

Identification of Endomycorrhizal Fungi Colonizing Roots by Fluorescent Single-Strand Conformation Polymorphism–Polymerase Chain Reaction

L. SIMON,^{1*} R. C. LÉVESQUE,² AND M. LALONDE¹

Centre de Recherche en Biologie Forestière, Faculté de Foresterie et de Géomatique,¹ et Département de Microbiologie, Faculté de Médecine,² Université Laval, Sainte-Foy, Québec G1K 7P4, Canada

Received 20 July 1993/Accepted 7 October 1993

A method to identify arbuscular endomycorrhizal fungi based on the amplification of portions of the nuclear gene coding for the small subunit rRNA is presented. By coupling the sensitivity of the polymerase chain reaction and the specificity afforded by taxon-specific primers, a variety of samples can be analyzed, including small amounts of colonized roots. Family-specific primers as well as generic primers are described and can be used to amplify small subunit rRNA fragments from endomycorrhizal fungi by polymerase chain reaction. The amplified products are then subjected to single-strand conformation polymorphism analysis to detect sequence differences. Among the advantages of this approach is the possibility of directly identifying the fungi inside field-collected roots, without having to rely on the fortuitous presence of spores. This technique should have obvious applications in the study of arbuscular endomycorrhizal fungi populations and allow closer examination of their host specificity.

The arbuscular endomycorrhizal fungi can colonize the roots of the vast majority of vascular plants, establishing a mutualistic symbiosis (11). These fungi are classified in the order Glomales (8) within the zygomycetes, among which three families are presently recognized. Morphological characters of the large (diameter, 80 to 500 μm) soilborne spores are the basis for the description of over 130 species of these obligatory biotrophs (10). Recently, sequences of the gene coding for the small subunit rRNA (SSU) were obtained from 12 endomycorrhizal fungal species, and comparisons with other 18S sequences indicated that the Glomales formed a phylogenetically coherent group that originated about 400 million years ago (2, 12).

Since we are unable to grow these fungi in pure culture, and because the spores that can be identified are produced in the soil outside the colonized roots, the identity of the endophyte inside field-collected colonized roots is difficult to determine with any degree of certainty. The development of DNA-based methods could allow the identification of endomycorrhizal fungi in otherwise intractable samples and provide a useful tool to complement the expertise of fungal taxonomists. Our approach was to combine the specificity of polymerase chain reaction (PCR) amplification of 18S gene fragments with the sensitivity of single-strand conformation polymorphism (SSCP) analysis to rapidly characterize the endophytes in colonized roots.

MATERIALS AND METHODS

Design of primers. A total of 12 nuclear genes coding for the SSU were recently sequenced from different species of endomycorrhizal fungi (12, 13). These were aligned, and regions potentially useful for the design of taxon specific probes or primers were visually identified. One such region is illustrated in Fig. 1, along with the primers VALETC, VAGLO, VAACAU, and VAGIGA that were designed to

discriminate among four distinct groups of endomycorrhizal species.

Another informative region was identified approximately 400 bp further downstream, and primers were designed from the flanking conserved stretches. Using VANS22 and VANS32, we should be capable of amplifying a 150-bp informative fragment from any endomycorrhizal fungi. These primers were not designed to be *Glomales* specific, and it is possible that they could also be useful for other fungi or eucaryotes.

Labeling and purification of primers. Primers were synthesized on a Pharmacia Gene Assembler Plus (Pharmacia LKB Biotechnology, Baie d'Urfé, Quebec, Canada), or obtained commercially. The VANS1 primer was labeled by incorporating a fluorescein-amidite (FluorePrime; Pharmacia LKB Biotechnology) at the 5' end. The VANS22 primer was obtained with a 5' amino linker and was labeled with the FAM (ABI, Mississauga, Ontario, Canada) fluorochrome (4). The following oligonucleotides were used as primers, and their location relative to the SSU gene is depicted in Fig. 2:

VANS1,	5'GTCTAGTATAATCGTTATACAGG
NS21,	5'AATATACGCTATTGGAGCTGG
VALETC,	5'ATCACCAAGGTTTAGTTGGTTGC
VAGLO,	5'CAAGGGAATCGGTTGCCCGAT
VAACAU,	5'TGATTACCAATGGGAAACCC
VAGIGA,	5'TCACCAAGGGAACCCGAAGG
VANS22,	5'TAAACACTCTAATTTTTC
VANS32,	5'AAGCTCGTAGTTGAATTTCCG
SS1492',	5'GCGGCCGCTACGGWACCTTGTTACGACTT

PCR. Unless specified otherwise, template DNA used in amplification assays consisted of portions of the 18S nuclear rRNA gene previously amplified from spores of endomycorrhizal fungi (12).

The reaction mixtures consisted of template solution and a master mix (20 mM Tris-HCl [pH 8.4], 100 mM KCl, 3 mM MgCl_2 , 0.02% gelatin, 60 mM each nucleotide) (1:1 [vol/vol]) containing 5 U of *Taq* DNA polymerase (Amplitaq, Perkin-

* Corresponding author. Electronic mail address: lsimon@alnu.s.ulaval.ca.

<i>Glomus etunicatum</i>	ACCAACAGTTGGGCAACCAACTAAACCTTGGTGATTCAT
VALETC	CGTTGGTTGATTTGGAACCACTA
<i>G. mossae</i>	ACCAATA-TCGGGCAACCGA-TTCCC-TTGGTGATTCAT
<i>G. intraradices</i>	ACCAATA-TCGGGCAACCGA-TTCCC-TTGGTGATTCAT
	----- -----
VAGLO	T-AGCCCGTTGGCT-AAGGG-AAC
<i>Entrophospora sp.</i>	ACCAATAGCTCCGCAAGGGTTTCCCATTGGTGAATCAT
<i>E. columbiana</i>	ACCAATAGCTCCGCAAGGGTTTCCCATTGGTGAATCAT
<i>Acaulospora rugosa</i>	ACCAATAACTCCTGNNGGGTTTCCCATTGGTGAATTCAT
<i>A. spinosa</i>	ACCAATAGCTCCTTCAGGGTTTCCCATTGGTGAATCAT
VAACAU	CCCCAAAGGGTAACCACCTTAGT
<i>Gigaspora margarita</i>	ACCAATAACCTTC---GGGTTTCCC-TTGGTGATTCAT
<i>G. gigantea</i>	ACCAATAACCTTC---GGGTTTCCC-TTGGTGATTCAT
<i>G. albidia</i>	ACCAATAACCTTC---GGGTTTCCC-TTGGTGATTCAT
<i>Scutellospora pellucida</i>	ACCAATAACCTTC---GGGTTTCCC-TTGGTGATTCAT
<i>S. dipapillosa</i>	ACCAATAACCTTC---GGGTTTCCC-TTGGTGATTCAT
VAGIGA	GGAAG---CCCAAAGGG-AACCACCT

FIG. 1. Partial alignment of SSU sequences. SSU sequences of 12 endomycorrhizal fungi available in GenBank were aligned. The region that was used for the design of the taxon-specific primers VALETC, VAGLO, VAACAU, and VAGIGA is depicted. -, gap; |, complementary base pair.

Elmer, Montréal, Québec, Canada) per ml and 50 pmol of each appropriate primer per ml in a 20- to 100- μ l total reaction volume, overlaid with light mineral oil when appropriate. Alternatively, Vent_R (exo-) polymerase (New England BioLabs, Mississauga, Ontario, Canada) was used with the buffer provided. The temperature profile was programmed on a DNA Thermal Cycler (Perkin-Elmer) to repeat 25 to 35 times a cycle of denaturation (60 s at 94°C), annealing (45 s at 50°C), and polymerization (60 s at 72°C), followed by a final extension step of 10 min at 72°C. PCR products were monitored by agarose gel electrophoresis on 2% Nusieve-1% Agarose, stained with ethidium bromide, and photographed.

SSCP. Fluorescent-labeled PCR products were diluted 5- to 10-fold in 95% formamide-10 mM NaOH. After 5 min of denaturation at 85°C, 3 μ l was loaded on a nondenaturing acrylamide gel (0.5 \times MDE; AT Biochem, Malvern, Pa.) and electrophoresed on an A.L.F. sequencer (Pharmacia LKB Biotechnology) fitted with an external thermostated circulator. Fragment Manager software (Pharmacia LKB Biotechnology) was used for data analysis and visualization.

Extraction of DNA from roots and sequencing. Leek roots (*Allium porum*) colonized with *Glomus vesiculiferum* were harvested, rinsed with tap water, and ground to a powder in liquid nitrogen (14). Total DNA was extracted from aliquots of ca. 100 mg of ground roots with the IsoQuick extraction kit (MicroProbe Corp., Bothell, Wash.). By using primers VANS1-SS1492', the nearly complete nuclear gene coding for the SSU of *G. vesiculiferum* was amplified. Portions of the genes were subsequently reamplified by asymmetric

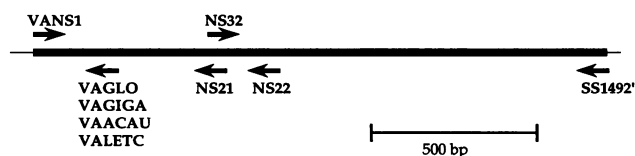


FIG. 2. Diagram of the location of the primers used in this study. Location and orientation of the primers (arrows) are shown on a schematic representation of the nuclear gene coding for the SSU RNA. Thin lines represent flanking intergenic spacers. Sequences of the primers are listed in Materials and Methods.

PCR with universal primers to yield overlapping single-stranded DNAs that were then sequenced as previously described (13). The sequence was deposited in GenBank (accession no. L20824).

RESULTS

Family-specific amplification. Four taxon-specific primers (VAACAU, VAGIGA, VAGLO, and VALETC) were designed and synthesized. They were designed to be used in conjunction with the previously described Glomales-specific VANS1 primer (13). The length of these primers was adjusted to compensate for their different base composition to yield primers of comparable melting temperatures. To verify the ability of these primer pairs to be used in a family-level identification test, purified SSU fragments previously amplified from spores of the different endomycorrhizal fungi were used as templates in PCR assays. Preliminary experiments using *Taq* polymerase and standard PCR conditions showed poor specificity; the expected products were always produced, but all primers also allowed amplification with some "incompatible" templates (sequenced SSU fragments that were predicted not to serve as efficient template for a given primer pair). Attempts to enhance the specificity by using higher annealing temperatures were unsuccessful. The use of a different enzyme, Vent (exo-) polymerase, allowed more specific amplification by these primers, using the same temperature profile (Table 1). Under these conditions, the combination of presence or absence of an amplified fragment in the four separate amplification reactions performed could be used to discriminate between any of the four groups considered (Acaulosporaceae, Gigasporaceae, Glomaceae, and *Glomus etunicatum*). For example, a sample that produced an amplified fragment when subjected to PCR with primers VANS1-VAGLO but not with VANS1-VAACAU, VANS1-VAGIGA, or VANS1-VALETC would be classified as belonging to the Glomaceae.

SSCP analysis of PCR fragments. We sought a methodology that could be used to characterize endomycorrhizal fungi without relying on prior availability of sequence information. SSCP analysis has been reported to be capable of detecting single-base substitution between homologous fragments of a few hundred base pairs (1, 3, 5, 7, 9). Briefly, differences in homologous fragments are detected by running them on a high-resolution polyacrylamide gel under nondenaturing conditions. Since the samples are denatured immediately before the gel is loaded, the two strands of each fragment migrate separately and their rate of migration is affected by the conformation that they are allowed to adopt in the gel. This technique was applied to the characterization of the fragments amplified with the family-specific primers. By using one fluorescently labeled primer in the PCR reaction, a single strand of the amplified product was labeled during the amplification process. Following denaturation of the amplified fragments, electrophoresis on a nondenaturing polyacrylamide gel was performed on an automated sequencer that allows real-time visualization of the fluorescent molecules. Fluorescently labeled standards were added to each sample to precisely compare the migration of the fragments of interest between any of the 40 lanes available on the slab gel. An electrophoregram (Fig. 3) illustrates the differences in migration that were observed between fragments amplified with primer pair VANS1-VAACAU. SSCP analysis of the fragments allowed the distinction between *Acaulospora spinosa*, *Acaulospora rugosa*, and *Entrophospora* spp. but not between *Entrophospora columbiana* and

TABLE 1. Sizes^a of PCR fragments obtained with different primer pairs

Species (accession number)	Size (bp)					
	VALETC ^b	VAGLO ^b	VAACAU ^b	VAGIGA ^b	VANS21 ^b	VANS22 ^c
<i>Glomus etunicatum</i> (Z14008)	195	— ^d	—	—	550	155
<i>Glomus mossae</i> (Z14007)	—	188	—	—	548	152
<i>Glomus intraradices</i> (X58725)	—	188	—	—	548	151
<i>Glomus vesiculiferum</i> (L20824)	—	188	—	—	548	151
<i>Entrophospora columbiana</i> (Z14006)	—	—	199	—	551	153
<i>Entrophospora</i> sp. (Z14011)	—	—	199	—	551	153
<i>Acaulospora rugosa</i> (Z14005)	—	—	198	—	550	152
<i>Acaulospora spinosa</i> (Z14004)	—	—	198	—	550	152
<i>Gigaspora margarita</i> (X58726)	—	(191) ^e	—	189	545	150
<i>Gigaspora gigantea</i> (Z14010)	—	(191)	—	189	545	150
<i>Gigaspora albida</i> (Z14009)	—	(191)	—	189	545	150
<i>Scutellospora pellucida</i> (Z14012)	—	(191)	—	189	545	151
<i>Scutellospora dipapillosa</i> (Z14013)	—	(191)	—	189	545	150

^a As predicted from the sequences of the endomycorrhizal fungi SSU deposited in GenBank (accession numbers are given in the table).

^b In conjunction with primer VANS1, using conditions described in Materials and Methods.

^c In conjunction with primer VANS32.

^d —, no visible fragment.

^e (), unexpected fragment can sometimes be amplified, even with Vent_R (exo-).

the other *Entrophospora* spp. available. This reflects the presence of six substitutions between the two *Acaulospora*-derived fragments, while the two *Entrophospora*-derived fragments were absolutely identical, as predicted from their published SSU sequences. Similarly, SSCP analysis of the VANS1-VAGIGA fragments could detect differences in migration among *Scutellospora dipapillosa*, *Scutellospora pellucida*, and the *Gigaspora* species (data not shown), but no migration difference was observed between fragments derived from the three *Gigaspora* species, as predicted from their sequences.

Since there appears to be more divergence in the family Glomaceae than previously recognized, and because a limited number of sequences were available for the design of the primers VAGLO and VALETC, there is a possibility that some endomycorrhizal fungi possess 18S genes that are refractory to amplification by the aforementioned primer

pairs. Consequently, another variable portion of the 18S gene was identified and primers VANS22 and VANS32 were designed to amplify a 149- to 155-bp fragment, comprising many variable sites. These primers were selected to be complementary to conserved regions of the 18S genes; the targeted fragments should be amplified from any endomycorrhizal fungi. After VANS22 was labeled with a suitable fluorochrome and the expected fragment was amplified from the 12 available endomycorrhizal fungi, SSCP analysis was conducted. Again, differences in migration were observed between fragments of similar size but differing in composition, as exemplified by the discrimination between *S. pellucida*, *S. dipapillosa*, and *Gigaspora* spp. (Fig. 4). Even though the fragments amplified with VANS22-VANS32 are shorter, they can be very informative: there are 12 substitutions and a 1-bp length difference between the *Scutellospora*-derived fragments.

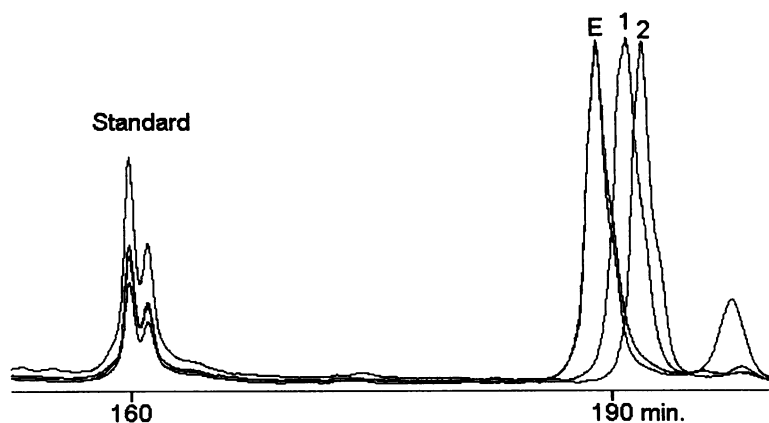


FIG. 3. SSCP analysis with VANS1-VAACAU. Previously purified SSU fragments from four species of Acaulosporaceae were amplified by using primer pair VANS1-VAACAU as described in Materials and Methods. The VANS1 primer was fluorescein labeled. Resulting amplification products were diluted in formamide-NaOH and spiked with a fluorescent size standard. After being heated at 85°C for 5 min, 3- μ l samples were loaded per lane on a 0.35-mm-thick polyacrylamide slab gel (MDE 0.5 \times ; AT Biochem). Electrophoresis was performed on an automated sequencer (ALF; Pharmacia) at 1,200 V for 4 h, using 0.6 \times TBE running buffer. A constant temperature of 30°C was maintained by an external thermostated circulator. Acquired data were analyzed with Fragment Manager software, which allows lane-to-lane migration standardization. The four traces depicted from adjacent lanes illustrate the identical migration rate of single-stranded fragments from both *Entrophospora* sp. and *E. columbiana* (line E), while those from *A. spinosa* (line 1) and *A. rugosa* (line 2) migrated at lower rates.

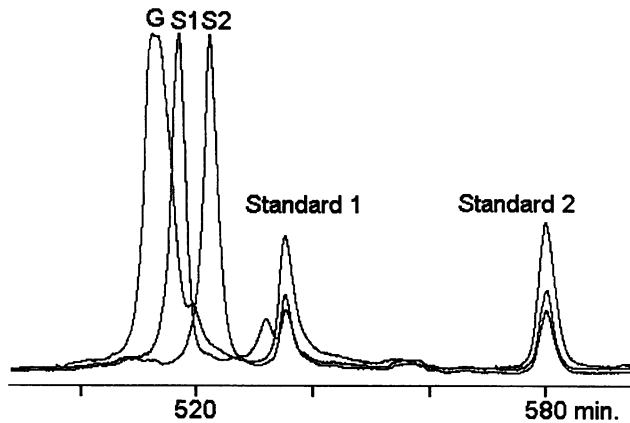


FIG. 4. SSCP analysis with VANS22-VANS32. Previously purified SSU fragments from three species of Gigasporaceae were amplified by using primer pair VANS22-VANS32 as described in Materials and Methods. The VANS22 primer was fluorescein labeled. The resulting amplification products were diluted in formamide-NaOH and spiked with two fluorescent size standards. After being heated at 85°C for 5 min, 3- μ l samples were loaded per lane on a 0.35-mm-thick polyacrylamide slab gel (MDE 0.5 \times ; AT Biochem). Electrophoresis was performed on an automated sequencer (ALF; Pharmacia) at 600 V for 10 h, using 0.6 \times TBE running buffer. A constant temperature of 30°C was maintained by an external thermostated circulator. Acquired data were analyzed with Fragment Manager software, which allows lane-to-lane migration standardization. The traces depicted from three adjacent lanes illustrate the different migration rates of single-stranded fragments from *Gigaspora gigantea* (line G), *S. dipapillosa* (line S1), and *S. pellucida* (line S2) in order of decreasing speed. No migration differences were observed between the single-stranded fragments from the three *Gigaspora* species analyzed (data not shown).

Characterization of an endomycorrhizal fungus colonizing roots. To demonstrate that this methodology could be applied to the characterization of an endophyte directly inside roots, leek root samples colonized by *G. vesiculiferum*, an endomycorrhizal fungus for which no sequence data were available, were analyzed. By using crude root DNA extracts, a fungal SSU fragment approximately 720 bp long was amplified with primer pair VANS1-VANS22. This amplified fragment was diluted and used as a template for subsequent amplifications with primer pairs VANS1-VAGLO, VANS1-VALETC, VANS1-VAGIGA, VANS1-VAACAU, and VANS22-VANS32. A fragment of the expected size was obtained following PCR with VANS1-VAGLO, and no PCR product was obtained with the other three taxon-specific primer pairs. SSCP analysis of this fragment indicated that it was different from homologous fragments of either *Glomus mossae* or *Glomus intraradices*. Similarly, SSCP analysis of the VANS22-VANS32 fragments confirmed that this endophyte was different from either *G. mossae* or *G. intraradices*. To verify this finding, the nearly complete 18S gene was amplified with VANS1-SS1492' and sequenced. The resulting sequence was compared with the 12 published sequences from endomycorrhizal fungi. Over the 1,631 bases compared, there were 34, 69, and 124 differences observed between *G. vesiculiferum* and *G. intraradices*, *G. mossae*, and *G. etunicatum*, respectively, while 131 to 137 differences were observed between *G. vesiculiferum* and the other endomycorrhizal fungi.

DISCUSSION

The availability of a number of sequences of the nuclear genes coding for the SSU RNA from endomycorrhizal fungi allowed the design of family-specific primers. A small region approximately 180 bp downstream from the VANS1 primer was found to be polymorphic at the interfamily level (Fig. 1). In this region, there were substantial differences in the DNA sequences of representatives of the three recognized families, Acaulosporaceae, Gigasporaceae, and Glomaceae, while intrafamilial differences were few, except for *Glomus etunicatum*, which was very different from the other members of the Glomaceae. We have already suggested that *G. etunicatum* might eventually be placed in a separate family (12). Consequently, four distinct groups were considered and taxon-specific amplification primers in the 18S sequences that could discriminate between them were designed. The specificity of these four primers, when used in conjunction with the Glomales-specific VANS1 primer, was tested in amplifications conducted on purified SSU fragments previously obtained from a number of endomycorrhizal fungi. When a proper polymerase is used, such as Vent_R (exo-), these primers reliably yield sufficient information to discriminate between the four groups of taxa considered. The observation that thermostable polymerases differ in their ability to catalyze taxon-specific amplification might be related to differences in the extent of primer mismatches tolerated. The Stoffel fragment of the *Taq* polymerase might also be useful as it was reported that it could only extend perfectly matched primers (6).

Similarly, other primers or probes targeting informative portions of this same gene could be designed to discriminate between some genera or species, which might be useful in the context of controlled experiments. This strategy is dependent on the availability of sequences from all species to be identified. Unfortunately, 18S sequences are currently available from only 12 species of endomycorrhizal fungi. Consequently, a methodology that could be used to characterize endomycorrhizal fungi without relying on prior knowledge of their DNA sequence was sought. SSCP analysis is a technique that could detect sequence variation, sometimes as small as a single-base substitution, between DNA fragments ranging from 100 to 450 bp (5). By using SSCP analysis, fragments amplified using a single primer pair could be further characterized without the need for other specific primers or probes. This was illustrated by the analysis of fragments amplified with primer pair VANS1-VAACAU, with which discrimination between related species was achieved. (Fig. 3)

In order for this strategy to be applicable to the widest range of endomycorrhizal fungi, even those that might not be amenable to amplification by using the four family-specific primers described in this paper, new primers, VANS22 and VANS32, were designed to amplify a variable portion of the 18S gene of any endomycorrhizal fungus. Even though the specificity of these primers is not restricted to endomycorrhizal fungi, adequate specificity can be achieved by first amplifying a larger fragment by using VANS1-VANS22, and then using VANS22-VANS32 (Fig. 2). The specificity of the VANS1 primer for members of the Glomales has already been established (13). By using this approach, it was possible to characterize an endomycorrhizal fungus present in leek (*A. porum*) roots. SSCP analyses of both the VANS22-VANS32 and VANS1-VAGLO fragments confirmed that this endophyte was different from previously characterized species, which prompted efforts to sequence its 18S gene.

Because it is based on PCR, the sensitivity of this identification procedure is theoretically sufficient to be used on very small samples of colonized roots. Extraction protocols might need to be optimized to consistently yield amplifiable fungal DNA from roots of the variety of plant species that can be colonized by endomycorrhizal arbuscular fungi. This methodology might be useful to rapidly characterize field-collected samples, allowing a preliminary identification of the endophytes, and to help single out those that seem novel and worth further analysis.

ACKNOWLEDGMENTS

We thank Sylvie Chabot for the gift of leek roots colonized with *Glomus vesiculiferum* and France Couture and Pierre Chrétien for their help with the A.L.F. and Fragment Manager software.

This work was supported in part by an operating grant from the Natural Sciences and Engineering Research Council of Canada to M.L. (No. A-2920). Work in R. C. Lévesque's laboratory was funded by the MRC and the Canadian Bacterial Diseases Network via the Centers of Excellence.

REFERENCES

1. Ainsworth, P. J., L. C. Surh, and M. B. Coulter-Mackie. 1991. Diagnostic single strand conformational polymorphism (SSCP): a simplified non-radioisotopic method as applied to a Tay-Sachs B1 variant. *Nucleic Acids Res.* **19**:405-406.
2. Bruns, T. D., et al. 1992. Evolutionary relationships within the fungi: analyses of small subunit rRNA sequences. *Mol. Phylogen. Evol.* **1**:231-243.
3. Dockhorn-Dworniczak, B., B. Dworniczak, L. Brommelkamp, J. Bulles, J. Horst, and W. W. Bocker. 1991. Non-isotopic detection of single strand conformation polymorphism (PCR-SSCP): a rapid and sensitive technique in diagnosis of phenylketonuria. *Nucleic Acids Res.* **19**:2500.
4. Giusti, W. G., and T. Adriano. 1993. Synthesis and characterization of 5'-fluorescent-dye-labelled oligonucleotides. *PCR Methods Appl.* **2**:223-227.
5. Hayashi, K. 1991. PCR-SSCP: a simple and sensitive method for detection of mutations in the genomic DNA. *PCR Methods Appl.* **1**:34-38.
6. Lawyer, F. C., S. Stoffel, R. K. Saiki, S.-Y. Chang, P. A. Landre, R. D. Abramson, and D. H. Gelfand. 1993. High-level expression, purification, and enzymatic characterization of full-length *Thermus aquaticus* DNA polymerase and a truncated form deficient in 5' to 3' exonuclease activity. *PCR Methods Appl.* **2**:275-287.
7. Makino, R., H. Yazyu, Y. Kishimoto, T. Sekiya, and K. Hayashi. 1992. F-SSCP: fluorescence-based polymerase chain reaction-single-strand conformation polymorphism (PCR-SSCP) analysis. *PCR Methods Appl.* **2**:10-13.
8. Morton, J. B., and G. L. Benny. 1990. Revised classification of arbuscular mycorrhizal fungi (Zygomycetes): a new order, Glomales, two new suborders, Glominae and Gigasporinae, and two new families, Acaulosporaceae and Gigasporaceae, with an emendation of Glomaceae. *Mycotaxon* **37**:471-491.
9. Orita, M., H. Iwahana, H. Kanazawa, K. Hayashi, and T. Sekiya. 1989. Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphism. *Proc. Nat. Acad. Sci. USA* **86**:2766-2770.
10. Pérez, Y., and N. C. Schenck. 1990. A unique code for each species of VA mycorrhizal fungi. *Mycologia* **82**:256-260.
11. Sanders, F. E., B. Mosse, and P. B. Tinker. 1975. *Endomycorrhizas*. Academic Press, London.
12. Simon, L., J. Bousquet, R. C. Lévesque, and M. Lalonde. 1993. Origin and diversification of endomycorrhizal fungi and coincidence with vascular land plants. *Nature (London)* **363**:67-69.
13. Simon, L., M. Lalonde, and T. D. Bruns. 1992. Specific amplification of 18S fungal ribosomal genes from vesicular-arbuscular endomycorrhizal fungi colonizing roots. *Appl. Environ. Microbiol.* **58**:291-295.
14. Simon, L., R. C. Lévesque, and M. Lalonde. 1992. Rapid quantitation by PCR of endomycorrhizal fungi colonizing roots. *PCR Methods Appl.* **2**:76-80.