The bands in lanes 12-14 from the extracts kept at 95°C were less pronounced and the bands of lanes 20-22 from the treatment with 99°C were even weaker. The negative controls or blanks (BL) all showed no bands. With these results it should be taken into consideration that setting a 10 min heating step right before the PCR program, at 90°C instead of 95°C (the usual practice in direct PCR) must be considered.

Testing the preservation condition of the extracts

To evaluate the influence of storage time on the DNA quality of the genomic extracts preserved in TE at -20°C, a SSU PCR (AmyxF/Rib2) was run again on a selection of extracts after 5 years. The complete extract (spores, TE buffer and beads) was stored in one tube, as is the case with samples in lanes 1, 3, 7 and 8. For a few of the extracts, the supernatant was stored and tested in lanes 2, 4 and 6.

The sample in lane 5 were the spores kept with the beads and a minimum of TE. None of the samples showed detectable loss of quality in comparison with the original PCRs.

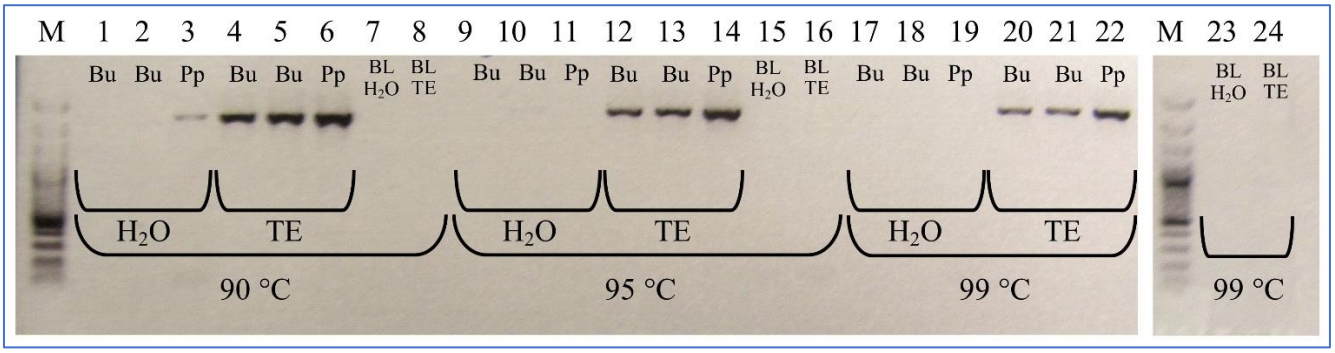

Figure 4. Color inverted image of 1% agarose gel stained with EthBr of 2 µL of the PCR products obtained from genomic DNA spore extraction of two species *Badhamia utricularis* (two repeats, Bu) and *Physarum polycephalum* (Pp) to test H_2O (sterile demineralized water) against TE (Tris-EDTA buffer pH 8.0), and to test the optimal extraction temperature (10 min at 90, 95 or 99 °C). Lanes indicated with BL are the results of blank extractions without spores but with H_2O or TE.

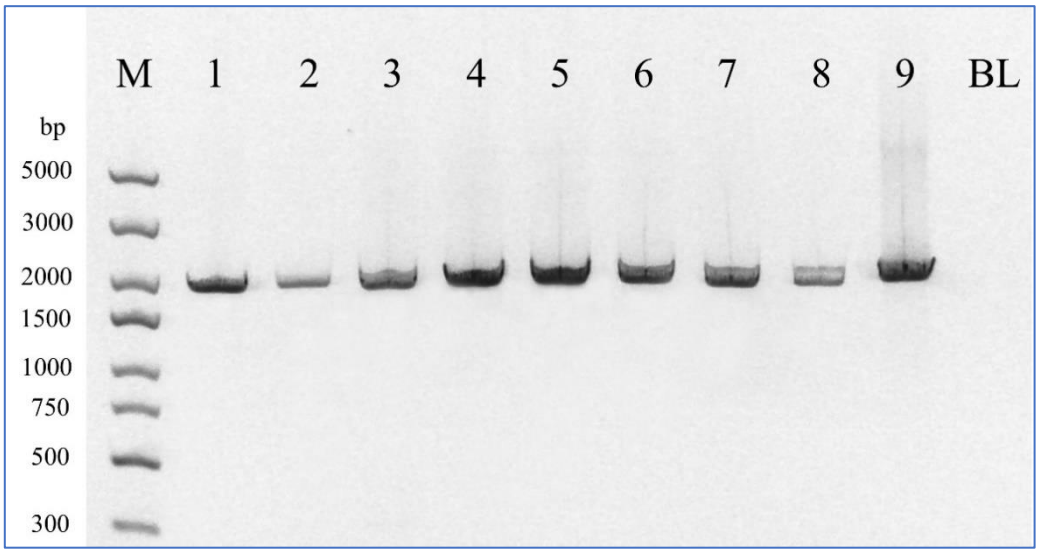

Figure 5. Color inverted image of a precast E-Gel EX 1% agarose gel pre-stained with SYBR Gold II DNA gel stain of 2 µL of the PCR products obtained from genomic DNA spore extraction performed 5 years previously. 1. *Badhamia utricularis* BUX2_TE. 2. Supernatant of *B. utricularis* BUX2_TE. 3. *Physarum polycephalum* PTE95. 4. Supernatant of *B. utricularis* BTE90_1. 5. Spores of *B. utricularis* BTE90_1. 6. Supernatant of *B. utricularis* BTE95_2, 7. *Craterium minutum* MdH1709004. 8. *Physarum didermoides* BW2348c. 9. Positive control M- Marker 1 Kb Plus Express DNA Ladder with a range of 100 to 5000 bp.

Conclusion

From the various tests described in this paper, the following combination was considered the optimal: extraction solution TE pH: 8, grinding with 1 mm Zirconia/Silica beads during 60 sec at 30 Hz, cell weakening at 90°C and storage at -20°C. This simple approach can serve as a fast, cheap and cleaner alternative to other extraction protocols, or it can be used as a preparation phase for these established methods. The obtained extracts can be used for analysis of multiple markers.

Including the ones listed in Table 1, several specimens (110) of a variety of taxa (50) have been successfully sequenced. The generated sequences are mostly from the SSU rRNA gene locus, however as extra multiple markers, the Elongation factor 1-alpha and Cytochrome c oxidase subunit 1 genes were also sequenced from a selection of the extracts (14). We intend to submit more of the obtained sequences in GenBank for public use and data of the corresponding specimens will be freely available on the MYXO-BE website (2020-2023).

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