

Phyton (Horn, Austria) Special issue: "Achenkirch II"	Vol. 36	Fasc. 4	[109]-[118]	1. 7. 1996
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Identification of Some Ectomycorrhizal Basidiomycetes by PCR-Amplification of their *gpd* (Glycerinaldehyde-3-phosphate Dehydrogenase Encoding) Genes

By

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Key words: Glycerinaldehyde-3-phosphate dehydrogenase, ectomycorrhiza, basidiomycetes, *Lactarius*, *Boletus*, *Amanita*, molecular identification.

S u m m a r y

N. KREUZINGER, R. PODEU, F. GÖBL, F. GRUBER & C.P. KUBICEK 1996. Identification of some ectomycorrhizal basidiomycetes by PCR-amplification of their *gpd* (glycerinaldehyde-3-phosphate dehydrogenase encoding) genes. - *Phyton* (Horn, Austria) 36 (4): 109 - 118.

Degenerated oligonucleotide primers, designed to flank an approximately 1.2-kb fragment of the glycerinaldehyde-3-phosphate dehydrogenase-encoding (*gpd*) gene from asco- and basidiomycetes, were used to amplify and sequence the corresponding *gpd*-fragment from several species of the ectomycorrhizal fungal taxa *Boletus*, *Amanita* and *Lactarius*. The amplified fragment from a given taxon could be distinguished from that of others by both restriction nuclease cleavage analysis as well as by Southern-hybridization. A procedure for labelling DNA-probes with fluoresceine-12-dUTP by the polymerase chain reaction was developed. These probes were used in a non-radioactive hybridization assay, which was able to detect 2 ng of chromosomal DNA of *L. deterrimus* specifically on slot-blots. Taxon-specific amplification could be achieved by the design of specific oligonucleotide primers. The application of the *gpd* gene for the identification of mycorrhizal fungi under field conditions was demonstrated, using *Picea abies* (spruce) mycorrhized roots harvested from a north-alpine forest area as well as from a plant breeding garden. We conclude that *gpd* can be used to detect ectomycorrhizal fungi during symbiotic interaction.

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Introduction

Ectomycorrhiza, a mutualistic plant-fungus symbiosis, plays a pivotal role in the growth and nutrition of forest trees (AGERER 1993, JASPER 1994, RYGIEWICZ & ANDERSEN 1994). The specificity of mycorrhizal interaction varies widely, yielding also varying patterns of efficacy under different ecological conditions. Identification of the fungal partner in this symbiosis can thus provide valuable information on the physiological condition of a tree. Unfortunately, these fungi are very difficult to identify, particularly during the early stages of interaction. In addition, they are often difficult to grow in pure culture, which is critical for morphological identification, under these conditions.

With the advent of PCR-techniques, several groups have employed molecular tools to identify ectomycorrhizal fungi by means of selective amplification of appropriate (such as rDNA-encoding) genes, followed by restriction analysis or hybridization (ARMSTRONG & al. 1989, EGGER & al. 1991, GARDES & al. 1990, 1991, MARMEISSE & al. 1992, BRUNS & GARDES 1993, GARDES & BRUNS 1993, HENRION & al. 1992, 1994). MEHMANN & al. 1994 recently discussed an alternative approach by amplifying fragments of a gene present only in the fungal partner (i.e. chitin synthase), followed by restriction analysis.

Theoretically, any gene may be used for the identification of the fungal symbiont of ectomycorrhizae, provided that it contains regions of high homology, suitable for amplification from a wide variety of fungi with the same set of primers, and regions of very low homology also, which would make differentiation by hybridization or restriction analysis possible. We will show here that the amplification of large (1.2-kb) fragments of the glyceraldehyde-3-phosphate dehydrogenase-encoding (*gpd*)-genes from the ectomycorrhizal fungal taxa *Boletus* sp., *Amanita* sp., and *Lactarius* sp. can be used to detect and distinguish mycorrhizal symbionts on the plant root.

Materials and Methods

Selection of ectomycorrhizal basidiomycetes and DNA extraction. Basidiomycetous taxa were chosen corresponding to the distribution of ectomycorrhizal species within fungal families in higher Alpine area according to MOSER 1984 and are given in Table 1 (HAUDEK & al. 1995). Confirmatory specimens of the fungi used are deposited at Institute of Microbiology, University of Innsbruck, Austria. To obtain fungal biomass for DNA preparation, pieces of the basidiocarp of individual fungi were grown on Moser 6-agar (MOSER 1963), which had been covered by a cellophane sheet. The plates were placed into closed Styropor-containers, into which also a beaker with wet cotton had been placed to avoid dehydration. DNA was isolated by the method of LEE & TAYLOR 1992.

Table 1. Mycorrhizal isolates investigated in this study.

Strain	Code	Origin	Year
<i>Amanita muscaria</i>	(AMUa)	Haggen, Tyrol ¹	1980
<i>Amanita porphyria</i>	(APO)	Matzenköpfl, Tyrol ⁺	1985
<i>Amanita rubescens</i>	(ARUa)	Klausboden, Tyrol ⁺	1974
<i>Boletus edulis</i>	(BEDt)	Schulterberg, Tyrol ^x	1990
<i>Boletus aereus</i> ¹	(BAR)	Borgotora, Italy [§]	1985
<i>Boletus erythropus</i>	(BER)	Paida, Tyrol ⁺	1978
<i>Boletus calopus</i>	(BOC)	Ehrwald, Tyrol ⁺	1987
<i>Lactarius deterrimus</i>	(LADg)	Schulterberg, Tyrol ^x	1990
<i>Lactarius deliciosus</i> ²	(LAL)	Mutters, Tyrol [§]	1983
<i>Lactarius porninsis</i> ³	(LAP)	Haggen, Tyrol [§]	1980
<i>Lactarius scrobiculatus</i>	(LAS)	Wattener Lizum, Tyrol ⁺	1992

¹ is a typical symbiont of *Fagus* sp. and *Quercus* sp.; ² of *Pinus* sp.; and ³ of *Larix* sp. All others are typical for *Picea abies*.

^x indicates isolates from habitats within the Achenkirch area;

⁺ indicates isolates from other parts of Tyrol or Italy;

[§] indicates species symbiotic for trees other than *Picea abies*.

Extraction of DNA from mycorrhized roots. Fine hairy roots were harvested from soil, freed from soil-clumps first by shaking and then by thoroughly washing with tap water. Once cleaned, they were usually processed within the following 24 h, but storage at -70°C for at least 12 months was possible. To extract DNA, only the root tips (2 - 5 mm length) were used. 5 - 50 mg (fresh weight) of root tip material were placed on filter paper to remove excess water (which would otherwise alter the concentration of additives in the following extraction steps), subsequently placed in Eppendorf vials and then homogenized in 0.5 ml CTAB-buffer (GARDES & BRUNS 1993), using a motor-driven conical grinder (Treff AG, Degersheim, Switzerland), exactly fitting into the Eppendorf tube, until a fine suspension was obtained. All subsequent steps were identical to those used by GARDES & BRUNS 1993.

PCR conditions. A DNA region within the open reading frame of glyceraldehyde-3-phosphate dehydrogenase-encoding genes was amplified. Primer CTK-052 (sense) and primer CTK-031_{rev} (antisense), corresponding to sequences in the 5'- and 3'-terminal area within the *gpd*-coding region were designed after comparing sequences of several other fungal *gpd*-genes for areas of homology, and extended by an additional *Bam*HI/*Nde*I/*Nsi*I-polylinker sequence to facilitate subsequent cloning (cf. Table 2). The reaction mix for amplification contained: 10 µl 10x reaction buffer (Promega); 2.5 mM MgCl₂, 50 nM in each dNTP, 0.1 µM primer CTK-052, and CTK-031_{rev}, respectively, 0.1 - 1 µg chromosomal DNA, 5 or 2.5 units of *Taq* polymerase (Promega, Madison, WI) or *BiTaq*-Polymerase (Biomedica, London, UK) in a final reaction volume of 100 µl (adjusted with bidistilled water). The amplification reaction was carried out in a Hybaid-thermal cycler (Hybaid, London, UK) as follows: 8 cycles consisting of 90 s denaturation (95 °C), 30 s primer annealing (54 °C), further 90 sec at 48°C, and 90 s extension (72 °C); followed by 40 cycles consisting of 60 sec at 95°C, 30 sec at 54°C, 50 sec at 48°C and 60 sec at 72°C; finally completed by a single cycle involving 60 sec at 95°C, 20 sec at 54°C, 20 sec at 50°C, and 10 min at 72°C. Hold temperature was 28°C. The amplification products were directly subjected to agarose gel electrophoresis.

For specific amplification of the *gpd*-fragment from *L. deterrimus* only, the *Lactarius*-specific primers CTK107 and CTK108_{rev} were designed (cf. Table 2). Amplification was carried out using the same reaction conditions as described above, but using the following PCR program:

35 cycles consisting of 35 sec at 95°C, 60 sec at 62°C and 60 sec at 72°C, followed by a single cycle of 90 sec at 95°C, 60 sec at 55°C and 10 min at 72°C.

Table 2. Oligonucleotide primers used in the present study.

Code	Purpose	Structure
CTK-052	General (5')	5'-ATG GAT CCA TAT GCA TCG GCC GTA TCG TCC TCC GTA ATG C-3'
CTK-031 _{rev}	General (3')	5'-ATG GAT CCA TAT GCA TGA GTA ACC GCA TTC GTT ATC GTA CC-3'
CTK-107*	<i>L. deterrimus</i> specific (5')	5'-GCT CCT AGA CCC TCG TGT CAA GG-3'
CTK-108 _{rev} *	<i>L. deterrimus</i> specific (3')	5'-ATG CCC TTG AGA GGG CCA TCG GC-3'
CTK-132*	Nested Primer (5')	5'-GTC TAC ATG TTC AAG TAC GAC TC-3'
CTK-133 _{rev} *	Nested Primer (3')	5'-CCG ATG AAG TCA GTT GAC ACT AC-3'

* Primers 52 and 31 were according to homologous nt-areas in the *gpd* genes of (EMBL-accession No. in brackets): *Aspergillus nidulans* (M19694), *Cryphonectria parasitica* (X53996), *Ustilago mayidis* (X07879), *Agaricus bisporus* 1 (M 81727), *A. bisporus* 2 (M 81728), *Phanerochaete chrysosporium* (M81754), and *Schizophyllum commune* (M81724).

To amplify fungal *gpd* from mycorrhizal roots, a PCR amplification with primers CTK-052 and CTK-031 was carried out as described above, followed by a second, nested PCR step with primers CTK132 and CTK133_{rev} under the following conditions: 10 cycles of 90 sec 95°C, 30 sec 55°C, 30 sec 52°C and 90 sec 72°C, followed by 30 cycles of 45 sec at 95°C, 60 sec at 54°C and 60 sec at 72°C, and terminated by a single final cycle of 60 sec at 95°C, 60 sec at 54°C, 10 min at 72°C and 10 min at 28°C.

Cloning and sequencing. PCR products were separated on low-melting point agarose gels, isolated, filled in with Klenow enzyme, and blunt-end cloned into pGEM-5Zf(+), previously linearized with *EcoRV* (Promega Biotec, Madison, Wis.). DNA Manipulations and transformation of *E. coli* were performed by standard methods (SAMBROOK & al. 1989). The glyceraldehyde-3-phosphate dehydrogenase gene fragments were sequenced by the method of SANGER & al. 1977, using universal sequencing primers, and primers designed according to obtained sequences. PCR primers and sequence specific primers for DNA-sequencing were synthesized by M. MÜLLER, Vienna Biocenter, Vienna, Austria.

Analysis of *gpd*-fragments by non-radioactive hybridization. Two different methods were used to detect strain specific *gpd*-fragments on gels, i.e. (a) Southern hybridization, and (b) slot blot hybridization. For (a), PCR-products were separated on agarose gels and transferred to nylon membranes (Hybaid, London, UK) according to standardized protocols. Hybridization was carried out for 1 h at 68°C, followed by 2 x 20 min washing at 68°C in 4xSSC + 0.1 % (w/v) SDS, and in 0.1 x SSC + 0.1 % (w/v) SDS. For (b), DNA samples were heated on a water bath for 3 min, and then applied in various dilutions onto nylon membranes, previously soaked in 20 x SSC (10 min) and inserted into a slot-blot apparatus (Hoefer Scientific Instruments, San Francisco, CA). After application, the membranes were dried on air and the DNA covalently linked to the membrane by a 4 min UV-treatment. Hybridization was carried out as described above. Fluoresceine-labelled DNA probes were used for hybridization in both cases. They were synthesized by PCR, using 3-5 µg of chromosomal DNA from *B. edulis*, *L. deterrimus* or *A. muscaria* as template, and 50 nM of dATP, dGTP and dCTP, 0.33 nM of dTTP and 0.17 nM of fluoresceine-12-dUTP. All other PCR conditions were essentially the same as those described above. After completion of the PCR program, 2 µl of 0.2 M EDTA and 2.5 µl of 1 M LiCl were added, and the DNA precipitated by the addition of 75 ml 96 % (w/v) ethanol. The DNA was washed with ethanol, dried *in vacuo* and finally taken up in 50 µl TE-buffer. This solution was directly used for hybridization without further treatment.

Immunostaining was used to detect the hybridizing fluoresceine-labelled probe, using a monoclonal anti-fluoresceine IgG, coupled to alkaline phosphatase, and 5-bromo-4-chloro-3-indolyl-phosphate and nitroblue tetrazolium salts as a chromogenic substrates, according to the manufacturers instructions (BOEHRINGER MANNHEIM 1989).

Results and Discussion

PCR was performed with chromosomal DNA extracted from mycelia or fruiting bodies of several species of the taxa *Boletus*, *Amanita* and *Lactarius*. Fragments were directly assessed by viewing the amplified fragments after gel electrophoresis (Fig. 1a): among the 11 ectomycorrhizal fungi, the amplification products derived from the two primers varied from 1-4 DNA fragments, among which a major band of 1.2-kb was always, and sometimes exclusively, present. The size of this 1.2-kb nt-band corresponded perfectly with the expected size of the fragment as calculated from the corresponding sequences of *A. bisporus*, *P. chrysosporium* and *S. communes* (HARMSSEN & al. 1992). Proof for the identity of this nucleotide band with a *gpd*' fragment was obtained by sequencing. The deduced aa-sequence showed all the hallmark-sequences typical for GPD-proteins from other sources (GRUBER & al. 1995, KREUZINGER & al. 1995). The *gpd*-genes of the three taxa showed homology to each other of about 75 %. The amplified putative *gpd*-fragment from three selected species corresponding to the 3 taxa - *B. edulis*, *A. muscaria*, and *L. deterrimus* - was eluted from the gel, subcloned and subjected to RFLP-analysis using *Bam*HI, *Nde*I and *Nsi*I. These species were chosen because

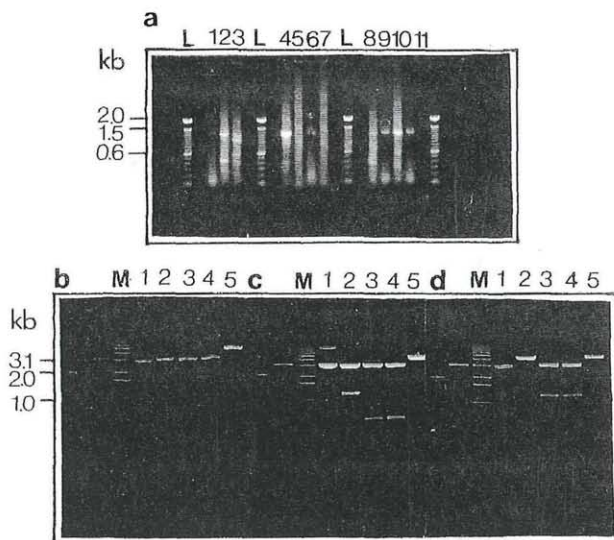


Fig. 1a. Amplification of the *gpd*-fragment by PCR, using primers 31 and 52, from chromosomal DNA of several mycorrhizal basidiomycetous species: L shows a 100-bp ladder. 1, *A. muscaria*; 2, *A. porphyrea*; 3, *A. rubescens*; 4, *B. aereus*; 5, *B. calopus*; 6, *B. edulis*; 7, *B. edulis*; 8, *L. deliciosus*; 9, *L. deterrimus*; 10, *L. porninsis*; 11, *L. scrobiculatus*. (b - d) Restriction cleavage analysis of the *gpd*-fragment of *A. muscaria* (b), *B. edulis* (c) and *L. deterrimus* (d) after cloning into the *Eco*RV-site of vector pGEM5z+. L indicates the 100-bp ladder; (1) shows intact pGEM5z+; (2) *Sal*I treated pGEM5z+; (3) intact pGEM5z+ containing the respective *gpd*-fragments; (4) shows the products of cleavage with *Bam*HI, (5) with *Nde*I, (6) with *Nsi*I, and (7) with *Sal*I, respectively.

they represent the majority of mycorrhizal symbionts of *P. abies* in the Austrian North-alpine forest areas. The three restriction nucleases already allowed a distinction between these three fungi (Fig. 1b), hence suggesting that RFLP-analysis of *gpd* may allow to distinguish between the three basidiomycetous species.

These roughly 25 % differences in the nt-sequences of *gpd* between *L. deterrimus*, *B. edulis* and *A. muscaria* suggested that stringent hybridization conditions could be used to specifically detect one of these genes in mycorrhizal roots. In order to simultaneously develop this method for routine analysis in an average laboratory, a non-radioactive assay was also adopted. Using carefully optimized high-stringency conditions (cf. Material and Methods), a *L. deterrimus* fluoresceine-12-dUTP labelled *gpd* fragment hybridized in Southern blots to its own amplification products only (Fig. 2). Similarly, *B. edulis* or *A. muscaria*-derived *gpd*-gene fragments were successfully used to detect amplification products of these species from DNA mixtures (data not shown).

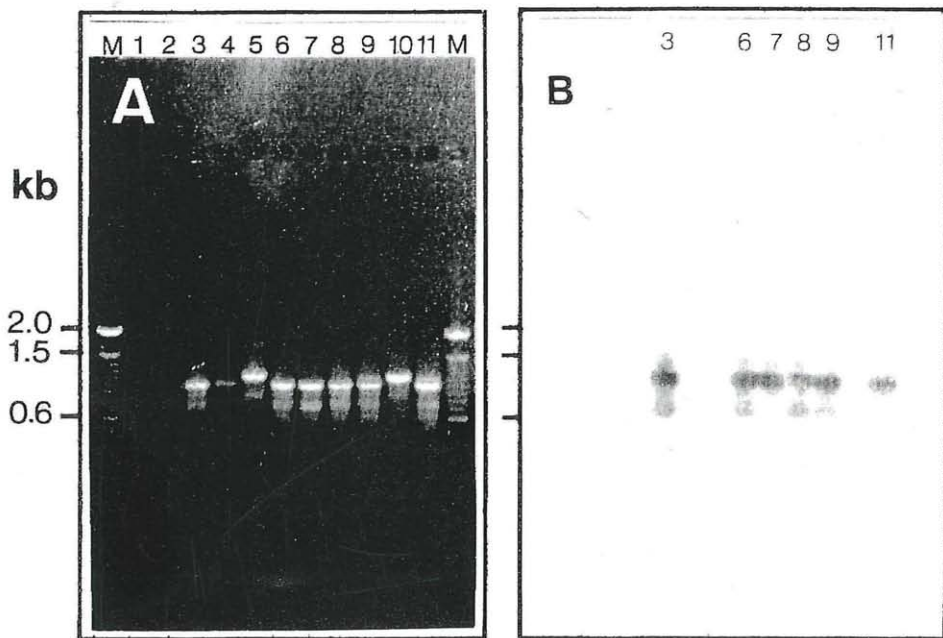


Fig. 2a. Agarose gel electrophoresis of PCR-reaction products with chromosomal DNA from following organisms as template (1 µg total concentration per organism): *A. muscaria* (1), *B. edulis* (2), *L. deterrimus* (3), *S. cerevisiae* (4), *T. reesei* (5), 1+3 (6), 2+3 (7), 3+4 (8), 3+5 (9), 1+2+4+5 (10), 1+2+3+4+5 (11). (b) Southern hybridization analysis of the PCR-generated *gpd*-fragment using a PCR-generated fluoresceine-dUTP-labelled probe.

To examine the sensitivity of hybridization by the fluoresceine labelled probe, serial dilutions of the *L. deterrimus gpd* fragment were carried out. In order

to eliminate electrophoresis and Southern transfer, slot blot analysis was used for this purpose. After hybridization, we detected 2 of DNA (Fig. 3a). Using a TLC-scanner, a standard calibration curve was obtained which showed that the quantification of the blotted DNA was possible between 1 - 10 ng of applied material (Fig. 3b). Owing to the high factor of amplification by PCR, and the presence of 1 % DNA per fungal biomass dry weight, this means that even traces of fungal mycelium can be detected by this method.

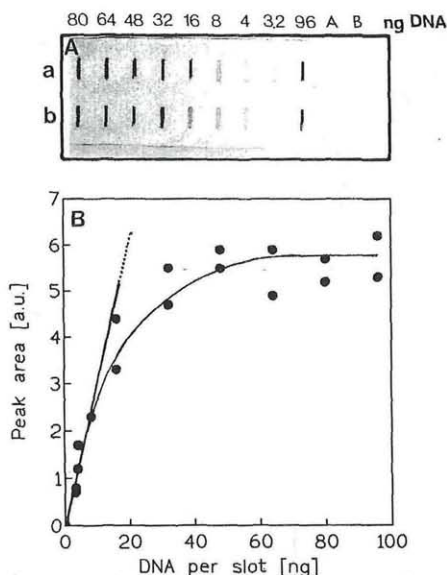


Fig. 3a. Slot-blot analysis using PCR-generated fluoresceine-dUTP labelled probe of the 1,2-kb *gpd*-fragment amplified from *L. deterrimus*. Rows (a) and (b) are duplicates of the sample. Lanes 1 - 9 contain the different concentrations of *L. deterrimus* DNA, applied to the slots, respectively, a and b contain DNA extracted from different samples of *L. deterrimus*. (b) calibration curve drawn from scanning the slots in (a) and plotting the peak area (in arbitrary units) vs. the applied concentration. The dotted line shows the continuation of the pseudolinear part of the graph, which can be used as a calibration curve.

Following the protocol given in Materials and Methods, we used this procedure to identify the fungal *gpd* genes selectively and species specific from mycorrhizal roots. The presence of unknown substances, which affected the polymerase chain reaction, were bypassed by nested PCR-amplification: a first one

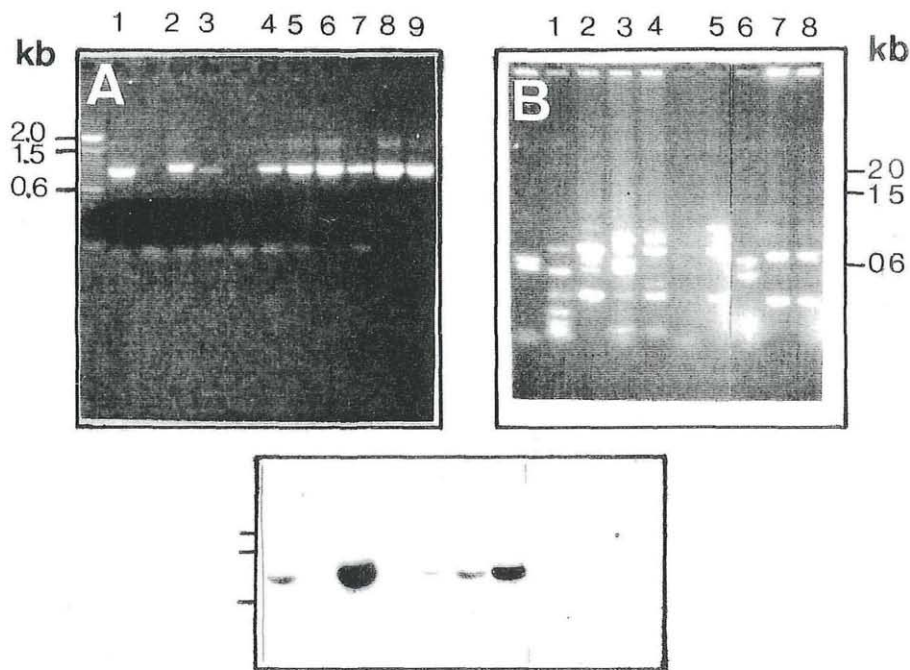


Fig. 4. Detection of basidiomycetous species in mycorrhizae by nested PCR (a), restriction analysis (b), and Southern hybridization (c). DNA was extracted from the root tips and subjected to PCR amplification with primers CKT-052 and CKT-031_{rev} as described in Materials and Methods. The amplification products were then directly used for a nested PCR reaction, using primers CKT 132 and CKT 133_{rev} (a) agarose gel electrophoresis of the products of the nested PCR amplification: Lanes 4 - 9, various samples collected throughout October and November 1993 on Schulterberg, Tyrol. Controls, i.e. chromosomal DNA from *B. edulis* (1), *L. deterrimus* (2) and *A. muscaria* (3) are also given; (b) restriction analysis of the nested-PCR products from (a) with *DdeI*. Numbering of samples is identical with (a). (c) shows the results from Southern hybridization of various samples with the amplified, random-primed fragment from sample 7 in (a). Lanes 2, 3 and 4 indicate controls with chromosomal DNA from *B. edulis*, *L. deterrimus* and *A. muscaria* (identical to 1-3 in a), resp., whereas the other lanes contain amplification products from various samples isolated on Schulterberg, Tyrol. Note that the numbering of these samples is different in this figure, but samples 1 and 6 are identical to 7 and 8 from (a) and (b).

using very diluted DNA concentrations, and a second, "nested PCR" step, to increase the concentration of the amplified fungal gene fragments. To this end, appropriate primers (cf. Table 2) were designed which corresponded to sequences located closely 3' and 5' of the sense- and antisense-primers, respectively, used in the first PCR amplification. Fig. 4 a shows that this procedure yielded the expected 1.2-kb *gpd*-fragment with all but one sample. Controls, using only one of both primers were negative, hence demonstrating the specificity of the "nested PCR reaction". In order to identify the fungal partners in these symbioses, the

amplification products were treated with *Dde*I (Fig. 4 b). From the RFLP it became evident that most of the mycorrhizae investigated apparently contained *L. deterrimus* as fungal partner. In order to provide further evidence for this, the fragment from sample #7 was labelled by Nick-translation, and used as a probe for high-stringency Southern hybridization of a blot containing genomic DNA of various *Lactarius* sp., *Boletus* sp. and *Amanita* sp. (Fig. 4c). Exclusive hybridization with *L. deterrimus* DNA only was achieved. Consistent results were further obtained by microscopical examination of the roots from which #7 had been extracted (GÖBL, unpublished results). Hence our data show that amplification of the *gpd*-fragment and subsequent analysis is able to identify the fungal partner in ectomycorrhizal symbiosis.

In summary, the success in using the *gpd*-gene for diagnosis of the fungal partners in mycorrhizal symbiosis complements the tools already available (i.e. fragments of the rDNA gene cluster, cf. HENRION & al. 1992, BRUNS & GARDES 1993, GARDES & BRUNS 1993); and chitin synthase encoding genes, (cf. MEHMANN & al. 1994), thereby offering the possibility to cross-check results from an analysis. The presentation of a nonradioactive method for hybridization will further aid to the use of molecular tools in mycorrhizal identification, as the whole procedure can now be carried out on an average laboratory bench and does not require sophisticated equipment.

Acknowledgements

The authors thank F. HERMAN and J. CAMBA for their interest and support, and J.G.H. WESSELS for disclosing the sequences of other basidiomycetes prior to their publication (HARMSSEN & al. 1992). This work was supported by grant 56.810/39-VA2/91 from the Austrian Federal Ministry of Agriculture and Forestry.

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Jahr/Year: 1996

Band/Volume: [36_4](#)

Autor(en)/Author(s): Kreuzinger N., Podeu R., Göbl Friederike, Gruber Franz, Kubicek C. P.

Artikel/Article: [Identification of Some Ectomycorrhizal Basidiomycetes by PCR-Amplification of their gpd \(Glycerinaldehyde-3-phosphate Dehydrogenase Encoding\) Genes. 109-118](#)