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A Reliable "Direct from Field" PCR Method for Identification of Mycorrhizal Fungi from Associated Roots

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A very reliable and specific method for the identification of fungi in ectotrophic mycorrhizal symbiosis was developed using a specific PCR assay based on the amplification of the ITS1 region. To obtain specific data, an ITS-diagnostic assay was carried out that reveals genera and species specific sequences. Here, an application of one method is presented, which covers the identification of pure mycelia, basidiocarps as well as mixed samples such as ectomycorrhizal roots that were mingled with remains of the host plant. For this purpose a protocol was established that allowed the extraction of DNA from single mycorrhizal roots. In order to perform a specific ITS analysis we generated a new ITS-primer (ITS8) by a multiple alignment of five different genera and species of mycorrhizal fungi. The utilization of ITS1 and ITS8 resulted in specific PCR amplicons, which were characterized by sequencing without purification steps, even when the template DNA was associated with roots.

KEYWORDS: **DNA extraction, Ectomycorrhizal roots, Fungi, Gel electrophoresis, ITS, PCR diagnostic assay**

More than 90% of all terrestrial plants live with mycorrhizal fungi either as facultative or as obligate symbionts. Without these fungi higher plants could not have colonized many inhospitable regions of the world. Plant roots exist commensally with several species of mycorrhizal fungi, which become established at the same time, but with apparent differences in the scope and specificity in their nutritional contribution to the higher plant. Many polymerase chain reaction (PCR) approaches have been published using the internal transcribed spacer regions (ITS1 and ITS2 regions) to discriminate the fungi. White *et al.* (1990) first described the two general primers ITS1 and ITS4, binding to the 18S and 28S rDNA, respectively, and amplifying both ITS1 and ITS2 regions as well as the 5.8S rDNA in between. However, the binding sites of these primers are strongly conserved among eucaryotic organisms. Very often, DNA from the host plant or other fungi, not associated with the mycorrhiza, has been coamplified with this procedure. Moreover, the ITS1 primer is vulnerable to dimerization when annealing occurred at low temperatures. As a first solution to these problems, Gardes and Bruns (1993) used the primers ITS1-F and ITS4-B, with supposedly enhanced specificity for basidiomycete analysis. However, the problem of coamplification of host DNA could not be eliminated by using their protocol, and most researchers still have to rely on pure cultures of their fungi of interest.

The purpose of this study was to develop specific PCR primers for use in detecting mycorrhizal fungi directly from the field without prior purification steps of the samples even when they are associated with plant roots. Here we describe an analysis protocol, which includes the use of an advanced DNA extraction technique combined with amplification of specific ITS sites. Hence, characterization of fungal ITS1 regions from a very broad range of the *Basidiomycota* from three different locations of the world were possible even when only small amounts of samples were used. This protocol has been established as a reliable standard method for the identification of ectomycorrhizal fungi.

Materials and Methods

Sample collection. Basidiocarps and root samples were collected under trees of Populus tremula near the University of Bremen, Germany. Further basidiocarp samples were collected in Mallorca, Spain, in the Tramontana Mountains near Col de Hono, at an altitude of about 600 m in a steep valley, dominated by Quercus ilex. Samples of basidocarps from Korea have been collected at Mount Wol Ak in Eastern Chung-Puk in stands of Pinus densiflora and *Quercus acutissima.*

Isolation of mycelia from basidiocarps. Fresh Basidiocarps of mycorrhizal fungi were collected and washed with tap water. Without sterilization of the surface the hymenium was carefully peeled off. Petri dishes with Modified Melin-Norkrans (MMN) medium were inocu-

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lated with 6~10 pieces of mycelium and incubated at room temperature in the dark (Molina and Palmer, 1982). Repeated transfer of the mycelia into sterile MMN petri dishes eventually resulted in pure cultures.

Isolation of mycelia from associated roots. Mycelia associated with roots were washed in tap water to remove soil particles, cut into short pieces and sterilized with 1.5~3% H_2O_2. The exact parameters of the sterilization procedure depended on the diameter of the mycorrhiza and the degree of contamination (Melin, 1936; Slankis, 1958), and had to be determined empirically for each sample. After additional washing, the roots were blotted on sterile filter paper and placed in MMN petri dishes. After 2~4 days, growing mycelia could be observed. Pure cultures of mycorrhizal fungi were obtained by repeated transfers of the mycelia into sterile MMN petri dishes (Marx, 1969).

DNA extraction. 0.05~0.2 g of fresh mycelium was removed with a sterile scalpel from either a MMN petri dish or from the interior of a basidiocarp. Samples of mycorrhizal roots were washed in tap water. Sample material were transferred to a sterile 2 *ml* Eppendorff cup filled with silanized sand and a ceramic sphere. DNA extraction was performed with the QiaGen DNEasy™ DNA Extraction Kit from Plants according to the manufacturers instructions. Purified DNA was separated in a 1% agarose gel stained with ethidium bromide and concentration was estimated by comparison with known length standards.

DNA samples from Korean fungi (accession number: AF438556, AF438557, AF438558, AF438560, AF438562, AF438563, AF438567, AF438566, AF438568, AF438575, AF438578, AF438579, AF438583, AF438582, AF438590, AF438554, AF438599, AF438600, AF438602, AF438605) were supplied by S.S. Lee and have been isolated as previously described by Gosselin *et al.* (1995).

PCR analysis. ITS-specific oligonucleotides, ITS1 (TCCGTAGGTGAACCTGCGG) which was described previously (Jackson *et al.*, 1999; Hamelin *et al.*, 1996; White *et al.*, 1990) and ITS8 (ACAGGCATGCTCCTCG-GAA) which was designed to amplify the internal transcribed spacer region ITS1 were synthesized by MWG Biotech. The ITS8 primer was obtained by performing a multiple sequence alignment of five mycorrhizal fungi with CLUSTAL and binds within the 5.8S rDNA of basidiomycetes. PCR amplification reactions were carried out in volumes of 50 μl containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 3% DMSO, 100 pmol of each primer ITS1, ITS8, 0.4 mM of each dNTP and 2.5 units of Taq Polymerase (Platinum Taq Polymerase, Gibco BRL) and 0.1~100 ng DNA. All reactions were carried out in a Stratagene Robocycler with an initial denaturation step at 95°C for 5 min followed by 35 cycles of 95°C for 1 min, 68°C for 1 min, and 72°C for 1 min. Reactions were completed by a final 2 min extension step at 72°C. After PCR amplification the amplicons were separated in a 1.5% agarose TAE gel stained with etidium bromide, cut out with a clean scalpel and recovered with the Millipore Ultrafree®-DA kit according to the manufacturers manual. PCR products have then been applied directly to sequencing (MWG-BIOTECH AG, D-85560 Ebersberg and Biozym Genomic Service, D-31833 Hessisch-Oldendorf, Germany), using ITS8 as the sequencing primer.

Data analysis. Sequences of PCR amplicons were aligned via Internet with the sequence blast service of *NCBI* using the blast program blast 2.1 (Altschul *et al.*, 1997). Alignment analyses to obtain sequence data for generating the ITS8 primer was performed by using ClustalV (Higgins and Sharp, 1988, 1989).

Results

Our method was performed on a total number of 52 sam-

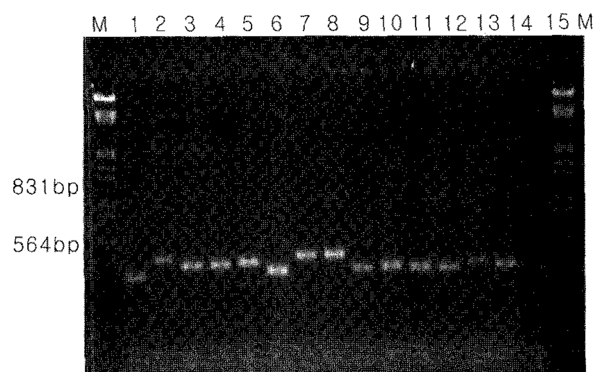

Fig. 1. Pattern of ethidium bromide-stained PCR fragments after gel electrophoresis of 15 μl of the PCR volume in 1.5% TAE agarose. PCR fragments obtained from pure mycelia or basidiocarps, respectively. (M1 and 2) Marker; length standard, *lambda* DNA digested with the restriction enzymes *Hin*dIII and *Eco*RI. Lane 1) *Pisolithus tinctorius mycelium*, Lane 2) *Paxillus involutus mycelium*, Lane 3) *Basidiomycetes sample 4, sporocarp*, Lane 4) *Basidiomycetes sample 5, sporocarp* Lane 5) *Amanita rubescens mycelium*, Lane 6) *Amanita muscaria, sporocarp*, Lane 7) *Agaricus bisporus, sporocarp*, Lane 8) *Agaricus bisporus, "Bella Gomba"*, Lane 9) *Suillus luteus mycelium*, Lane 10) *Suillus bovinus mycelium*, Lane 11) *Xerocomus badius, sporocarp* Lane 12) *Xerocomus badius mycelium*, Lane 13) *Letinus edodes, sporocarp* Lane 14) *Rhizopogon rubescens, sporocarp* Lane 15) negative control; bidest. All fragments derived from specific ITS analysis were extracted from the gel and were sequenced with the ITS8 primer.

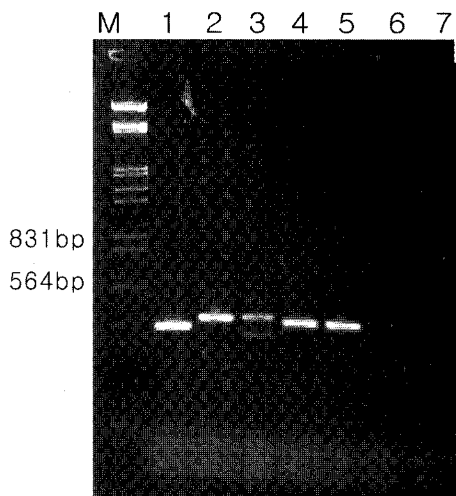

Fig. 2. Comparison of PCR products using template DNA obtained from mycelia associated with roots. Pattern of ethidium bromide-stained fragments after gel electrophoresis of 15 μl of the PCR volume in a 1.5% TAE agarose gel. (M1 and 2) Marker; length standard, *lambda* DNA digested with the restriction enzymes *Hind*III and *Eco*RI. Lane 1) *Amanita muscaria, sporocarp extract*, Lane 2) Root sample P1, *Hebeloma helodes*, Lane 3) Root sample P2, *Hebeloma mesophaeum*, Lane 4) Root sample Pt, *Rhizopogon rubescens*, Lane 5) Root sample 1M, *Rhizopogon rubescens*, Lane 6) extraction without sample, Lane 7) negative control.

ples. The amplification of DNA isolated from mycelia and basidiocarps resulted in single PCR products of variable sizes (300~560 bp) (Fig. 1), whereas in some cases the DNA of mycelia associated roots resulted in two products as shown in Fig. 2 lane 4. For these sequences, a two-dimensional agarose gel electrophoresis was conducted using a special dye, which is described elsewhere (Harms *et al.*, 2000; Mueller *et al.*, 1997). After gel electrophoresis dominant bands with a light smear underneath and above the fragment of interest occurred (data not shown). Characteristic spots were excised with a toothpick and were further amplified with PCR according to the core amplifying method (core sample PCR). In brief: DNA was stabbed out of agarose from the centre of the bands with a sterile yellow pipette tip. The extracted DNA was used directly for a second PCR reaction with an initial denaturation step at 95°C for 5 min using the same primers as mentioned before. As for the root samples, three out of four reactions resulted in single PCR products (Fig. 2). Only one sample (lane 4 in Fig. 2) showed three discernible bands, of which the dominant band has been applied to sequencing (*Hebeloma mesophaeum*, AF438582). After electrophoresis and recovery from the gel, amplicons were directly characterized by sequencing using ITS8 as the sequencing primer. All sequences were aligned using the software ClustalV and GenBank to confirm their identity.

Discussion

The work we present here shows the potential for identifying mycorrhizal fungi from pure cultures and basidiocarps as well as from mycorrhizal roots from the field. Analysis of cultured mycelia and basidiocarps using ITS primers (ITS1 and ITS4 and their derivatives) and RAPD PCR analysis have been previously described (Gardes and Bruns, 1993; Hamelin *et al.*, 1996; Fujimori and Okuda, 1994; Egger, 1994). Both the RAPD PCR and the specific ITS PCR analysis are useful tools to identify mycorrhiza according to their fragment patterns and their variability within the ITS region. The disadvantage of the RAPD PCR method is the requirement of pure mycelia grown under controlled conditions. Despite the very important results that have been obtained in the identification of mycorrhizal fungi with the mentioned ITS1/ITS4 primers, the problems in co-amplification of contaminating DNA material is observed when these primers were used on mycorrhizal roots. The use of the newly designed primer (ITS8) resulted in clearly distinguishable PCR fragments (Fig. 1), which are easily applied to further sequence characterization. All sequences obtained have been registered at GenBank[1].

One obvious practical problem that appears to limit this technique in a direct identification assay is the production of PCR fragments of equal size amplified from DNA of phenotypically similar mycorrhizal fungi (Fig. 2 lane 4). To solve this problem the use of a bisbenzimide-PEG conjugate (H.A.-yellow) has been shown to be capable of distinguishing DNA fragments differing in base composition. We applied a modified agarose gel electrophoresis method that contained the dye to retard AT-rich DNA clusters (Harms *et al.*, 2000 and Mueller *et al.*, 1997) and revealed a finer differentiation between PCR products.

The work presented has several advantages for future research. First, this method is applicable to a very broad systematic range of species within the *Basidiomycota*, with no apparent differences regarding the actual region of sampling. In all analyses conducted using pure culture mycelia as well as fruit bodies, singular PCR products were obtained. Second, DNA extraction, PCR setup and

[1]GeBank accession numbers are: AF438561, AF438557, AF438558, AF438559, AF438560, AF438593, AF438562, AF438563, AF438567, AF438565, AF438566, AF438568, AF438569, AF438570, AF438591, AF438582, AF438575, AF438576, AF438577, AF438578, AF438579, AF438583, AF438582, AF438580, AF438581, AF438584, AF438585, AF438586, AF438587, AF438588, AF438590, AF438594, AF438595, AF438596, AF438574, AF438554, AF438597, AF438598, AF438599, AF438600, AF438601, AF438602, AF438604, AF438603, AF438605, AF438606, AF438607, AF438609, AF438608

recovery of the reaction product can be completed within a single working day, sequences are usually obtained within additional two days. Furthermore, the applied DNA isolation method allows the processing of very small samples. For the root samples presented in Fig. 2, lanes 3, 5 and 6, mycorrhizal rootlets with a size of only 3~5 mm were used in each extraction. This is important for analysis in the field, where the roots of a single plant individual are often colonized by more than one mycorrhizal fungus. As an additional advantage, no organic solvents are required for DNA extraction, and polymerase inhibition by phenolic substances from the plant roots has become a very rare phenomenon. Our method will be useful for other researchers working with unknown ectomycorrhizal fungi collected direct from the field.

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