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Current Trends in Biotechnology and Pharmacy
Vol. 7 (4) 825-836 October 2013, ISSN 0973-8916 (Print), 2230-7303 (Online)

Fast Microwave-based DNA Extraction from Vegetative Mycelium and Fruiting Body Tissues of Agaricomycetes for PCR Amplification

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Abstract

In this study, we tested a microwave-based DNA extraction method for subsequent DNA amplifications by PCR on vegetative mycelia and mushrooms of different Agaricomycetes. The extraction method requires tiny amounts of fungal material, is rapid and achieved within minutes, why it is superior to classical extraction methods which are work-intensive and require larger amounts of starting material, hours of time for performance and in addition specific expensive and hazardous chemicals for cell lysis and DNA purification. The microwave method with highest reliability is suitable for vegetative mycelium harvested from fresh and also from aged fungal cultures. It is especially attractive for slow-growing species of which larger amounts of mycelium are difficult to obtain from. The method is further applicable with success rates between 76.9% and 90.9% to fleshy mushrooms over a wide range of families of Agaricomycetes, both in fresh as well as in dried form, and also to firm young and older fruiting bodies of more robust leathery, corky and woody textures. Also non-cultivable species can thus be accessed for DNA analyses. Finally, we show that also fungal infested plant material such as millet, straw, wood and bark can be used. However, here the success depends on freshness of the material and on presence of sufficient surface mycelium.

Keywords: Agaricomycetes, mushrooms, mycelium, microwaving, DNA extraction, PCR amplification

Introduction

Classically, fungal DNA isolation involves cultivation of individual clones, harvesting the mycelium and isolating the DNA from usually frozen or freeze-dried samples (1-6). Depending on the growth capacity of a species the whole process can take up several weeks, provided that a fungus can be cultured. Due to the rigid character of fungal cell walls, the applied isolation protocol requires an effective method to break the cells and thereby release the DNA. Conventional DNA isolation protocols include an initial mechanical grinding and a successive treatment with chemicals to disrupt the fungal cell walls and membranes. The released DNA is subsequently purified from cell wall debris, proteins and other molecules, mostly applying a mixture of phenol-chloroform followed by centrifugation and ethanol precipitation of the DNA from the watery supernatant. The obtained genomic DNA is used for a broad spectrum of molecular biological applications such as DNA library construction, sequence analyses, subcloning of (PCR-amplified) DNA fragments, Southern blot analyses, screening of genetic transformants, fungal species and strain identification (barcoding), fungal population analyses, and more. However, conventional isolation protocols apply some hazardous chemicals like liquid nitrogen, CTAB (cetyltrimethylammonium bromide), SDS (sodium dodecyl sulfate), phenol:chloroform and β-mercaptoethanol. Furthermore, common methods require sufficient amounts of starting

material, are time consuming due to extensive handling and purification steps, involve costs for chemicals and are an extra investment when applied in form of commercial kits.

Not all applications require high quality, not much sheared DNA. In recent years, alternative short-protocols were developed for isolation of DNA from fungi for applications in connection with PCR (7-15). Some of these alternatives use microwave irradiation for breaking up the fungal cell walls and membranes to release the DNA from the cells without former mechanical grinding (11-15) and in some instances this is also done without any specific lysis buffer (12-15). To our knowledge, there are currently only two reports on higher basidiomycetes (Agaricomycetes) using such technique (14,15). Nakazawa *et al.* (14) applied microwaving for quick screening of collections of transformants of the fungus *Coprinopsis cinerea* whereas Izumitsu *et al.* (15) presented a protocol for rapid DNA isolation from young mycelium grown on artificial medium and from fresh fleshy mushrooms of a range of Agaricomycetes. Here, we test these methods on fungal cultures and diverse biological materials taken from nature.

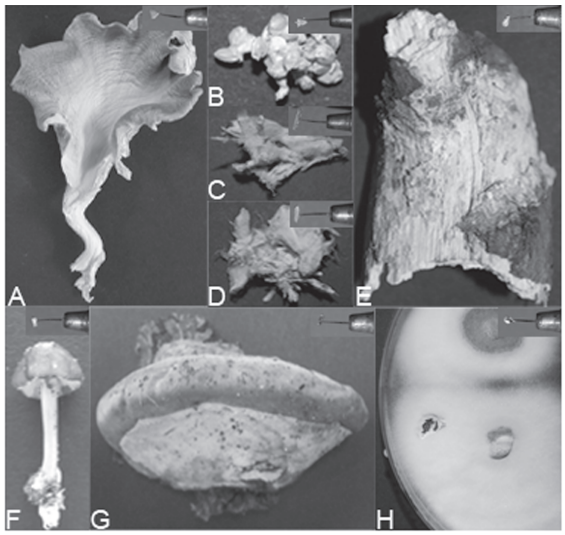

Fig. 1. Exemplary fungal materials used in this study. **A.** Dried cultivated mushroom of *Pleurotus sajor-caju*. **B.** Millet infested with *Pleurotus djamor*. **C.** Wheat straw and **D.** poplar wood chips infested with mycelium of *Coprinellus micaceus*. **E.** White-rotting lilac branch infested with *Daedaleopsis* sp.2. **F.** Fleshy *Entoloma* sp. mushroom and **G.** firm *Fomes fomentarius* fruiting body collected from the wild. **H.** Fresh mycelium of *Coprinopsis cinerea* FA2222 grown on a YMG/T agar plate. Insets in subfigures indicate amounts of material collected for microwaving.

Material and Methods

***Fungal materials*:** *C. cinerea* strain FA2222 (16) was cultivated at 37°C on solid YMG/T medium (17), *Heterobasidion irregulare* strain TC-32-1 (18) at 25°C on 2% malt extract agar (1% agar) and some unknown slow-growing basidiomycetes (kindly supplied by Prof. F. Schauer, Greifswald, Germany) at 25°C on either solid YMG/T, rich YMPS (3 g/l yeast extract, 3 g/l malt extract, 5 g/l peptone, 10 g/l sucrose, solidified by 20 g/l agar) and *Ustilago* complete medium (19). Fresh and air-dried fruiting bodies of *Pleurotus* species (*calyptratus*, *cystidiosus*, *djamor*, and *sajor-caju*) and *P. cystidiosus* and *P. djamor* mycelium grown on millet (Fig. 1A,B) were kindly supplied by A.A. Shnyreva, straw and birch and poplar wood chips infested with mycelium of *Coprinellus* species (*micaceus*, *radians* and *xanthrothrix*) by J. Barb (Fig. 1C,D). Miscellaneous fleshy and firm mushrooms grown on wood or in meadows (Fig. 1E-G) were collected from the grounds of the Goettingen University North Campus and from forests of the surroundings of Goettingen, Germany. Genera and, where possible, also species names were determined using the guide books of Breitenbach and Kränzlin (20). Purified genomic DNA of *C. cinerea* strain AmutBmut (16) was kindly supplied by Dr. B. Pickel.

***Microwaving and PCR*:** Fungal mycelium, tissue sections from basidiocarps (where possible separated in cap and stipe), and infested millet, straw and wood (Fig. 1) were directly used for DNA extraction in sterile dd$H_2$0 or TE-buffer (10 mM Tris, 1 mM EDTA; pH 8.0). A pinhead sample of mycelium from fungal cultures (ca 2 x 2 mm^2, corresponding to about 2 mg) was scraped from the surface of plates thereby taking care to avoid

the agar (Fig. 1H). Similar small samples from basidiocarps (about 5 mg; Fig. 1A,F,G) and from infested plant materials (Fig. 1B-E) were obtained by cutting with a flamed razorblade or by picking with a sterile needle. Individual samples were transferred into 1.5 ml sterile micro-centrifuge tubes (E-tubes; Sarstedt AG & Co., Nümbrecht, Germany) and 100 µl sterile ddH_2O or TE buffer were added. In standard reactions, the closed tubes were microwaved for 1 min at 600 W in a household microwave oven (MS 197 H 700W, LG Electronics Deutschland GmbH, Ratingen, Germany), shortly vortexed, stored for 30 sec at room temperature (RT) and microwaved again for 1 min at 600 W. Afterwards, tubes were transferred for about 10 min to -20°C and then centrifuged at 9.300 x *g* for 5 min at RT. Typically, 1 µl of resulting supernatants was directly used in PCR in a reaction mixture of 25 µl containing PCR buffer (final concentration: 10 mM Tris pH 8.8, 50 mM KCl, 0.1% Triton X-100), 1.5 mM $MgCl_2$, 0.2 mM dNTPs (Fermentas GmbH, St. Leon-Rot, Germany), 0.4 µM of each primer, and 1 U of *Taq* DNA polymerase. The fungal universal primers ITS1 (5'-TCCGTAGGTG AACCTGCGG-3') and ITS4 (5' -TCCTCCGCTTA TTGATATGC-3') were used for amplification of the ITS1-5.8S-ITS2 ribosomal DNA regions (21,22). Control reactions contained no DNA (negative controls) or 1.25 ng of purified genomic DNA of *C. cinerea* AmutBmut. PCR conditions were 2-min initial denaturation at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at annealing temperature of 55°C, and 30 s for primer extension at 72°C, and a final extension step at 72°C for 10 min. 5 µl per reaction were analyzed by electrophoresis on 1% agarose gels in TAE buffer (23). Gels were photographed with a Gel Doc 2000 Imaging and Documentation System and band intensities were estimated with Quantity One 4.2 software (Bio-Rad GmbH, Munich, Germany). Resulting DNA amounts were multiplied by 5 to calculate the total DNA amounts in the respective 25 µl PCR samples.

***Sample boiling and PCR*:** Mycelial and fruiting body samples of similar size than in the microwaving approach were transferred into 1.5 ml E-tubes with 100 µl sterile ddH_20. E-tubes were transferred for 1 min into boiling water, then 30 s stored at RT, a second time transferred for 1 min into boiling water and finally for 10 min into -20°C. After centrifugation at 9.300 x *g* for 5 min at RT, 1 µl of respective supernatants was applied in PCR reactions as described above.

Results and Discussion

Microwaving fungal mycelium from laboratory cultures on agar-medium in Petri dishes: Initially, we used fresh mycelium of fully grown *C. cinerea* FA2222 cultures on YMG/T plates to test the two protocols described in literature for microwaving basidiomycete samples (14,15). Main differences were the type of liquid and its volume (30 µl ddH_20 versus 100 µl TE, pH 8), the time (10 min versus 2x 1 min) and the applied power (500 W versus 600 W) of microwaving. First trials following the protocol of Nakazawa *et al.* (14) lead to explosive opening of the E-tube lids with loss of liquid and also melting of the E-tubes already after about 2 to 5 min of microwaving (Fig. 2). Such effects were not encountered with the 2x 1 min short time microwave exposure. Thus, we continued with the protocol of Izumitsu *et al.* (15). Alterations were done to change from TE buffer to ddH_20

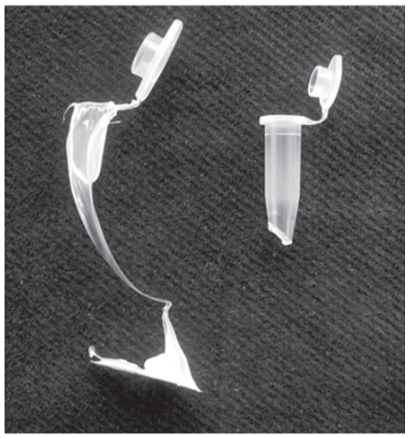

Fig. 2. 1.5 ml and 0.5 ml E-tubes after microwave treatment for 5 min at 500 W. Lids were opened, liquids were lost and tubes were molten.

with no negative consequences for subsequent PCR amplifications with primers ITS1 and ITS4 (not shown). Visible PCR bands in agarose-gels were obtained from up to 100fold dilutions of DNA-H_20 samples from fresh *C. cinerea* mycelium (Fig. 3A). Considering 60-90 copies of the rRNA gene clusters per *C. cinerea* genome (24) and a genome size of 36.2 Mb (25) and a DNA amount of 145 pg after 35 cycles of PCR amplification of the 599 bp-long ITS region from a 10fold dilution of the original sample (the dilution that still gave a reasonably visible band in a not fully saturated PCR reaction, compare Fig. 3A), about 47-70 fg genomic DNA (i.e. about 1.2 to 1.8 copies of the complete *C. cinerea* genome) should be present in 1 µl of the undiluted solution after microwaving of mycelium. In the PCRs with 1 µl of the 1:10 dilution of DNA (i.e. about 4.7-7.0 fg DNA), we expect thus originally about 7.7 - 10.6 copies of the ITS region which proofed to be sufficient for amplification of the fragment with primers ITS1 and ITS4. Similar amounts of genomic DNA have already been shown in previous studies with other lower eukaryotes to be sufficient for amplification of ITS regions by PCR (26,27). Furthermore, we even amplified a fine band in PCRs of 1 µl of the 1:100 DNA dilution (Fig. 3A) that by the above calculation originally should only have had 1.1 copies of the ITS region. Theoretically, this should result in 35 cycles in 2.5 ng DNA in total. However, the DNA amount of the fine band in Fig. 3A is possibly twice as that (5 ng) which suggests that our calculation is probably a 10fold underestimation in actual original DNA concentration.

The standard method was further used to extract and compare DNA from fresh and from 4-month old, cold-stored mycelium of *C. cinerea* from fully-grown Petri dishes, from fresh small 2-day old *C. cinerea* colonies (ca. 1 cm in Ø), from 4-month old, cold-stored mycelium from fully grown plates of *H. irregulare* and from 1 to 4 cm sized colonies of very slow growing unknown basidiomycetes that were cultivated on different media (see Material and methods) for 2 weeks to 2 months at 25°C. In total, 24 different mycelial samples of *C. cinerea*, 2 samples of *H. irregulare* and 6 samples from three unknown species were analysed. In all instances (100% of reactions), the respective ITS regions of the fungi were successfully amplified with primers ITS1 and ITS4 after microwaving, indicating that neither the age of the mycelium nor the size of colonies or the media used for cultivation of the fungi hindered DNA isolation and subsequent PCRs (Fig. 3B). Furthermore, PCR amplifications to saturation of reaction with other primers of single copy genes on fragments sized up to 3.5 kb were also possible without difficulty with 0.5 to 1 µl solution of the undiluted samples (experiments not further shown). Also these results suggest that the DNA concentration in the microwaved samples is likely somewhat higher than the amounts calculated above.

Izumitsu *et al.* (15) in their studies used always fresh mycelium (4 to 6 days old). These authors applied the technique to mycelium of the Agaricales *Agaricus bisporus*, *Lentinula edodes*, *Flammulina velutipes*, *Pleurotus eryngii*, *Pleurotus pulmonarius*, *Hypsizygus marmoreus*, *Grifola frondosa*, and *Mycena chlorophos*. Our analysis expands the range of species to which the technique was successfully applied to. Important new findings from our study are that quite old mycelium and also very small cultures can be used for microwave-preparation of DNA. The latter is especially beneficial for species determination of fungi that are difficult to grow and of which therefore only relative old mycelium in minute amounts can be available.

Microwaving mycelium from infested plant substrates: Basidiomycetes often infest dead or living recalcitrant plant materials and it is not easy to obtain fungal DNA in quantity and of quality from such substrates. Co-extracted ingredients from the complex organic substrates such as organic acids or phenolic compounds can have an inhibiting effect on the PCR reaction (28-31). Therefore, small samples of infested millet, straw, wood and bark samples (Fig. 1B-E) were directly used in DNA extraction by microwaving as

Vol. 7 (4) 825-836 October 2013, ISSN 0973-8916 (Print), 2230-7303 (Online)

examples of fungal infected plant materials. ITS regions were successfully amplified by PCR from all microwaved samples of the two tested *Pleurotus* species (Fig. 3C) cultivated for 3 months at RT on millet (each species was tested 2x) and of the three different *Coprinellus* species (Fig. 3D, *C. micaceus* is exemplarily shown) kept for 4 months under laboratory conditions in jars on moisturized straw, on birch and on poplar wood chips (each two samples per substrate were tested for *C. micaceus* and each one sample per substrate for *C. radians* and *C. xanthrothrix*).

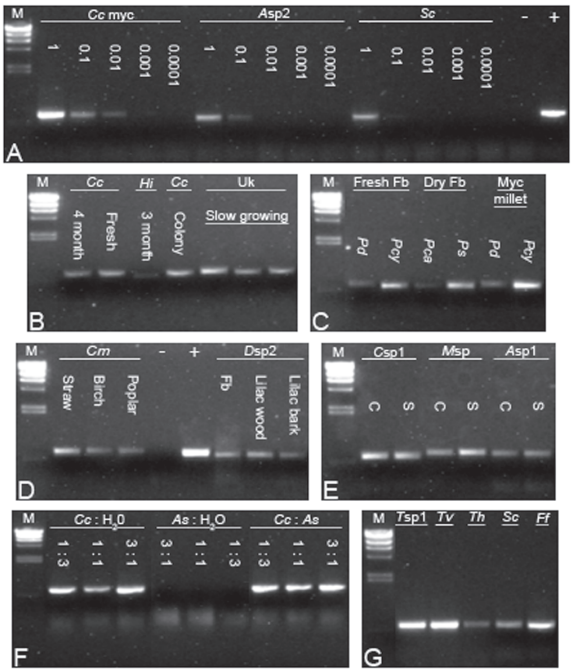

Fig. 3. Analysis of PCR amplifications of fungal ITS regions with primers ITS1 and ITS4 on 1% agarose gels, using per reaction 1 µl of solution after microwaving fungal materials by the standard method if not otherwise stated. **A.** Serial dilution of DNA solution (1 to 10^{-4} µl) from fresh *Coprinopsis cinerea* FA2222 mycelium (*Cc* myc), a fresh fleshy *Agrocybe* sp.2 fruiting body (*A*sp2) and a firm *Schizophyllum commune* fruiting body (*Sc*). **B.** Samples from a 4-month old *C. cinerea* culture, fresh mycelium from the outer region of a fully grown 9 cm Petri dish culture of *C. cinerea*, a 4-month old *Heterobasidion irregulare* (*Hi*) culture, an 1 cm sized 2-day-old *C. cinerea* colony, and three different unknown slow-growing basidiomycetes (Uk). **C.** Samples from fresh fruiting bodies (Fb) and mycelium (Myc) on millet of *Pleurotus djamor* (*Pd*) and *Pleurotus cystidiosus* (*Pcy*) and dried fruiting bodies of *Pleurotus calyptratus* (*Pca*) and *Pleurotus sajor-caju* (*Ps*). **D.** Samples from straw and birch and poplar wood chips infested with *Coprinellus micaceus* (*Cm*) and a sample of a firm *Daedaleopsis* sp.2 (*D*sp2) fruiting body compared with samples of lilac wood and bark infested by the same fungus. **E.** Samples from exemplary fleshy mushrooms: *Conocybe* sp.1 (*C*sp1), *Mycena* sp. (*M*sp) and *Agrocybe* sp.1 (*A*sp1). **F.** PCR reactions of 1 µl from 1:3, 1:1 and 3:1 ddH_2O diluted samples of pure genomic DNA of *C. cinerea* (*Cc*) AmutBmut (which corresponds to 0.3 , 0.6 and 0.9 ng, respectively) and of mere solution (0.75, 0.5 and 0.25 µl, respectively) of microwaved *Amanita strobiliformis* cap tissue (*As*) in comparison with 1:3, 1:1 and 3:1 mixtures of both. **G.** Samples of exemplary tough mushrooms: *Trametes* sp.1 (*T*sp1), *Trametes versicolor* (*Tv*), *Trametes hirsuta* (*Th*), *S. commune* (*Sc*) and *Fomes fomentarius* (*Ff*). Lanes marked M = 200 ng Lambda DNA/*Hin*dIII marker, + = positive PCR control performed with 1.25 ng purified genomic AmutBmut DNA, - = negative control. Positive and negative controls were done in all series of PCR amplifications although the controls are shown here only in some cases.

Microwave based DNA extraction

The samples from the laboratory were from pure cultures of single organisms and characterized by presence of surface mycelium, factors which both could ease the release of enough fungal DNA into solution. Therefore, we also tried unsterile bark and white-rotted wood from infested dying branches of *Ribes sanguineum* (red-flowering currant) and *Syringae tigerstedtii* (lilac) bushes (each two samples per type of substrate and plant species). Only one bark and one wood sample of the lilac functioned subsequently in PCR (Fig. 3D). Samples of decaying deadwood of beech were also unsuccessfully applied in microwaving and PCR. In total, only 1/6 wood samples and 1/4 bark samples from wild collections resulted in PCR bands. Positive samples had strong fresh mycelium on the surface. Samples with some dried mycelium on the surface and samples with no surface mycelium were ineffective. The results indicate that the microwave technique has only limited application for wood samples. However, this differs not from other techniques applied in fungal DNA isolation from wooden substrate for PCR amplifications (30,31).

Microwaving tissues from fleshy fruiting bodies: DNA was extracted by the standard microwaving method from cap samples of fresh *P. cystidiosus and P. djamor* fruiting bodies cultivated in the lab and from cap and stipe samples from fresh fleshy fruiting bodies from seven different species (*Amanita strobiliformis*, *Agrocybe* sp.1, *Agrocybe* sp.2, *Conocybe* sp.1, *Conocybe* sp.2, *Entoloma* sp., *Mycena* sp., *Psathyrella* sp.) taken from nature. From all species except *A. strobiliformis* (Fig. 3F), it was possible with primers ITS1 and ITS4 to amplify in PCR the respective ITS-regions after microwaving (see examples in Fig. 3A,E). Izumitsu *et al.* (15) reported in their studies that success with wild mushrooms was greater with stipe than with gill tissue [95.3% (41/43 tested cases) versus 73.5% (25/34 of tested cases) positive reactions]. Instead of gills, we used always inner pileus tissue from the caps but success rates were similar to those obtained by Izumitsu *et al.* (15) with gills. Apart from *A. strobiliformis*, 76.9% (10/13) of all cap samples and 90.9% (10/11) of all stipe samples from wild mushrooms gave positive results in PCR. Furthermore, ITS bands were obtained in PCR from all *Pleurotus* cap samples (2 per species) (Fig. 3C). In conclusion from this series of experiments, we can confirm the earlier observation of Izumitsu *et al.* (15) that the microwave technique can easily be applied to most fresh fleshy mushrooms. Izumitsu *et al.* (15) successfully isolated DNA from fleshy mushrooms of cultivated species (*A. bisporus*, *L. edodes*, *F. velutipes*, *P. eryngii*, *G. frondosa*, *H. marmoreus*) and of species collected from the wild [*Agrocybe chaxingu*, 9 different *Amanita* species (*flavipes*, *farinosa*, *fritillaria*, *fuliginea*, *oberwinklerana*, *imazekii*, *sychnopyramis* f. *subannulata*, *vaginata*, sp.), *Boletus rubropunctus*, *Cantharellus cibarius*, *Hymenopellis raphanipes*, 2 different *Inocybe* species (*sphaerospora*, sp.), 4 different *Russula* species (*crustosa*, *mariae*, *subnigricans*, cf. *subnigricans*), *Tricholoma bakamatsutake*]. Together with our study, this gives in total 35 different species from 18 different genera and 17 different families of which DNA of sufficient amount and quality for PCR amplifications of ITS regions was obtained from microwaving fresh fruiting body tissues.

Following the standard protocol, we unsuccessfully tested in total 3 cap samples and 2 stipe samples from fresh mushrooms of *A. strobiliformis* collected at two different occasions from underneath a birch tree. Also PCR with 10 fold sample dilutions did not lead to amplification of the ITS region and neither PCRs with 5 µl and with 10 µl of undiluted solution per sample. The ITS4 primer (combined with a primer ITS1F) has previously been shown to work on isolated DNA of this species (32) and the ITS1 primer in combination with ITS4 on several closely related *Amanita* species (33) and both primers work perfectly with multiple species over the broad range of Agaricomycetes and also other fungi (21,22,31-34, this study). It is therefore not likely

that the failure in PCR with *A. strobiliformis* samples based simply on unsuitability of the two primers. As another possibility, some type of metabolic product(s) of the fungus could hinder the PCR reactions. Therefore, a set of PCR reactions was done in which aliquots from microwaved *A. strobiliformis* cap tissues (0.75 to 0.25 µl) and purified genomic *C. cinerea* AmutBmut DNA (0.3 ng to 0.9 ng) were mixed, parallel to PCRs with only respective aliquots of *A. strobiliformis* material and PCRs with respective amounts of pure *C. cinerea* DNA. All PCR reactions with *C. cinerea* DNA gave strong bands of identical size (corresponding to the *C. cinerea* ITS region) in agarose gels unlike the PCRs with only aliquots from microwaving *A. strobiliformis* tissues (Fig. 3F), implying that the cause of failure in PCR is not an inhibitor within the samples. As another reason, it might just be that microwaving did not freely release the *A. strobiliformis* DNA from the hyphal cells into solution or that released DNA was quickly degraded (see discussion below).

Fleshy mushrooms tend to have only a very short life time due to fast onset of decay, even when stored at colder temperature. Not surprisingly therefore, we were not anymore able to obtain DNA functional in PCR from disintegrating mushrooms such as *Conocybe* sp. when stored for one week in the fridge (not further shown). However, experiments with pileus tissues from air-dried mushrooms from *P. sajor-caju* and *P. calyptratus* (stored for 4 weeks at RT) showed that it is also possible to employ freshly harvested and directly dried mushrooms for later use in DNA preparations for PCR (Fig. 3C).

Microwaving tissues from fruiting bodies of tough structure: New in this study, DNA was extracted by the microwave-based method also from cap tissues of collections of firm durable bracket fungi that are characterized by a tougher and fairly dry consistency. With increasing degree of toughness, we divided mushrooms collected in nature from wood logs and infested branches into fruiting bodies with leathery structure (*Daedaleopsis* sp.1, *Schizophyllum commune*, *Trametes versicolor*, *Trametes* sp.1), corky texture (*Trametes hirsuta*, *Trametes* sp.2) and woody appearance (*Daedaleopsis* sp.2 grown on the *S. tigerstedtii* branch analysed above, *Ganoderma* sp., *Fomes fomentarius*). Microwaving for DNA isolation was done when the fruiting bodies were freshly collected as well as after storage for two and a half months at 5°C, resulting in 2 to 4 reactions per species. PCR reactions were successful for 23 of the 25 samples analysed in total (92%) and from every species at least one of two samples were positive regardless of their degree of rigidity (see positive results of five exemplary species in Fig. 3G). Notably, the size of the amplified ITS fragment of the *Daedaleopsis* sp.2 fruiting body was identical to those obtained from the bark and the branch of *S. tigerstedtii* on which the fruiting body grew on (Fig. 3D).

Taken the same parameters as used above for the calculation of the *C. cinerea* DNA concentrations, DNA amounts obtained from bracket fungi were comparable to amounts from fleshy mushrooms (ca. 8 fg/µl) but about 10fold lower than DNA amounts obtained from fresh mycelia (Fig. 3A). The experiments also revealed that tough, fairly dry bracket fungi can be accessible for DNA extraction by microwaving even after longer storage. However, we also tested tissues of two different decomposing *Trametes* fruiting bodies of rubbery-crumby consistency (collected from rotting wood) but the decay of the specimens was apparently too advanced to still result in quality DNA isolation.

Further analysis of the method: Microwaving can affect the biological material in two ways, by its electromagnetic field ("non-thermal-effect") and by the heat it produces (35-41). Heat can also be applied to cells just by boiling. Accordingly, some rapid DNA extraction procedures for PCR applications appoint (repetitive) quick heating (e.g. to 95-100°C between 1 and 30 min) and rapid cooling (8,9). Here, we also tested the effect of boiling on DNA-release from *C. cinerea* mycelium and from a

selection of mushrooms of different textures. In subsequent PCRs, no major difference was observed between DNA obtained from a material by the microwaving procedure and by the boiling procedure described in detail in the methods section (Fig. 4). In conclusion, heat must be a main factor for the release of DNA from the cells and both methods might be used.

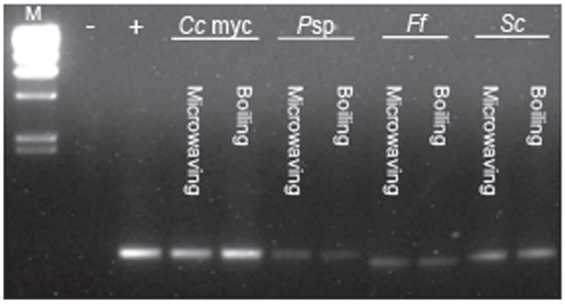

Fig. 4. Comparison of PCR amplifications of fungal ITS regions with primers ITS1 and ITS4 on 1% agarose gels, using per reaction 1 µl of solution after microwaving and after boiling of samples from fresh *Coprinopsis cinerea* mycelium (*Cc* myc), a fresh fleshy *Psathyrella* sp. mushroom (*P*sp), and firm fruiting bodies of *Fomes fomentarius* (*Ff*) and *Schizophyllum commune* (*Sc*). Lanes marked M = 200 ng Lambda DNA/*Hin*dIII marker, + = positive PCR control performed with 1.25 ng purified genomic AmutBmut DNA, - = negative control.

Comparing the two principles, the general handling of microwaving is easier and much faster since no bath of boiling water has to be prepared and there is no danger of burning oneself with the hot water when moving samples in or out. Importantly, Stroop and Schaefer (35) pointed out that microwaves are less aggressive in breaking down DNA and preserve the DNA chain lengths better than heat treatment at the boiling point of water. In cells, electromagnetic fields produced by microwaves are understood in non-thermal manner to affect the cell membrane integrity and to cause pore formation. This eventually leads to cellular leakage of proteins, electrolytes and DNA (36-39). Microwaves in dose-dependent manner also cause opening of DNA double-strands below their melting temperature (40) and may result in single- and double-strand breaks such as under influence of certain metal ions (41). We tested different irradiation schemes on fresh *C. cinerea* mycelium and on tissue samples of fresh fleshy, corky and woody mushrooms. Time of irradiation mattered for good yields in PCR amplification in case of the mushrooms but less in case of the generally more effective vegetative mycelium (Fig. 5). Shorter 1-min irradiation was better for mushroom tissues than in total 2-min irradiation, regardless of whether done as in the standard protocol in two 1-min steps or whether done consecutively. Since there was a decrease up to failure in DNA amplification by PCR upon longer microwaving (Fig. 5), it must be assumed that (parts of) released DNA will be degraded through microwaving in the liquid. Izumitsu *et al.* (15) stated that the 10-min -20°C freezing step in the protocol was optional. In our reactions, application of a freezing step appeared sometimes sub-optimal compared to non-freezing (Fig. 5). Since the quality assay in our study for DNA release is a PCR reaction that does not depend on double-stranded DNA, it is not as likely that due to quick freezing a problem was generated by lack of reformation of DNA double-strands. More likely appears that slower cooling down to RT will allow longer permeability of the membranes made porous by the rotational energy transferred onto dipole molecules within the membranes through the microwaves (38) and, in consequence, more leakage of DNA from the cells. Such longer DNA release may then compensate former decline by microwave-induced DNA degradation. If such different effects function at the same time, the results will be more visible in samples from mushrooms releasing less easily higher amounts of DNA than in samples from vegetative mycelium being more effective in the DNA release. In conclusion, while the standard protocol of microwaving as given in the methods might be applied for vegetative mycelium as it is, for mushroom samples a single 1-min treatment with cooling down to RT appears to be the better choice for future studies.

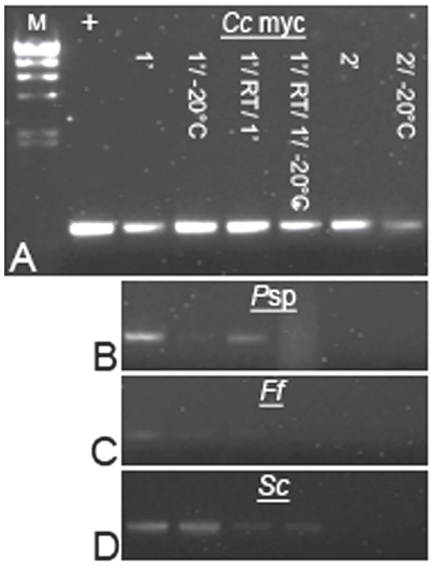

Fig. 5. Comparison of PCR amplifications of fungal ITS regions with primers ITS1 and ITS4 on 1% agarose gels, using per reaction 1 µl of solution after microwaving under different irradiation schemes, with and without subsequent 10-min freezing at -20°C. **A.** Samples from fresh *Coprinopsis cinerea* mycelium (*Cc* myc), **B.** a fresh fleshy *Psathyrella* sp. mushroom (*P*sp), and firm fruiting bodies of **C.** *Fomes fomentarius* (*Ff*) and **D.** *Schizophyllum commune* (*Sc*), loaded in the same order of irradiation schemes. Applied irradiation schemes: 1 min irradiation (1'); 1 min irradiation with subsequent 10-min freezing at -20°C (1'/ -20°C); 1 min irradiation followed by 30 sec at RT and another 1 min irradiation (1'/ RT/ 1'); 1 min irradiation followed by 30 sec at RT, 1 min irradiation and subsequent 10-min freezing at -20°C (1'/ RT/ 1'/ -20°C); 2 min of continued irradiation without (2') and with freezing (2'/-20°C). Lanes marked M = 200 ng Lambda DNA/*Hin*dIII marker, + = positive PCR control performed with 1.25 ng purified genomic AmutBmut DNA. Negative control not shown.

Conclusions

In this study, we successfully tested the rapid and cheap microwaving-protocol recently published by Izumitsu *et al.* (15) for DNA extraction from vegetative mycelium and fleshy mushrooms of Agaricomycetes for subsequent applications in PCR amplifications. As Izumitsu *et al.* (15), we observed highest success rates with this method. 100% of all mycelial samples and 76.9 to 90.9% of samples from fresh fleshy mushrooms gave positive results in subsequent PCR amplifications. In addition to verifying the observations of the former publication, we show here that the technique can also be applied to very old mycelium of well growing species, to poorly growing mycelium, and to mycelium growing on diverse plant materials. Low success rates in subsequent PCR amplifications were only encountered with infested wood and bark collected from nature but such material presents a general resisting problem in fungal DNA isolation (30,31). In this study, the method was furthermore effectively applied to dried mushrooms and to fruiting bodies of tough and dryer structures. In total, 92% of samples of firm bracket fungi performed positively in PCR amplifications.

In summary, the standard protocol is suitable for extraction of fungal DNA for PCR amplifications of non-cultivable species, slow and poorly growing species, old cultures, and of fresh and dried fleshy and tough fruiting bodies. However, the results show that shorter times of microwaving (1x1 min) should be applied for mushrooms and that the 10-min freezing at -20°C is better omitted in favour of slowly cooling down to RT. Overall, the method of DNA extraction by microwaving is rapid and easily performed within minutes, and needs only tiny amounts of fungal material and no expensive and hazardous chemicals. Negative effects by release of inhibitors of PCR amplifications were not observed. Furthermore, the ease of handling with minimal labour and time input enables simultaneous treatment of large numbers of samples.

Acknowledgements

We are indebted to Prof. F. Schauer for sending us slow growing unknown fungal strains and to colleagues and students from our group who supplied material for this study (Dr. B. Pickel, A.A. Shnyreva, J. Barb) and observations on first experimental trials of the methods (Y. Yu) and who helped to cultivate fungi (M.-C. Hsieh, M.

Zomorrodi) and in collecting mushrooms (K.K.R. Lakkireddy, A.-K. Straten).

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