15 Use of Short Oligonucleotide Primers in Random Amplified Polymorphic DNA Techniques for Species Identification

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15.1 Introduction

The introduction of molecular techniques in biology has been a major force in the areas of systematics and population biology of the fungi. The introduction of PCR-based methods has significantly increased the level of activity. The simplicity of the techniques, coupled with the general use of particular regions of the genomes, has resulted in many important advances in our understanding of taxonomic grouping as well as the evolutionary histories and functional properties associated with them. The nuclear genomes of fungi are small, intermediate between that of prokaryotes and the higher eukaryotic plants and animals. Compared with higher plants and animals, fungi have a much lower percentage of redundant DNA. Typically 10–20% of the DNA in fungi is redundant, while as much as 80% of the DNA may be redundant in other eukaryotes (Dutta 1974). The baker's yeast *Saccharomyces cerevisiae* contains a genome of 16 chromosomes, including 13.4 million bases. Its genome displays significant redundancy, with 53 duplicated gene clusters among the 16 chromosomes. These duplicated regions represent more than 30% of the entire genome (Mewes et al. 1997). Fungi have extrachromosomal genetic elements, the most important of which are found in mitochondria. Mitochondrial (mt) genomes provide another source of genetic variability that is independent of sexual reproduction. The mitochondrial genome in fungi is usually uniparentally (maternally) inherited. The mtDNA is the useful tool for the taxonomic studies because it is relatively small, making it possible to analyze the entire genome, and its composition is not complicated by the recombination that occurs (Taylor 1986).

Variation within species can be assayed using the random amplified polymorphic DNA (RAPD) method (Welsh and McClelland 1990; Williams et al.

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1990), in which arbitrary short oligonucleotide primers, targeting unknown sequences in the genome, are used to generate amplification products that often show size polymorphism within species. RAPD analysis offers the possibility of creating polymorphism without any prior knowledge of the DNA sequences of the organism investigated. The method is fast and economic for screening large number of samples. The RAPD band pattern has been used to define some fungal species in which species-specific bands or combinations of bands have been considered. In these techniques there is the assumption that bands with identical mobility and staining intensity are of the same or very similar sequences. Characterization of species at morphological and protein level is not fully reliable since environmental conditions influence the nature of the organism to a great extent. The use of molecular markers such as RAPDs along with morphological and protein traits may provide a more clear concept of the species. RAPD markers are randomly distributed through out the genome and can be efficiently and randomly sampled using established procedures. The RAPD procedure developed by Welsh and McClelland (1990) and Williams et al. (1990) involves simultaneous amplification of several anonymous loci in the genome and has been used for genetic, taxonomic and ecological studies of several fungi (Zinno et al. 1998).

PCR-based techniques have already been applied to endo-and ectomycorrhizal fungi where morphological characters are in conflict, ambiguous and missing (Podila and Lanfranco 2004). This approach has allowed the development of molecular tools for their identification and increased the level of understanding in the molecular taxonomy of microorganisms (Solaiman and Abbott 2004; Varma et al. 2004). The most commonly used PCR-based techniques include amplification of variable regions in the ribosomal genes, restriction fragment length polymorphism (RFLP), amplification of short repeated sequences (microsatellites) and random amplification of polymorphic DNA (RAPD; Erlich et al. 1991). These techniques provide a different degree of resolution in the study of genetic polymorphisms. RAPD reveals intraspecific differences by originating DNA fingerprints, which may be unique for a single isolate (Perotto et al. 1996). Identification of individual clones is essential for the better understanding of the diversity, structure and dynamic of populations of ectomycorrhizal fungi. Unfortunately, this approach is time-consuming. RAPD (Welsh and McClelland 1990; Williams et al. 1990) has therefore been used for the analysis of populations of *Suillus granulatus* (Jacobson et al. 1993) and *Laccavia bicolor* (Buschena et al. 1992). However, this technique has been reported to be very sensitive to experimental variables and the RAPD assay conditions described for one species may not be suitable for another. Huai et al. (2003) studied the genetic variation and spatial distribution of the ectomycorrhizal fungus *Tricholoma terreum*. The 33 sporophores studied belonged to distinct genotypes, based on the analysis of RAPD markers. The genets of *T. terreum* were small and not larger than 0.5 m. Two major phenetic groups, i.e., eight individuals in group 1 and 25 in group 2, were identified by principal component analysis and by the unweighed pair group method with arithmetic means of simple matching coefficients,

respectively. The application of RAPD analysis was investigated for the identification of ectomycorrhizal symbionts of spruce (*Picea abies*) belonging to the genera *Boletus*, *Amanita* and *Lactarius* at and below the species level. Using both fingerprinting [M13, $(GTG)_5$, $(GACA)_4$] and random oligonucleotide primers (V1, V5), a high degree of variability of amplified DNA fragments (band-sharing index 65–80%) was detected between different strains of the same species, hence enabling the identification of individual strains within the same species. The band-sharing index between different species of the same genus (*Boletus*, *Russula*, *Amanita*) was in the range 20–30% and similar values were obtained when strains from different taxa were compared. Thus RAPD is too sensitive at this level of relationship and cannot be used to align unknown symbionts to a given taxon. They therefore conclude that RAPD is a promising tool for the identification of individual strains and could thus be used to distinguish indigenous and introduced mycorrhizal strains from the same species in natural ecosystem. The genetic variability of *Trichoderma* isolates using the RAPD were analyzed by Góes et al. (2002), who found high intra-specific genetic variation among those fungi.

15.2 Polymorphism between *Piriformospora indica* and *Sebacina vermifera*, Members of the Order Sebacinales

Piriformospora indica, a new endophyte (Verma et al. 1998) has the ability to grow axenically. The cultivability of this fungus in different synthetic media, like *Aspergillus* medium (Malla et al. 2002; Pham et al. 2004), provided an opportunity to study the comparative isozyme polymorphism and a molecular marker like RAPD to establish variability in between *P. indica* and the closely related organism *Sebacina vermifera* (Malla et al. 2004b). The analysis of enzymes, isozymes like laccase, malate dehydrogenase, esterase, peptidase, peroxidase, acid and alkaline phosphatase and non-enzymic proteins and their mobility displayed clear variations among different species (Malla et al. 2004a).

Proteomics and genomics data about this fungus were recently described (Peskan et al. 2004; Kaldorf et al. 2005; Shahollari et al. 2005). *S. vermifera* sensu stricto consists of a broad complex of species possibly including mycobionts of jungermannioid and ericoid mycorrhizas. Extrapolating from the known rDNA sequences in the Sebacinaceae, it is evident that there is a cosm of mycorrhizal biodiversity yet to be discovered in this group (Weiß et al. 2004).

The acid phosphatases (ACPases; Fig. 15.1) in *P. indica* and *S. vermifera* sensu stricto are similar in their molecular mass. The antibody raised against the ACPase of *P. indica* showed a maximum ELISA reading with *S. vermifera* sensu stricto, supporting a strong relationship between these two fungi. The immunoblot analysis showed a strong reactivity of *P. indica* antiserum with *S. vermifera*

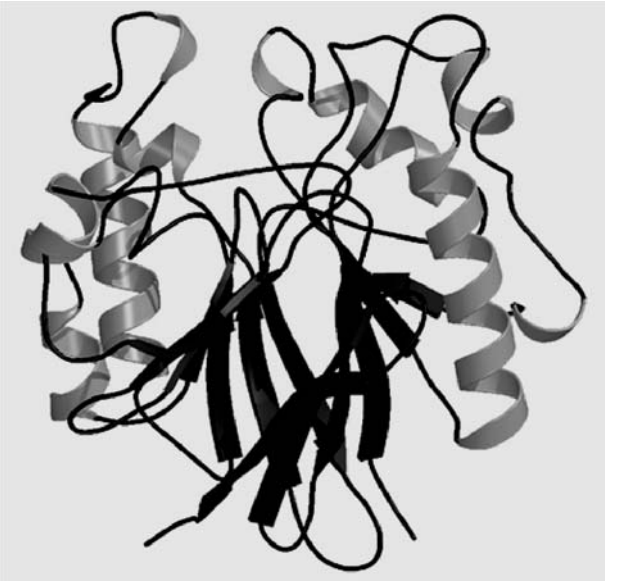

Fig. 15.1 3D structure of acid phosphatase

sensu stricto. The antiserum blotted bands at 66 kDa with *S. vermifera* separated in denatured PAGE and at a similar location with *P. indica* in non-denatured PAGE. The antiserum also localized the enzyme in *S. vermifera* by an immunofluorescence technique, showing a strong relationship of this fungus with *P. indica* (Fig. 15.2). The immunogold labeling of antiserum from *P. indica* precisely localized the enzyme in the cytoplasm and vacuoles of *S. vermifera*, supporting the strong immunological link between these two fungi. Two-dimensional maps of crude protein of these two fungi showed some differences in minor proteins. *P. indica* and *S. vermifera* sensu stricto belonging to same taxonomic group show similar morphology, functions and isozymes. However, they show distinct genetic variation based on the RAPD analysis and can be considered to be placed within species from the same ancestral root.

The study aimed to establish genetic diversity between the two species *P. indica* and *S. vermifera* sensu stricto belonging to the same order, Sebacinales. Seven random 10-bp oligonucleotide primers of different origin were used. Clustering of similarity matrices was done by the un-weighted pair group method with arithmetic mean (UPGMA) and projection by the TREE program of NTSYS-pc (Numerical Taxonomy System, Applied Biostatistic). Out of seven primers, six gave scorable, reproducible DNA products (bands) suitable for establishing a genetic diversity. UPGMA cluster analysis clustered the isolates into two distinct groups. The average genetic similarity between both fungi was 0.58 (i.e., 58%) and can be considered to place them within species from the same ancestral root. These results illustrated the potential value of RAPD techniques for detecting polymorphism among fungal isolates.

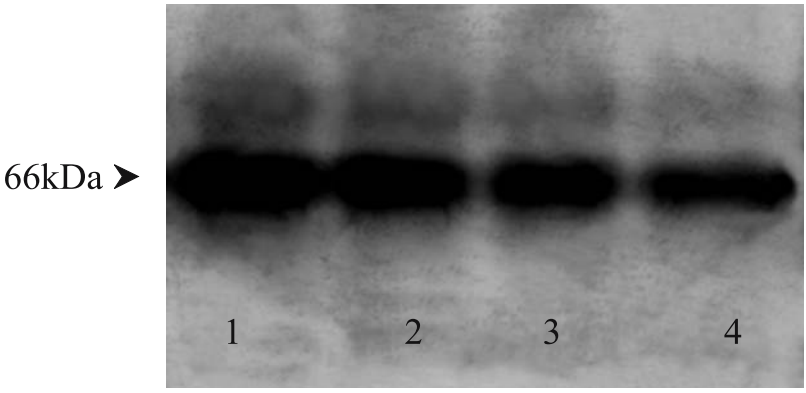

Fig. 15.2 Western blot analysis of *P. indica* and *S. vermifera* sensu stricto. Protein separated by 10% SDS PAGE transferred to nitrocellulose membrane by electroblotting. The blot were blocked using 5% bovine serum albumin and reacted with acid phosphatase antiserum and peroxidase conjugated secondary antibody, visualized using DAB. *Lanes 1, 2* Cytoplasmic fraction (CF; *lane 1*) and wall membrane fraction (W/MF; *lane 2*) of *P. indica* reacted with homogenous antiserum. *Lanes 3, 4* CF (*lane 3*) and W/MF (*lane 4*) of *S. vermifera* sensu stricto cross-reacted with *P. indica* antiserum. The result shows precisely defined bands in all samples. All blotted bands represent similarities in their molecular mass, supporting immunologically highly related species

15.3 General Protocol for RAPD Technique to Show Polymorphism

1. Equipment: thermal cycler, gel electrophoresis apparatus, band analysis software, UV transilluminator and gel documentation system. Caution: UV rays are dangerous. Protect eyes with a plastic shield.
2. Reagents (all the chemicals, primers and enzymes were obtained from Operon Technology): DNA isolation buffer (Moller et al. 1992), 2% hexadecyltrimethyl ammonium bromide (CTAB), NaCl (1.4 M), EDTA (20 mM), Tris HCl (100 mM), chloroform:isoamylalcohol (20:1), isopropanol, sodium acetate, ethanol (70%), Tris EDTA (TE, pH 8.0), Tris-HCl (pH 8.0, 10 mM), EDTA (pH 8.0, 1 mM), DNA amplification mixture for PCR, RNase A, loading buffer, bromophenol blue (0.25%), sucrose in water (40%, w/v; store in small aliquots at 4 °C), primers (short oligonucleotide), ethidium bromide (caution: ethidium bromide is a powerful mutagen; wear gloves and masks when handling and weighing). Note: all buffers, pipette tips and Eppendorfs should be sterilized at 121 °C for 15 min. Sterilize by autoclaving at 15 psi (ca. 103 kPa) for 15 min.
3. DNA amplification mixture for PCR (25 μl; Operon Technologies, Alameda, Calif.): 10× buffer (2.5 μl), $MgCl_2$ (2.5 μl), dNTPs (10 mM; 0.8 μl), primer (30 ng/μl; 1.0 μl), Taq polymerase (3 units/μl; 0.5 μl), template DNA (1 μl), Milli Q water (ultrapure, 16.7 μl).

15.3.1 Experimental Procedures

15.3.1.1 DNA Extraction

1. Carry out isolation and purification of fungal DNA following the modified CTAB protocol of Moller et al. (1992).
2. Inoculate flasks containing 100 ml Hill and Kafer medium with axenic culture of *P. indica* and place in a growth chamber at 28 °C for 6–8 days. Collect the hyphae by filtration. Keep the mycelial network at –20 °C.
3. Grind the freeze-dried mycelia (5 g) using liquid nitrogen and transfer the powdered mycelium into Eppendorf tubes (2 ml). Add equal amounts of pre-warmed isolation buffer (2% CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl) as fast as possible and incubate for 30 min at 60 °C in a water bath. Gently mix after every 10 min. Add one volume of chloroform: isoamyl-alcohol (24:1).
4. Cap the tubes and shake for 10 min by hand. Mix gently but thoroughly to ensure emulsification of the phase.
5. Centrifuge the emulsion for 10 min (15 000 rpm at room temperature). Extract the upper aqueous phase with fresh chloroform isoamyl alcohol and transfer the final aqueous phase to a new Eppendorf tube using a large bore pipette.
6. After adding 0.6 vol. of ice-cold isopropanol and 0.1 vol. of sodium acetate, cap the tubes and place at –20 °C overnight and then centrifuge again at 15 000 rpm for 10 min.
7. Transfer the precipitated whitish network of DNA-CTAB complex to a new Eppendorff tube. Add washing solution (70% ethanol) and incubate for 30 min.
8. Mix gently but thoroughly by hand and centrifuge for 5 min at 8000 rpm at 4 °C. Remove residual CTAB at this step.
9. Decant the washing solution and dry the pellet at 37 °C for 3 h to ensure the removal of all parts of ethanol.
10. Add appropriate volume of 1× TE buffer and allow the pellet to dissolve at 4 °C without agitation.
11. After extraction, purify the DNA by using RNase A. Dilute the DNA in TE buffer (1×) for RAPD and store at –20 °C for further use.
12. DNA concentration can be quantified by UV spectrophotometer at 260 nm and by comparison to DNA standards by agarose gel electrophoresis.

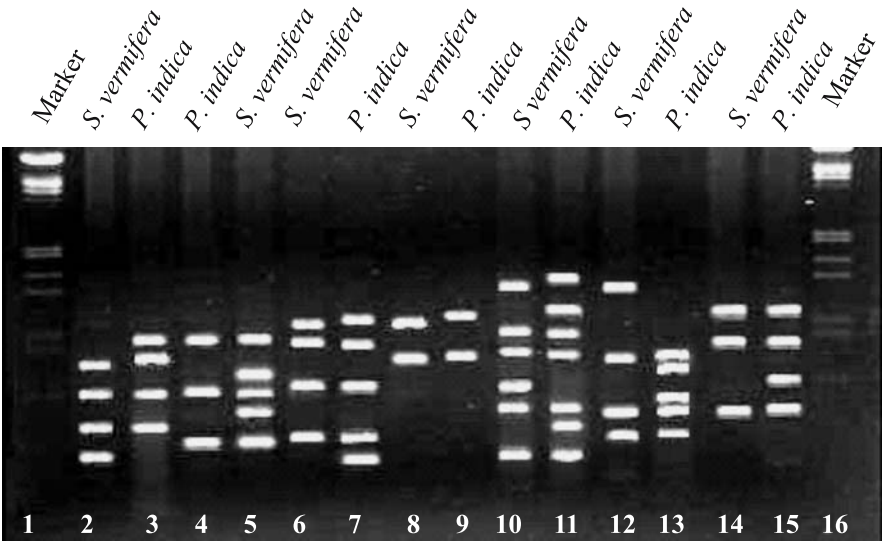

Fig. 15.3 The RAPD analysis of *P. indica* and *S. vermifera* sensu stricto to show genetic variation between these two fungi. Out of seven primers used for amplification, six have given a productive polymorphism. *Lanes 1, 16* Marker (λDNA *Eco*R1, *Hin*dIII). *Lanes 2, 3* Primer OPA10, *lanes 4, 5* OPD01, *lanes 6, 7* OPC06, *lanes 8, 9* OPC10, *lane 10, 11* OPC01, *lanes 12, 13* OPI04, *lanes 14, 15* OPI10. No polymorphism was observed when the genomic DNA was amplified with OPC10 (*lanes 8, 9*)

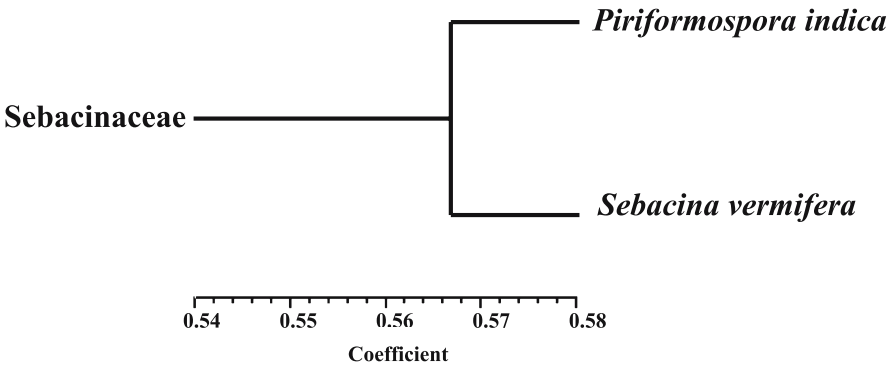

Fig. 15.4 Dendrogram showing phylogenetic relationship between *P. indica* and *S. vermifera* sensu stricto. The NTSYS-pc computer program (Numerical Taxonomy System, Applied Biostatistics) was used for data analysis

15.3.1.2 RAPD Analysis

RAPD analysis is done following Zinno et al. (1998).

1. DNA amplification is done in a total volume of 25 μl, containing 2.5 μl buffer (10× without $MgCl_2$), 2.5 μl $MgCl_2$, 0.8 μl dNTPs (10 mM), 1.0 μl primer (30 ng/μl), 0.5 μl *Taq* polymerase (3 units/μl) and DNA according to concentration use. Random 10-bp oligonucleotide primers (Operon Technologies Alameda, Calif.) are used to produce amplification: OPA10 (GTGATCGCAG), OPD01 (ACCGCGAAGG), OPC06 (GAACGGACTC), OPC10 (TGTCTGGGTG), OPC01 (TTCGAGCCAG), OPI04 (CCGCCTAGTC), OPI10 (ACAACGCGAG).
2. Each isolate is tested at a range of DNA concentrations from 0.5 μl to 2.5 μl and the clearest amplification of RAPD bands is used.
3. DNA is amplified in a PTC-200 thermal cycler (Techne, UK) with the following thermal profile: 95 °C for 5 min (initial denaturation cycle), then 36 cycles of 94 °C for 30 s (denaturation cycle), 36 °C for 2 min (annealing) and 72 °C for 2 min (extension); and a final extension at 72 °C for 5 min.
4. For separation, the amplified DNA samples are mixed with 6× loading dye and electrophoresed on 1.5% agarose gel in 1% TBE at 3.5 V/cm for 2 h, then stained with ethidium bromide and photographed under a transilluminator (Figs. 15.3, 15.4).

15.4 Troubleshooting

Only amplification products that are reproducible over two amplifications should be included. The variation in the intensity of fluorescence of different ethidium bromide-stained PCR products across the isolates was not considered for the purpose of data analysis.

15.5 Conclusions

The RAPD data confirmed that, even between these two species of Sebacinales belonging to same morpho-zymographical groups and with minor protein differences shown by 2-D PAGE, the level of variation was substantially high according to RAPD. Thus, it is suggested that such isolates should be considered as separate species. Molecular characterization offers an alternative approach for

more reliable and reproductive identification at species level. By using molecular markers like RAPDs, genetic polymorphism within species can be assayed. The use of immunological, molecular and enzymological techniques has opened an important area of research in *P. indica*. This study has opened up several novel pathways which can be explored to fill some lacunae in the molecular aspects of arbuscular mycorrhizal research, since *P. indica* is an axenically cultivable fungus mimicking various AM characters.

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