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DNA extraction and PCR amplification method suitable for fresh, herbarium-stored, lichenized, and other fungi

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Abstract: This paper presents a DNA extraction method suitable for fresh, herbarium-stored, lichenized and other fungal specimens. The method is fast and reliable, comparatively inexpensive and is suitable for obtaining PCR amplification quality DNA from large numbers of samples in a short time. The method has been tested with over 300 samples of *Ascochyta*, *Phyllosticta*, *Ramalina*, *Parmelia* and *Physconia*. Amplifiable fungal DNA was extracted from pure cultures, leaf lesions, whole thalli and dissected "only-fungal" sections of lichenized fungi. In addition, the method has proved suitable for use with herbarium specimens of both lichenized and non-lichenized fungi, stored as dried pure cultures or dried infected plant material.

The application of molecular tools to the analyses of DNA from lichen-forming fungal symbionts has met with difficulties due to problems in obtaining nucleic acids of sufficient purity for manipulation with different DNA modifying enzymes. The main reasons for these difficulties are the abundance of inhibitors such as polysaccharides and phenolic compounds which are difficult to eliminate and which inhibit the action of these enzymes, the necessity of separating the genomes of both bionts and the scarcity of available material in some cases (for examples see GRUBE & al. 1995, CRESPO & al. 1997). The major problem of extracting purified DNA of a single origin from lichen-forming fungi has largely been overcome through PCR amplification with selective primers (e.g. CRESPO & al. 1997). However, the purity of the original starting DNA can be crucial, and so there remains a need to be able to extract DNA from lichen samples that is free from contaminating polyphenols, tannins, proteins and polysaccharides. A wide variety of DNA extraction techniques have been developed for extracting purified DNA from fungal and plant materials. Those adopted for work with lichens have tended to use either guanidine thiocyanate and/or phenol extractions (e.g. ARMALEO & CLERC 1991; GRUBE & al. 1995), or chloroform and/or phenol extractions from detergent containing buffers (LEE & al. 1988, ARMALEO & CLERC 1995, CRESPO & al. 1997). Guanidine thiocyanate, phenol and β-mercaptoethanol (used in many

detergent buffers) are very toxic and so substitutes should be considered wherever possible, one alternative being to incorporate polyvinylpyrrolidone into a detergent containing buffer in place of β-mercaptoethanol (Rogers & Bendich 1994). Protocols with ethanol precipitations are unable to eliminate polysaccharides or require centrifugation at room temperature or low velocity, both of which can result in a decreased yield, and a further alternative is to use a semiquantitative isopropanol precipitation (Raeder & Broda 1985).

DNA has been isolated from a wide range of museum and herbarium materials (Rogers & al. 1989, Bruns & al. 1990, Pääbo 1990, Höss & Pääbo 1993, Cooper 1994, Taylor & Swann 1994, Savolainen & al. 1995). The extraction of DNA from herbarium specimens of lichen-forming fungi can pose further significant problems, both in relation to those encountered generally with older dried material (see Taylor & Swann, 1994, Bruns & al. 1990), and those that may be more specific to lichens, such as the presence of secondary products, and large amounts of polysaccharide (Armaleo & Clerc 1995).

We present here an optimised CTAB (Cetyl-trimethyl ammonium bromide) extraction protocol. The method is similar to that of Rogers & Bendich (1994), but it has been optimised for fungi and lichen-forming fungi by the reduction of CTAB reagents, and the inclusion of nuclease deactivating and semi-quantitative precipitation steps. This method has been found suitable for the examination of large numbers of both lichenized and non-lichenized samples, and has enabled the extraction of PCR amplification quality DNA from fresh lichen material, recent herbarium specimens of lichenized and non-lichenized fungi, and older specimens of up to 100 years in age. The procedure includes polyvinylpyrrolidone and avoids the use of guanidine thiocyanate, phenol and β-mercaptoethanol.

Materials and methods

Samples. Several kinds of fresh and dried materials were used in order to test the usefulness of the protocol, these were fresh samples of lichenized fungi and herbarium specimens of both lichenized and non-lichenized organisms.

Fresh lichenized material was obtained from the peripheral lobules from more than 250 fresh samples of *Physconia* spp., peripheral lobules from more than 20 fresh samples of *Parmelia* spp. and *Parmelina* spp., rhizines from *Physconia* spp. and *Parmelia* spp. and apothecia from *Physconia* spp.

Herbarium specimens of lichenized fungi comprised lacinia from five one year old dried samples of *Ramalina* spp., and 2 to 25 mg (average 12 mg) of dry peripheral lobules from 16 samples from *Parmelia* and *Physconia* herbarium specimens (age range from 26 to 15 years, average age 22 years).

Herbarium specimens of non-lichenized fungi comprised samples of mycelium, pycnidia and conidia from specimens of *Phoma minutispora* (IMI 13695; 32 years old) and *Ascochyta fabae* (IMI 336944; nine years old); and also pycnidia from *A. boltshauseri* (107 years old), *A. rabiei* (130 years old) and *A. pinodes* (more than 97 years old). Isolations were also made of leaf lesions from two herbarium samples containing fruiting bodies of *A. pisi* (30 years old) and plant tissue associated with more recent collections of *Phyllosticta sphaeropsoidea*, *A. rabiei* and two specimens described as *Ascochyta* sp. on *Vicia* (all collected in the past seven years).

PCR amplification. The suitability of the DNA obtained by the extraction method was tested by PCR amplification with specific primers ITS1F (GARDES & BRUNS 1993) and ITS4 (WHITE & al. 1990). This primer pair has been demonstrated as being largely "fungal-specific" (GARDES & BRUNS 1993, CRESPO & al. 1997) and amplifies a segment which includes both the ITS 1 and ITS 2 regions, the 5.8S gene, the 3′ terminus of the SSU rDNA and the 5′ terminus of the LSU rDNA.

PCR conditions were 2.5 mM $MgCl_2$, 0.4 mM each dNTP, 0.2 µM each primer, 0.2 U Taq, 1–5 ng DNA; in 25 µl final volume Cycling parameters were 40 cycles of a denaturation at 94 °C for 1 min, annealing at 53 °C for 45 s and elongation at 72 °C for 1 min. PCR products were separated by electrophoresis in 2% agarose gels in Tris-acetate-EDTA buffer (TAE; SAMBROOK & al. 1989) and stained with ethidium bromide (0.5 µg ml^{-1}).

DNA extraction. The final optimised extraction protocol is described below and 30–40 samples can be extracted by a single operator in one day.

1. Material disruption. Best results were obtained from liquid nitrogen frozen samples. 3–100 mg of material were put into 1.5 ml tubes and placed in a container with liquid nitrogen for 5–10 min. The tubes are then removed from the container and placed in an insulated rack to maintain the temperature and avoid the projection of the powdered material. Clean liquid nitrogen was added to the tube and a sterile precooled sharp glass bar was used to grind the material. This process takes only some seconds for each sample. Glass bars can be flame sterilised immediately prior to use.

Samples can be disrupted without liquid nitrogen by grinding the material in powdered glass. DNA extracted in this way gave good amplifications, although the total DNA yield was reduced compared to liquid nitrogen preparations. A mortar and pestle can also be used without additional abrasives although this did not prove practical for either large numbers or small amounts of material.

2. CTAB precipitation. 0.5 ml of extraction buffer (1% w/v CTAB; 1M NaCl; 100 mM Tris; 20 mM EDTA; 1% w/v polyvinyl polypyrolidone, PVPP) were added to the ground material. The buffer was prewarmed before addition to avoid CTAB precipitation. PVPP was added to the buffer immediately prior to use. The tubes were mixed by inverting them several times and then heated in a waterbath for 30 min at 70 °C before adding one volume of chloroform: isoamyl alcohol (24:1 v/v). The resulting complex was mixed by inverting the tube, and centrifuged for 5 min at 10000 g at room temperature. The upper aqueous phase was collected in a new tube and the slurry and lower layers were discarded.

Two volumes of precipitation buffer (1% w/v CTAB; 50 mM Tris-HCl; 10 mM EDTA; 40 mM NaCl) were added to the supernatant and mixed well by inversion for 2 min. The mixture was centrifuged for 15 min at 13000 g at room temperature and the pellet was collected.

3. Alcohol precipitation. The pellet was resuspended in 350 µl of 1.2 M NaCl, to which one volume of chloroform:isoamylalcohol (24:1) was added. This was mixed vigorously and centrifuged for 5 min at 10000 g at room temperature. The upper phase was removed to a new tube and 0.6 volume of isopropanol was added. This was mixed by inversion and the tube was placed at −20 °C for 15 min. The final pellet was collected by centrifugation for 20 min at 13000 g at 4 °C.

If RNA-free DNA is required then 2 µl of 10 mg·ml^{-1} RNAase A can be added to the sample and incubated at 37 °C for 30 min before the second chloroform treatment.

4. Final wash. The final pellet was washed with 1 ml of 70% ethanol and recollected by centrifugation for 3 min at 13000 g at 4 °C. The pellet was drained and dried at 50 °C prior to resuspension in either PCR grade water or TE buffer (10 mM Tris pH 7.4, 1 mM EDTA). Usually extractions from less than 20 mg starting material were resuspended in 10–25 µl TE.

The quality and quantity of extracted DNA samples was monitored routinely by electrophoresis of 5 to 25% of the material in a 1% agarose gel. Quantification was performed through comparison with known dilutions of lambda phage DNA. Aliquots with 5 ng DNA μl^{-1} were prepared for PCR reactions and stock DNAs were stored at −20 °C.

Results

Lichenized fresh material. High molecular weight DNA bands were observed in nearly all the extractions performed. RNA bands were also clearly observed from some fresh material but they became degraded after storage of the samples as no RNAase inhibitors were used. Strongly stained PCR products were obtained in more than 90% (>250) of the reactions (Fig. 1). In most cases where samples initially failed to amplify, successful amplification could be obtained by a 1:10 DNA dilution.

No discrepancies were seen in PCR products obtained from DNA from the whole thallus or isolated rhzines from a single specimen even when extracting from polymorphic species in *Parmelia* and *Physconia* (CUBERO & CRESPO 1997).

Lichenized herbarium material. DNA extractions from herbarium specimens of lichenized material were resuspended in 10 µl TE. Initial test samples of 5 µl were checked in 1% agarose gels and the rest were diluted tenfold. Of the 16 samples tested, only three showed apparent DNA bands but all showed a low weight RNA band (degraded RNA).

PCR reactions were undertaken with 1 µl DNA from all samples and 13 of the 16 samples gave visible PCR products, sometimes accompanied by a DNA smear, probably indicating some degree of degradation of the target DNA or the existence of inhibitors. No attempt was made to optimise the PCR conditions against inhibitors or to perform chained PCR, although both of these methods may increase the effectiveness of the method.

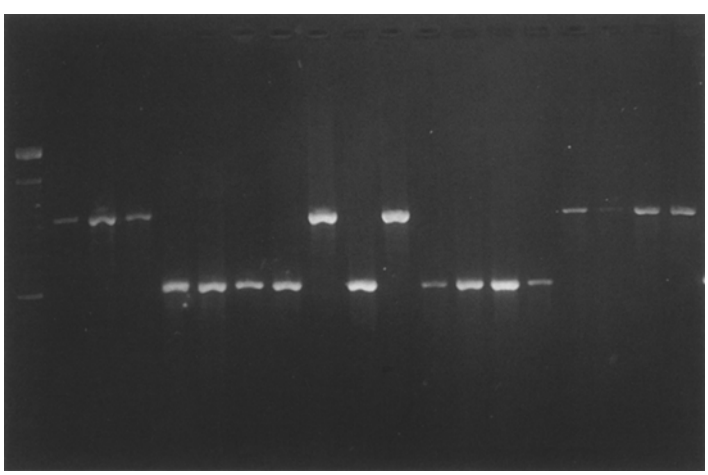

Fig. 1. PCR products from lichenized fungi. Lane 1: size markers. Lanes 2 and 3: product obtained from rhizines and thallus of a single specimen of *Physconia* sp. Lanes 4–19: products obtained from thalli of different specimens/isolates of *Physconia* spp

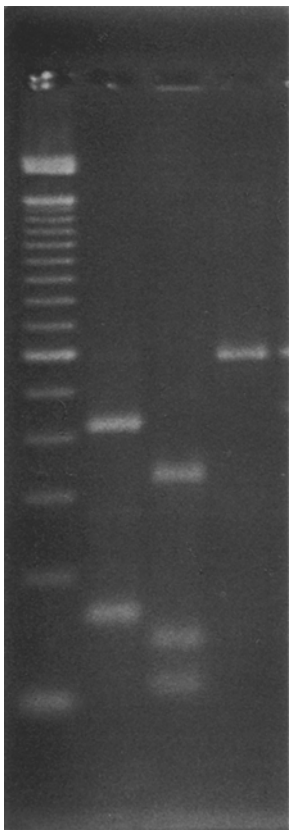

Fig. 2. Restriction digestion fragments of PCR products obtained from herbarium specimen of *Ascochyta fabae* (IMI 336944). Lane 1: size markers. Lane 2: *Dra*I digestion. Lane 3: *Hha*I digestion. Lane 4: *Ava*II digestion

Non-lichenized material. Eleven of the 12 non-lichenized fungal specimens gave a visible PCR product of the expected size. The authenticity of this product was checked by restriction digestion patterns of the product which were compared to those obtained from freshly isolated material (Fig. 2).

Discussion

The protocol described here permits the extraction and amplification of most herbarium samples. The technique has been used with up to 30 samples in a single day. However, larger sample numbers can be processed by dividing the extraction procedure into three steps and leaving both alcohol precipitations. This can give rise to an increase in yield, and permits the extraction of more than 100 samples over three days.

CTAB based DNA extraction was designed as an efficient and inexpensive way of avoiding polysaccharides (Jones 1963), and it has since been used widely in the extraction of DNA from fungi (e.g. Paterson & Bridge 1994, Armaleo & Clerc 1995). The use of CTAB avoids the use of guanidine thiocyanate and also minimises the SDS carry-over sometimes encountered in other methods (Bruns & al. 1990). In the procedure detailed here, the first stage of the process involves a drop in the NaCl concentration from 1.4 M to about 0.4 M, which causes the CTAB-DNA complex to precipitate, however, the absence of alcohol prevents the

coprecipitation of polysaccharides. The inclusion of PVPP (polyvinyl polypyrrolidone) is intended to assist in the elimination of mainly polyphenolic inhibitors, and this will also inhibit polyphenol oxidases (Rogers & Bendich 1994). The major differences between this procedure and method of Rogers & Bendich (1994) are the reduction in the number of CTAB solutions required, the incubation at 70 °C and the inclusion of a semiquantitative isopropanol precipitation as the first alcohol precipitation. The incubation step was found to be necessary as fungal extracts can show considerable nuclease activity. The isopropanol step will preferentially precipitate DNA, as compared to proteinaceous material (Raeder & Broda 1985). However, isopropanol precipitated DNA can prove troublesome in subsequent enzymic reactions, and a further ethanol wash is required to compensate for this P. Bridge, unpubl. results). The use of β-mercaptoethanol is avoided as dangerous, inconvenient and unnecessary because the elimination of disulphide bridges is not usually necessary in this type of material. Incubation and centrifugation time are minimised but strong centrifugation is used in order to optimise efficiency and reduce pellet loss.

In addition to the results presented here, this protocol has also been found to produce amplifiable DNA from the small apothecia from *Graphidaceae* and central cords of *Usnea* species (B. Bauer, M. Grube, pers. comm.).

Only one of the 28 herbarium specimens used in this study consistently failed to amplify; this was the 130 year old specimen of *A. rabiei*. This sample gave a very faint band of comparable size to others in the initial verification gel immediately after DNA extraction, and the reason for the repeated failure to amplify is not clear. Full records of herbarium sterilization and disinfection procedures are not available. However, with older samples it is very likely that they will have been treated with mercuric chlororide or exposed to fumigation by either methyl bromide or lindane at some time. The potential effects of herbarium insecticides have been considered by Taylor & Swann (1994); however, in the absence of full curatorial records for these specimens we are unable to add to those comments.

This protocol has proved useful when working with different lichen species and "only fungal" containing structures, as well as non-lichenized fungi and infected plant material. Amplifiable DNAs from herbarium samples can be extracted from extensive collections in major institutions and so this method provides a single technique suitable for use with such material in both current and historic population biology.

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