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Research Article

Isolation of DNA for Sequence Analysis from Herbarium Material of Some Lichen Specimens

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Abstract: An improved protocol for the isolation of DNA from herbarium material of some lichen specimens is described. The isolated DNA is suitable for PCR reactions for DNA sequence analysis. The hexadecyltrimethylammonium bromide (CTAB) based protocol defined in this study provides a number of advantages, mainly speed and reliability. In addition, different DNA extraction protocols were examined to determine the yield of DNA from the thallus of lichen specimens. The methods examined include a CTAB based protocol and a sodium dodecyl sulphate (SDS) based protocol. Although these procedures yielded DNA of suitable purity for PCR analysis, the improved protocol yielded DNA of higher quality. The recovery of DNA with an average yield of 25 mg/g of herbarium material was possible with this procedure.

Key Words: DNA isolation, herbarium material, lichen, sequence analysis

Bazı Likenlere ait Herbaryum Örneklerinde Dizi Analizi için DNA İzolasyonu

Özet: Bazı likenlerin herbaryum örnekleri için geliştirilmiş bir DNA izolasyon yöntemi belirlenmiştir. Bu yöntemle izole edilmiş DNA, DNA dizi analizinin PCR reaksiyonları için uygundur. Bu çalışmada tanımlanmış olan hekzadesiltrimetilamonyum bromür (CTAB)'e dayalı bu protokol başta hız ve güvenilirlik olmak üzere birçok avantaj sağlamaktadır. Buna ek olarak liken türlerinin talluslarından elde edilen DNA'yı belirlemek için farklı DNA ekstraksiyon yöntemleri kullanılmıştır. Denenen metodlar hekzadesiltrimetilamonyum bromür (CTAB)'e ve sodyum dodesil sülfat (SDS)'a bağlı birer yöntemi kapsamaktadır. Bu prosedürler PCR analizi için uygun saflıkta DNA sağlamalarına karşın, geliştirilen protokol daha yüksek kalitede DNA sağlamaktadır. Bu protokol ile herbaryum materyalinden ortalama 25 mg/g DNA elde edilmesi mümkün olmuştur.

Anahtar Sözcükler: DNA izolasyonu, herbaryum materyali, liken, dizi analizi

Introduction

Lichens, symbiotic associations between an exhabitant fungal partner (mycobiont) and an extracellular population of green-algal and/or cyanobacterial photobionts (Hawksworth & Honegger, 1994), are distributed from the polar to the tropical regions of the world and they often grow in extreme environments. Fungi require external sources of carbohydrates, which are provided by photobionts in lichen. If cyanobacteria participate in this symbiosis, the lichen has also the ability to supply nitrogen as an internal source (Walser et al., 2003).

Genetic variation is generally assumed to be an important factor in determining the ability of species to adapt and occupy new habitats, but knowledge about the processes that generate and maintain genetic variation in lichens is limited. In recent years, studies in this field have generally focused on the development of suitable molecular markers to study population structures (DePriest, 1993; Zoller et al., 1999; Högberg et al., 2002), breeding systems (Murtagh et al., 1999; Kroken & Taylor, 2001), and dispersal strategies of lichen-forming fungi (Walser et al., 2001) and also lichen systematics (Gargas et al., 1995; Grube & Kroken, 2000; Lutzoni et al., 2001).

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A number of different methods for the isolation and amplification of DNA from lichens and fungi have been published (Lee et al., 1988; Bruns et al., 1990; Lee & Taylor, 1990; Armeleo & Clerc, 1991, 1995; Grube et al., 1995; Landvik et al., 1996; Crespo et al., 1997; Cubero et al., 1999; Grube, 2005). With the aid of PCR amplification with selective primers the major obstacle of extracting DNA of a single origin from the fungal part of lichen has largely been overcome (Crespo et al., 1997). However, as the purity of the original starting DNA is crucial, to extract DNA from lichen samples free from contaminants, such as polyphenols, tannins, proteins and polysaccharides, remains a major concern for conducting PCR based molecular techniques.

In some species, separating DNA from naturally occurring plant cell contaminants, such as polysaccharides and phenolic compounds, is difficult. Furthermore, isolating intact DNA from herbarium species is a more difficult task, and a smear appearance of PCR products is usually visible on the gel. Current studies indicate that extraction of DNA is not always routine and simple, and conventional methods are not necessarily reproducible for all species, especially for herbarium material (Cubero, 1999; Aras, 2003). Although a number of extraction protocols are published for herbarium material of lichens belonging different genera (Cubero, 1999), the protocol in the current study provides a new strategy, which is fast and reliable and yielding DNA of higher quality compared with other methods (Table 1). The objectives of this study were to establish a DNA isolation protocol suitable for herbarium material of different genera of lichenised fungi to make comparisons with other published protocols, and to obtain a PCR optimisation for sequence analysis with the isolated DNA.

Table 1. Mean DNA yield obtained from spectrophotometric analysis (A260).

Extraction Method	Yield mg/g
SDS (Martin & Winka, 2000)	7-12 mg/g
CTAB (Cubero et al., 1999)	15-25 mg/g
Defined CTAB method for lichens	25- >25 mg/g

Materials and Methods

Lichen material: Herbarium materials of the genera species from A*spicilia, Collema, Flavoparmelia, Hypogymnia, Melanelia, Melanohalea, Parmelia, Parmelina, Pleurosticta, Ramalina, Rhizoplaca,* and *Xanthoparmelia* were used. Only *Flavoparmelia caperata* species was used from *Flavoparmelia* genus; 6 species of *Melanelia* used were; *Melanelia disjuncta, M. glabratula, M. septentrionalis, M. stygia, M. subargentifera, M. subaurifera.* Two species of *Melanohalea* used were; *Melanohalea exasperata, M. exasperatula.* Four species of *Parmelia* used were; *Parmelia omphalodes, P. saxatilis, P. sulcata, P. taractica.* Three species of *Parmelina* used were; *Parmelina pastillifera, P. quercina, P. tiliacea*. One species of *Pleurosticta* used was; *Pleurosticta acetabulum*. Four species of *Xanthoparmelia* used ere; *Xanthoparmelia conspersa, X. pulla* var. *pulla, X. pulla* var. *delisei, X. tinctina*. In addition to these genera three *Rhizoplaca* species were analysed: *Rhizoplaca melanopthalma, R. chrysoleuca, R. peltata* and 4 *Collema* species: *Collema flaccidum, C. nigrescens, C. tenax, C. undulatum.* Eight species of *Aspicilia; A. hispida, A. desertorum, A. esculenta, A. fruticulosa, A. calcarea, A. aspera, A. contorta* subsp. *contorta*, *A. contorta* subp. *hoffmanniana.* 4 *Hypogymnia* species were; *Hypogymnia laminisorediata, H. tubulosa, H. physodes, H. farinacea.* The 5 species of *Ramalina* were; *R. fraxinea, R. polymorpha, R. fastigiata, R. capitata, R. pollinaria.*

DNA isolation

- Lichen herbarium material (0.1 g) was ground to a fine powder in liquid nitrogen.
- Prewarmed extraction buffer [50 mM Tris-HCl (pH 8). 50 mM EDTA, 10 ml of LiCl (4 M), 1% CTAB, 2% PVPP (addition of PVPP is optional)] in the amount of 1 ml was added to the samples and ground once more in the buffer.
- After the samples were taken into 1.5-ml Eppendorf tubes, 0.2% of β-mercaptoethanol was added.
- The solution was incubated at 65 °C in a water bath for 15 min.
- Following these incubation periods, samples were cooled to room temperature, 0.5 ml of chloroform/isoamyl alcohol (24:1 [v/v]) was added and mixed well (no vortex).

- Then the samples were centrifuged at 17,000 x *g* (14,000 rpm) for 2 min, and the supernatant was transferred to a fresh tube (~0.8 ml).
- Supernatant was transferred to a fresh tube and an equal volume of isopropanol was added, followed by gentle mixing by inversion several times. Samples were incubated for at least 15 min on ice to increase the efficiency of DNA yield.
- The samples were then centrifuged for 2 min at 17,000 x *g* (14,000 rpm).
- Supernatant was discarded.
- 1 ml of 70% ethanol was added.
- The samples were then centrifuged for 1 min at 17,000 x *g* (14,000 rpm).
- The pellet was once more washed with 70% ethanol optionally and air-dried until the ethanol was removed.
- The obtained pellet was dissolved in an appropriate amount of TE buffer (10 mM Tris-HCl (pH 8), 1 mM EDTA) (30-60 µl).
- The solvent in TE buffer was treated with ribonuclease (RNase, 10 mg/ml) and stored at –20 °C until use.

DNA was quantified via spectrophotometric measurement of UV absorption at 260 nm (Specord UV-200). DNA was also quantified by means of agarose gel electrophoresis with ethidium bromide fluorescence and a 100-bp DNA ladder was used (Fermentas) as the DNA size marker.

PCR Amplification

Different parameters were detected for the optimisation of PCR reactions. PCR reactions for sequence analysis were performed in a 50 µL volume containing 200 ng of genomic DNA, 5 µL of 10 x reaction buffer, 2,5 mM $MgCl_2$, 20 mM dNTPs, 0,2 mM of each ITS F1 primer and ITS 4 primer, and 1 U *Taq* DNA polymerase (Promega). Amplification was performed in a Techne Progene thermal cycler (Techne Cambridge Ltd.). The reactions were heated in an initial step of 94 °C for 2 min and then subjected to 35 cycles of the following programme: 94 °C for 30 s, 55 °C for 1 min, and 72 °C for 1 min 45 s. After the last cycle, the temperature was maintained at 72 °C for 8 min. Amplified DNA was electrophoresed in a 1.2% agarose gel containing 0.5 mg/ml ethidium bromide.

Results

In the current study, an improved protocol for DNA isolation for amplification for sequence analysis from herbarium material of many different lichen species from different genera was established. Figure 1 displays the results of DNA on agarose gel electrophoresis. The PCR products obtained using ITS1-F (Gardes & Bruns, 1993) and ITS 4 (White et al., 1990) are shown in Figure 2. Figure 2 shows the results of PCR reactions conducted using *Aspicilia* as an example. Although all reactions yielded only 1 PCR band, the *Aspicilia calcarea* sample in the sixth lane gave 2 bands. The second band is probably due to the presence of a group I intron in the 3´- end of the SSU rDNA (DePriest, 1993).

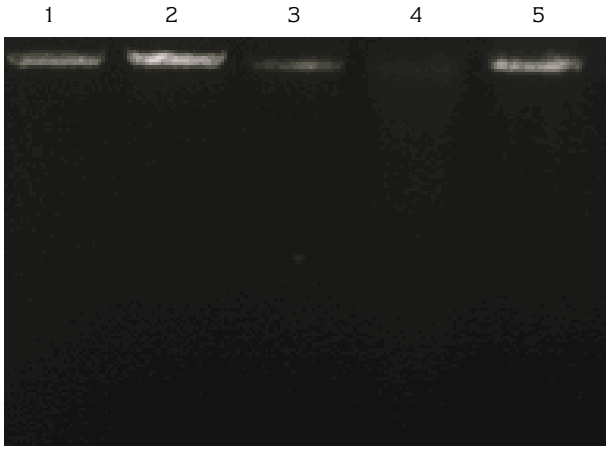

Figure 1. DNA assay conducted. Lane 1: *Aspicilia calcarea*, Lane 2: *Rhizoplaca chrysoleuca*, Lane 3: *Collema tenax*, Lane 4: *Melanelia glabratula*, Lane 5: *Parmelina tiliaceae*.

Discussion

During DNA isolation from lichens, the high concentration of polysaccharides found in many species must be taken into consideration. Different approaches have been used for the elimination of such inhibitors. For this purpose, Armaleo & Clerc (1991) used column purification and, in subsequent studies, CTAB (cetyl-trimethyl ammonium bromide) addition was shown to be very effective for DNA isolation from lichens (Bridge et al., 1998).

Although an optimal yield of DNA is obtained from fresh material of lichens, using herbarium material is

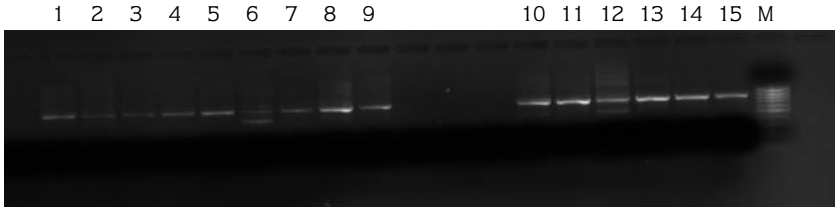

Figure 2. PCR assays conducted using the primers ITS 1F and ITS 4. Lane 1: shows the amplification product belonging to *Aspicilia contorta* subsp. *hoffmanniana*, Lane 2: *A. contorta* subsp. *hoffmanniana*, Lane 3: A. *esculenta*, Lane 4: *A. contorta* subsp. *hoffmanniana*, Lane 5: *A. contorta* subsp. *hoffmanniana*, Lane 6: *A. calcarea,* Lane 7: *A. desertorum*, Lane 6: *A. calcarea*, Lane 7: *A. desertorum*, Lane 8: *A. desertorum*, Lane 9: *A. calcarea*, Lane 10: *A. desertorum*, Lane 11: *A. calcarea*, Lane 12: *A. contorta* subsp. *hoffmanniana*, Lane 13: *A. calcarea*, Lane 14: *A. desertorum,* Lane 15: *A. contorta* subsp. *hoffmanniana*, M: Molecular weight marker (100 bp ladder).

often necessary since fresh samples may not be available. In this study, DNA was isolated from herbarium material of 45 species from 12 different genera.

Different methods already exist for DNA isolation from lichens, but this protocol provides a number of advantages, mainly speed and reliability. Approximately 1 h was sufficient to isolate 24 species, depending on centrifuge capacity. Although very short centrifugation and incubation times were employed, extraction and precipitation of DNA without any pellet loss were achieved by employing strong centrifugation (17,000 xg) and spinning times were prolonged when necessary. It is also possible to separate the protocol into 2 parts by incubating the samples in isopropanol for prolonged times, for example overnight. By doing so, it could be possible to isolate hundreds of samples at most within 2 days.

The protocol defined in this study is a CTAB based protocol. There are a number of CTAB based protocols in the literature for lichens and for other plants (e.g., Doyle & Doyle, 1987; Martin et al., 2000) as they provide an efficient and relatively inexpensive way to avoid polysaccharides. In the protocol PVPP (polyvinyl polypyrolidone) was also used to eliminate phenolic compounds, secondary plant metabolites that may constitute a major problem in DNA isolation. In order to avoid the probable troubles of of isopropanol precipitation an ethanol wash step was also employed (Cubero et al., 1999). Another observation encountered during extraction studies was the reduction in DNA amount and quality when phenol was applied together with chloroform and isoamyl alcohol.

The protocol defined in this study proved useful for DNA isolation from different genera and the DNA yielded was consistently suitable for PCR amplification.

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References

Aras S, Duran A & Yenilmez G (2003). Isolation of DNA for RAPD analysis from dry leaf material of some *Hesperis* L. specimens. *Plant Mol Biol Rep* 21: 461a-461f.

Armaleo D & Clerc P (1991). Lichen chimeras: DNA analyses suggest that one fungus forms two morphotypes. *Exp Mycol* 15: 1-10.

Armaleo D & Clerc P (1995). A rapid and inexpensive method for the purification of DNA from lichens and their symbionts. *Lichenologist* 27: 207-213.

Bridge PD, Arora DK, Reddy CA & Elander RP (1998). *Applications of PCR in Mycology*. New York: CABI, pp. 85-105.

Bruns TD, Fogel R & Taylor JW (1990). Amplification and sequencing of DNA from fungal herbarium specimens. *Mycologia* 82: 175-184.

Crespo A, Bridge PD & Hawksworth DL (1997). Amplification of fungal rDNA-ITS regions from non-fertile specimens of the lichen-forming genus Parmelia. *Lichen* 29: 275-282.

Cubero OF, Crespo A, Fatehi J & Bridge PD (1999). DNA extraction and PCR amplification method suitable for fresh, herbarium-stored, lichenized, and other fungi. *Plant Syst Evol* 216: 243-249.

DePriest PT (1993). Small subunit rDNA variation in a population of lichen fungi due to optional group-I introns. *Gene* 134: 67-74.

Doyle JJ & Doyle JL (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem Bull* 19: 11-15.

Gardes M & Bruns TD (1993). ITS primers with enhanced specificity for Basidiomycetes - application to the identification of mycorrhizae and rusts. *Mol Ecol* 2: 113-118.

Gargas A, DePriest PT, Grube M & Tehler A (1995). Multiple origins of lichen symbioses in fungi suggested by SSU rDNA phylogeny. *Science* 268: 1492-1495.

Grube M, DePriest PT, Gargas A & Hafellner J (1995). DNA isolation from lichen ascomata. *Mycol Res* 99: 1321-1324.

Grube M & Kroken S (2000). Molecular approaches and the concept of species and species complexes in lichenized fungi. *Mycol Res* 104: 1284-1294.

Grube M (2005). Nucleic acid isolation from ecological samples-fungal associations, lichens. *Method Enzymol* 395: 48-57.

Hawksworth DL & Honegger R (1994). The lichen thallus a symbiotic phenotype of nutritionally specialized fungi and its response to gall producers. In: Williams MAJ (Ed.). Plant Galls. Clarendon Press, Oxford, pp: 77-98.

Högberg N, Kroken S, Thor G & Taylor JW (2002). Reproductive mode and genetic variation suggest a North American origin of European *Letharia vulpine*. *Mol Ecol* 11: 1191-1196.

Kroken S & Taylor JW (2001). Outcrossing and recombination in the lichenized fungus *Letharia*. *Fungal Genet Biol* 34: 83-92.

Landvik S, Shailer NFJ & Eriksson O (1996). SSU rDNA sequence support for a close relationship between the Elaphomycetales and the Eurotiales and Onygenales. *Mycoscience* 37: 237-241.

Lee SB, Milgroom MG & Taylor JW (1988). A rapid, high-yield mini-prep method for isolation of total genomic DNA from fungi. *Fungal Genet Biol* 35: 23-24.

Lee SB & Taylor JW (1990). Isolation of DNA from fungal mycelia and single spores. In: Innis M.A., Gelfand D.H., Sninsky J.J., White T.J. (eds) *PCR Protocols:* a Guide to Methods and Applications. Academic Press, San Diego, pp. 282-287.

Lutzoni F, Pagel M & Reeb V (2001). Major fungal lineages are derived from lichen symbiotic ancestors. *Nature* 411: 937-940.

Martin MF & Winka K (2000). Alternative methods of extracting and amplifying DNA from Lichens. *Lichenologist* 32: 189-196.

Murtagh GJ, Dyer PS, McClure PC & Crittenden PD (1999). Use of randomly amplified polymorphic DNA markers as a tool to study variation in lichen-forming fungi. *Lichenologist* 31: 257-267.

Walser JC, Zoller S, Büchler U & Scheidegger C (2001). Species-specific detection of *Lobaria pulmonaria* (lichenized ascomycete) diasporas in litter samples trapped in snow cover. *Mol Ecol* 10: 2129-2138.

Walser JC, Sperisen C, Soliva M & Scheidegger C (2003). Fungus-specific microsatellite primers: application for the assessment of genetic variation on different spatial scales in *Lobaria pulmonaria. Fungal Genet Biol* 40: 72-82.

White TJ, Bruns TD, Lee S & Taylor J (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *In PCR Protocols* (Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds). New York: Academic Press.

Zoller S, Lutzoni F & Scheidegger C (1999). Genetic variation within and among populations of the threatened lichen *Lobaria pulmonaria* in Switzerland and implications for its conservation. *Mol Ecol* 8: 2049-2059.