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Applications of PCR for Studying the Biodiversity of Mycorrhizal Fungi

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6.1 Introduction

Mycorrhizal fungi are a heterogeneous group of about 6000 species belonging to the *Zygo*-, *Asco*- and *Basidiomycotina*. They are grouped into a number of different types depending on their morphological relationships with the host plants, which comprise about 240,000 plant species. Mycorrhizal fungi occupy different niches during their life cycle: they reside in the rhizosphere as spores, hyphae and propagules, they occupy the rhizoplane during their interaction with the root, and finally develop inside the root tissues during the symbiotic phase (Bianciotto and Bonfante, 1998). Despite the diversity of the taxa involved, mycorrhizal fungi share substantial features. They live in close association with the roots and accomplish their life cycle due to the establishment of symbiotic relationships. During the interaction, a bidirectional transfer of mineral nutrients and carbon occurs, ensuring a continuous flow of nutrients between the partners (Smith and Read, 1997). There is extensive literature on the molecular, cellular and physiological aspects of mycorrhizal fungi (Allen 1992; Gianinazzi and Schuepp, 1994; Bonfante and Perotto, 1995; Martin *et al.*, 1995; Harrison, 1997). The positive effects of mycorrhizal fungi on plant nutrition, health and soil stability have valuable agro-biotechnological importance for low-input agriculture. However, previous knowledge of the biological diversity of mycorrhizal fungi in the rhizosphere is important to the potential exploitation of these fungi in agro-biotechnological systems. For example, the physiological traits and effectiveness of mycorrhizal fungi differ widely, depending on their taxonomic position and (at a lower rank) on the individual isolate. PCR-

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based techniques have been used to provide molecular tools for the identification of both endo- and ectomycorrhizal fungi when their morphological characters are ambiguous or missing. They have also been used to examine relations between closely related species and populations of a single species, a level of resolution usually beyond the reach of classic morphological studies.

6.2 Molecular Tools to Study Biodiversity

According to Bruns and Gardes (1993), the ideal region of choice for PCR amplification should be: (i) present in all fungi of interest; (ii) easy to amplify; (iii) preferentially amplified from fungi, when plant and fungal DNAs are mixed; and (iv) variable enough to enable probes to be designed for several taxonomic hierarchies (species, genera, families). Genes coding for ribosomal RNA (rDNA) satisfy many of these criteria, and have been extensively analysed. Repeated copies of rDNA genes are present in the genome of both prokaryotes and eukaryotes. They are highly conserved and composed of different regions (Fig. 6.1). The ribosomal coding regions are the best conserved, the internal transcribed spacers (ITS) display a certain degree of variation, and the intergenic spacers (IGS) are the most variable regions. Amplification of these regions is normally followed by restriction fragment length polymorphism (RFLP) analysis of the PCR-generated fragments.

Other PCR-based techniques have also been used to study the biodiversity of mycorrhizal fungi. Random amplification of polymorphic DNA (RAPD) is based on the use of short oligonucleotides of random sequence. This technique has proved to be a powerful tool in revealing genetic differences between closely related organisms, and has been extensively used in ecological studies to investigate the biodiversity of natural populations (Hadrys *et al.*, 1992; Foster *et al.*, 1993).

PCR amplification of regions containing microsatellites has been less commonly used in mycorrhizal research. Microsatellites are a special class of tandem repeats that involve a base motif of 1–10 bp repeated up to 100 times (Tautz, 1993) and dispersed over many genomic loci. They are present in eukaryotic genomes and have been used in individual identification, parentage testing, population genetic studies, genome mapping and in the screening

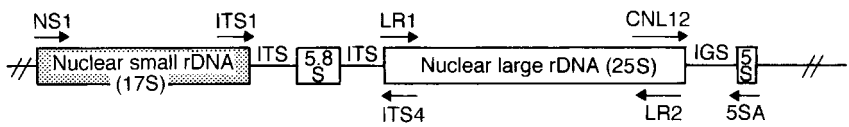

Fig. 6.1. Scheme showing a rDNA repeat. The positions corresponding to the annealing sites for several primers are shown by arrows.

of genomic libraries. Their exceptionally high mutation rate makes microsatellites very informative for determining relationships between closely related species (Bowcock *et al.*, 1994; Goldestein and Clark, 1995).

The aim of this chapter is to review the recent literature on PCR techniques applied to mycorrhizal symbionts, and to discuss their implications in soil microbial ecology.

6.3 PCR-based Methods to Investigate Population Dynamics of Ectomycorrhizal Fungi

More than 5000 species of ectomycorrhizal (ECM) fungi are associated with the secondary roots of Gymnosperms and Angiosperms (Molina *et al.*, 1992). One of the major objectives of current research is to understand the structure and dynamics of these fungal communities (Dahlberg and Stenlid, 1995). ECM fungi in fact display a great diversity even in small monoculture forests, which are typically occupied by 20–35 species (Bruns, 1995). Prior to the introduction of PCR methods, community studies focused on the presence of sporocarps with the assumption that their production reflected the abundance of symbiotic mycelia (Dahlberg and Stenlid, 1995). The presence of ECM fungi can also be evaluated through their morphological identification on mycorrhizal root apices: however, caution should be used because the extent of intraspecific morphological variations between taxa and in the same taxon on different hosts or in different environments is not well known (Egger, 1995). The PCR methods have advantages when applied to any stage in the life cycle of a mycorrhizal fungus including fruiting bodies, mycorrhizas, extraradical mycelia and isolated mycelia growing *in vitro*.

The nuclear and mitochondrial genes encoding structural rRNAs have been used to identify ECM fungi (Gardes *et al.*, 1991). Bruns and Gardes (1993) designed probes by the alignment of partial sequences of the mitochondrial large subunit rRNA gene (mt-LSU), which were specific for several taxa within the suilloid group of the *Boleta*. The probes were targeted at *Suillus*, *Rhizopogon*, and *Gomphidius*, and their specificity was determined by testing their ability to hybridize to PCR amplified fragments from 84 basidiomycete species. The probes were mostly useful in the identification of suilloid fungi at the generic level.

The original universal primers designed by White *et al.* (1990) to amplify the nuclear ITS region have been followed by primers for the amplification of specific taxa. Gardes and Bruns (1993) designed two taxon selective primers intended to be specific for fungi (ITS1-F) and Basidiomycetes (ITS4-B) respectively, though they can often amplify plant DNA. One limitation in the use of the ITS region is that the level of intraspecific variability is not uniform for all species. On the other hand, the ITS region is, in general, sufficiently variable to allow the clear discrimination between distantly related species and related genera. The increasing number of available

sequences of ribosomal genes has also allowed the construction of phylogenetic trees. For example Kretzer *et al.* (1996) have revised the genus *Suillus* by analysing 38 isolates belonging to many species. On this basis, they proposed a phylogenetic tree and suggested interesting relationships between the EMF and their host plants.

The development of PCR-based protocols has initiated many programmes for the investigation of the extent of biological diversity in field conditions. In a study carried out in a Norwegian spruce forest in Sweden, Erland (1995) used the universal primers ITS1 and ITS4 to amplify the ITS region from DNA extracted from mycorrhizal tips. Five major RFLP patterns were found from the amplified products and one was identified as typical of *Tylospora fibrillosa*. This fungus was present in at least 21% of the mycorrhizal roots tested, and was regarded as one of the most abundant mycorrhizal fungi. Similarly, Karen *et al*. (1996) compared RFLP patterns of ITS products amplified from mycorrhizal roots with those obtained from fruiting bodies of reference species. In this investigation, the fruiting body inventory indicated that the clear-cut planted forest had the lowest diversity and species richness, despite the fact that it showed the highest number of fruiting bodies. A forest of 150- to 200-year-old trees produced less fruiting bodies, but examination of mycorrhizal tips revealed several species which did not form epigeous fruiting bodies. In conclusion, the results suggested that forestry management affects fruiting body diversity more than mycorrhizal diversity (Karen *et al.*, 1996). Another interesting study of natural ECM populations was performed by Gardes and Bruns (1996). They examined the species diversity of a community in a forest of *Pinus muricata* in order to determine the above- and below-ground correspondence, in terms of species composition, spatial frequency and abundance. ITS-RFLP, taxon-specific oligonucleotide probes, and sequence analysis were applied to fruiting bodies and mycorrhizal tips sampled over a 4-year period. Over 45 fungal species were identified and grouped according to three patterns: (i) some, such as *Russula xerampelina*, were well represented both above and below ground; (ii) some, such as *Suillus pungens*, were mostly found as epigeous fruiting bodies; (iii) some, such as *Russula amoenolens* were not represented in the above-ground species. These results demonstrated that ECM fungal species change their resource allocation towards the production of either fruiting bodies or ectomycorrhizae in the same environment. Moreover, it is clear that there is no correspondence between above- and below-ground fungal structures (Gardes and Bruns, 1996).

The fate of an introduced ECM strain has been followed in a long-standing study by the group of Martin and Le Tacon, who monitored the presence of *Laccaria bicolor* strain S238N, an isolate known to improve the growth of Douglas fir and Norwegian spruce in nurseries. To evaluate the competitivity and persistence of the isolate after transplantation, the mycotrophic status of the forest trees was examined morphologically and determined by ribotyping between 1 and 10 years after planting out. *Laccaria*

was present in 90% of the mycorrhizal tips after 1 year, but in only 3% after 4 years, since it had been widely replaced by local strains. However, in other forest sites, *Laccaria* persisted for at least 10 years. These results showed that the destiny of an inoculated strain depends on the host plant, the presence of competitive local strains, and/or the climatic conditions (Henrion *et al.*, 1994b; Di Battista *et al.*, 1996; Selosse *et al.*, 1996).

In southern Europe, truffles have been the subject of intense application studies since the 1970s due to the outstanding commercial value of some species (Chevalier, 1994). Identification of truffles during their symbiotic phase is one of the major topics in this kind of research. The fruiting bodies are usually identified on the basis of the structure of the peridium and gleba, size and shape of their spores and asci, and their wall ornamentation. However, these features are lost when the hyphae of the mantle and Hartig net develop on the plant root. Many fruiting bodies have been easily identified with primers which amplify the ITS regions (Henrion *et al.*, 1994a; Lanfranco *et al.*, 1995a; Paolocci *et al.*, 1995), whereas many difficulties remain when mycorrhizal roots are considered. ITS primers often amplify plant DNA and, therefore, can give rise to complex patterns (Mello *et al.*, 1996).

Strategies can be developed to allow the exclusive amplification of fungal DNA from species of interest. For example, sequences of ITS fragments amplified from fruiting bodies have been analysed in order to design specific primers which permit the exclusive detection of the fungus of interest (Mello *et al.*, 1997) (Fig. 6.2). These specific sequences generally work at the species level, but only when the degree of intraspecific variation of the ITS region is low, as has already been demonstrated for some truffle species (Guillemaud *et al.*, 1996).

PCR amplification of single copy genes is an alternative approach. For example, Kreuzinger *et al.* (1996) have successfully used this method on a 1.2 kb fragment of the gene encoding glyceraldehyde-3-phosphate dehydrogenase. The amplified fragments from *Boletus*, *Lactarius*, and *Amanita* were distinguished by RFLP analysis and Southern blot hybridization, while fluorescent DNA probes could easily be used in slot-blot experiments. This approach is an original way for quickly developing inexpensive probes to detect ECM fungi of ecological and economic value.

6.4 Arbuscular Mycorrhizal Fungi: from the Development of Specific Probes to Ecological and Genetic Analysis

Arbuscular mycorrhizal (AM) fungi are obligate endosymbionts that colonize the roots of almost 80% of land plants. They belong to the Order *Glomales* (Morton and Benny, 1990), and consist of least 150 species, usually identified by their spore morphology (Walker, 1992). Simon *et al.* (1992, 1993a, b) were the first to apply PCR techniques to AM fungi in a study of

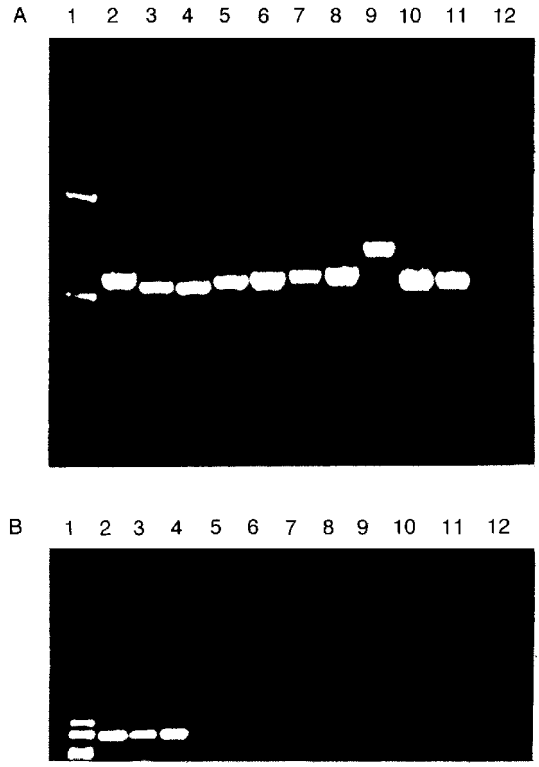

Fig. 6.2. Design of specific primers for *Tuber* species. (A) Amplification products with ITS1 and ITS4 primers on DNA extracted from the fruiting bodies of ten *Tuber* species. Lanes: 1, pBR 322 digested with *Hin*fI; 2, *T. magnatum*; 3, *T. borchii*; 4, *T. maculatum*; 5, *T. melanosporum*; 6, *T. macrosporum*; 7, *T. rufum*; 8, *T. aestivum*; 9, *T. brumale*; 10, *T. excavatum*; 11, *T. ferrugineum*; 12, no DNA. (B) On the basis of the ITS sequence, specific primers were designed for *T. borchii* and used to amplify a single band of 432 bp from fruiting bodies (lane 2), ectomycorrhizae (lane 3) and mycelium (lane 4). No amplification is obtained from other truffle species (lanes 5–11).

the nuclear gene encoding the small subunit rRNA. From the complete 18S sequence of two AM species (*Glomus intraradices* and *Gigaspora margarita*), they designed a primer (VANS1) that only amplified *Glomales* species, when used in combination with universal primers. This combination has successfully amplified the fungal DNA in mycorrhizal roots colonized by *Glomus vesiculiferum* (Simon *et al.*, 1992). When more 18S sequences from other AM species became available, family specific primers were also designed and used on mycorrhizal roots. The amplified products were then subjected to single-strand conformational polymorphism (SSCP) analysis to detect sequence differences (Simon *et al.*, 1993b).

The sequence data of the small ribosomal subunit from 12 isolates of

glomalean fungi have been used to map the phylogenetic relationships between AM fungi (Simon, 1996). The results confirmed the validity of the taxonomic grouping and allowed Simon *et al.* (1993a) to construct a molecular clock, by estimating the divergence times between glomalean families and genera.

The development of molecular markers for the identification of individual spores has been an important step for investigating species diversity of AM fungi in the rhizosphere of natural communities (Clapp *et al.*, 1995; Sanders *et al.*, 1995, 1996; Dodd *et al.*, 1996). Sanders *et al.* (1995) developed a protocol for PCR amplification with the universal primers ITS1 and ITS4, followed by RFLP to characterize the ITS region from single AM fungus spores. The ITS region, which is approximately 600 bp long, showed clear differences between species only after RFLP; the restriction patterns were reproducible for individual spores of a given species. However, when the technique was applied to spores from a Swiss grassland, the results were much more complex: ten individual spores belonging to the same morphological *Glomus* group produced ten different patterns, suggesting a high genetic diversity between individual spores.

Clapp *et al.* (1995) used PCR and genus-specific primers designed from 18S rDNA to investigate the fungal composition of mycorrhizal roots collected in the field. They introduced the selective enrichment of amplified DNA (SEAD) technique, based on the principle of subtractive hybridization, to remove interfering plant-derived DNA. Three genera of AM fungi, *Acaulospora*, *Scutellospora* and *Glomus*, were detected in bluebell roots. While the presence of the first two genera was expected on the basis of the spores found in the soil around the roots, *Glomus* spores were very rare in the rhizosphere, suggesting that the presence of an endophytic fungus does not always correlate with the presence of propagules.

Alternative PCR-based strategies have been devised to identify AM fungi during the sporal and symbiotic phases. Short arbitrary oligonucleotides were used as primers for the amplification of DNA extracted from spores of AM fungi in RAPD experiments (Wyss and Bonfante, 1993; Lanfranco *et al.*, 1997). RAPD analysis is a very sensitive tool for the detection of genetic differences between individuals. A non-polymorphic band was identified as a marker for eight isolates of *Glomus mosseae*. The fragment (about 650 bp long) was cloned and sequenced, and a pair of specific primers were designed (Lanfranco *et al.*, 1995b). These specifically amplified not only the DNA from *G. mosseae* spores, but also from roots of pea, clover, leek and onion plants when they were colonized by *G. mosseae* isolates. In addition, they allowed *G. mosseae* to be distinguished from the closely related species *G. coronatum*: interestingly, the two species were not distinguishable by ITS amplification techniques (Fig. 6.3) (Dodd *et al.*, 1996).

Highly repeated sequences are also widespread in the fungal genome. Their presence has been demonstrated in AM fungi by two approaches:

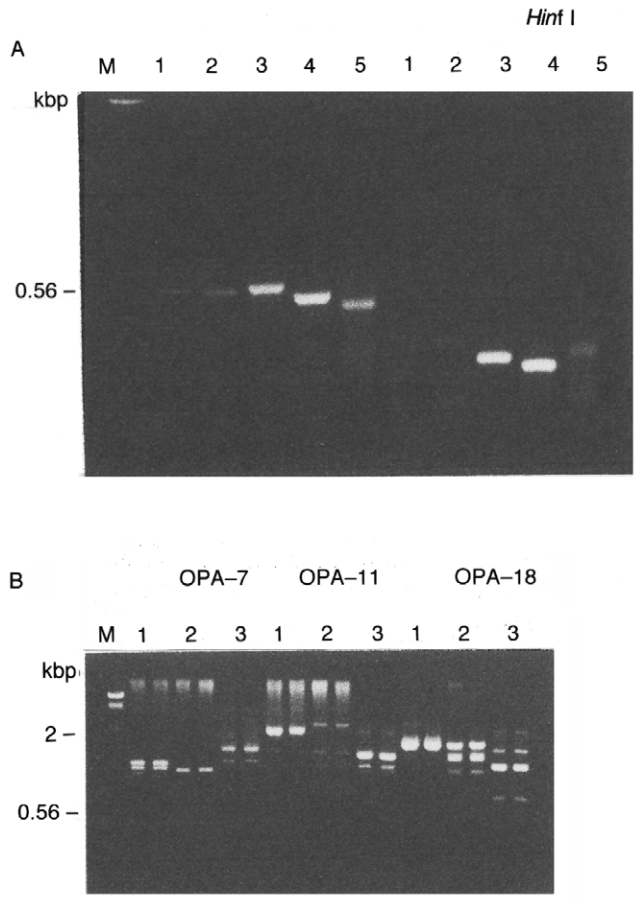

Fig. 6.3. Genome analysis on AM fungi. (A) Amplification products with ITS1 and ITS4 primers on DNA extracted from AM fungi. A single band of about 500–560 bp was obtained when the following isolates were investigated: 1, *Glomus coronatum*; 2, *G. mosseae*; 3, *G. versiforme*; 4, *G. flavisporum*; 5, *Gigaspora margarita*. Different patterns were obtained after RFLP analysis with *Hin*fI. (B) RAPD analysis performed using the primers OPA-7, OPA-11 and OPA-18 (Operon Technologies, USA) on: 1, *Glomus coronatum*; 2, *G. mosseae*; 3, *Gigaspora margarita*. Highly polymorphic patterns, which allow a clear separation between *G. coronatum* and *G. mosseae* were obtained. M, λ DNA digested with *Hin*dIII and *Eco*RI (from Dodd *et al.*, 1996 by permission of *New Phytologist*, Cambridge University Press).

Longato and Bonfante (1997) have used primers designed on microsatellite sequences, such as $(CT)_8$, $(GACA)_4$, $(TGTC)_4$, to obtain species-specific amplification profiles. This approach has proved to be a reliable and technically simple method for assaying genetic variability. Zézé *et al.* (1996) have isolated a highly repeated sequence from a partial genomic library of

Scutellospora castanea. This DNA fragment has been shown to be specific for this species by Southern blot hybridization, and by PCR assays with oligonucleotides derived from the sequence.

Molecular markers targeted to the ribosomal genes have provided new information. Sanders *et al.* (1995) have shown that a single spore of *G. mosseae* contains at least two different ITS sequences. Moreover, a third ITS variant has been found by Franken and Gianinazzi-Pearson (1996) by sequencing a ribosomal clone from a genomic phage library of *G. mosseae*. Lloyd-MacGilp *et al.* (1996) have made it clear that ITS heterogeneity is normal within *Glomus* spores. They obtained two sequences from single spores of three *G. mosseae* isolates and one isolate of *G. dimorphicum*, and three sequences from a single *G. coronatum* spore. Interestingly, the sequence divergence between isolates from distant continents is scarcely greater than that within single spores. The authors concluded that the sequence variants have evolved over a long time-scale relative to the rate at which these fungi spread across the world.

Some recent data obtained from the *Gigasporaceae* have shown a similar situation for this family. Three ITS sequences have been identified by cloning the amplified ITS fragment from *Gigaspora margarita* (L. Lanfranco, unpublished work), although it should be noted that these were obtained from a multispore DNA preparation. More compelling is the finding of different 18S sequences in a single spore of *Scutellospora* collected in an oak woodland in the north of England (J.P. Clapp, unpublished work, cited by Sanders *et al.,* 1996). These results show a high level of genetic variability in genes belonging to the same genome (Sanders *et al.*, 1996) and in organisms thought to be asexual. The many questions they raise have been discussed in detail by Sanders *et al.* (1996) who suggest that the multinucleate spores and hyphae of Glomales may be the products of heterokaryotic processes, and the variability of ITS sequence may be related to asexual reproduction.

In conclusion, PCR-based molecular markers for the identification of AM fungi allow both the investigation of their biodiversity in the rhizosphere and within the roots, and the elaboration of a phylogenetic framework. These methods have also opened the way to an understanding of AM fungal genetics.

6.5 PCR-based Methods to Analyse the Complexity of Ericoid Mycorrhizal Fungal Populations

Ericaceous plants associate with soil fungi to form a distinctive type of mycorrhiza, termed ericoid mycorrhiza (Perotto *et al.*, 1995). The specific features of this symbiosis make ericoid mycorrhizal plants successful in colonizing low-mineral, acidic organic soils high in toxic metal ions, where a crucial role in plant nutrition has been ascribed to the saprotrophic capabilities of the mycorrhizal endophyte. The endophytes isolated so far

belong to the ascomycetes, although basidiomycetes have been observed by electron microscopy inside naturally colonized roots (Bonfante, 1980). Few fungal species have been identified as partners of this mycorrhiza: *Hymenoscyphus ericae* was the first (Read, 1974), and several *Oidiodendron* species have also been demonstrated to be mycorrhizal in several parts of Europe and North America (Couture *et al.*, 1983; Dalpé, 1986; Douglas *et al.*, 1989; Perotto *et al.*, 1996a). However, the taxonomic position of most isolates is unknown, because they do not form identifiable reproductive structures. Isolates of *H. ericae* are usually hard to identify, as they often grow as sterile mycelia in pure culture.

Investigation of the genetic diversity of ericoid fungi has greatly benefited from PCR techniques as a means of overcoming the difficulties of morphological identification. By coupling molecular methods and traditional taxonomy, new tools have been provided for the study of ericoid mycorrhizal fungi. PCR–RFLP analysis of different regions of the ribosomal genes has been used to investigate the identity and diversity of ericoid fungi. Examination of the small subunit of the ribosomal genes of the hyphomycete *Scytalidium vaccinii* revealed its close taxonomic relationship with *H. ericae* (Egger and Siegler, 1993), while RFLP analysis of the ITS region amplified from mycelia colonizing *Calluna vulgaris* roots has demonstrated that the root of a single plant harbours several populations of mycorrhizal and non-mycorrhizal fungi (Perotto *et al.*, 1996a). Investigation of mycelia isolated from *Gaultheria shallon* roots (Monreal *et al.*, 1996) has also revealed the contemporary presence of fungi with different ITS restriction patterns.

RAPD analysis has enabled a higher resolving power to be used in the investigation of the genetic polymorphism of isolates sharing the same ITS-RFLP pattern (Perotto *et al.*, 1996a). Results of PCR amplification with about ten random primers on about 80 mycorrhizal mycelia isolated from *C. vulgaris* growing in five neighbouring sites have shown a high polymorphism in *Oidiodendron maius* isolates, even those derived from the same plant, and a lower variability within populations of sterile mycelia (Fig. 6.4). These data indicate that the root system of a single plant of *C. vulgaris* is a complex mosaic where several populations of mycorrhizal fungi coexist, each represented by a variable number of genetically distinct individuals (Perotto *et al.*, 1996a). The ecological significance of this promiscuous association is not clear. However, several ericoid isolates produce specific isoforms of extracellular enzymes useful for nutrition *in vitro* (Perotto *et al.*, 1997). Thus, an association with several fungi may be a way of broadening the metabolic capabilities of mycorrhizal roots in the exploitation of difficult substrates.

Analysis of ribosomal genes in ericoid fungi has revealed an unusual feature in their organization in many isolates. Amplification using universal primers designed to both the 18S and the 28S subunits has yielded DNA fragments which were often much larger in size than expected (Egger *et al.*, 1995; Perotto *et al.*, 1996b). Sequencing of these fragments has revealed that this discrepancy was due to the insertion of group I introns.

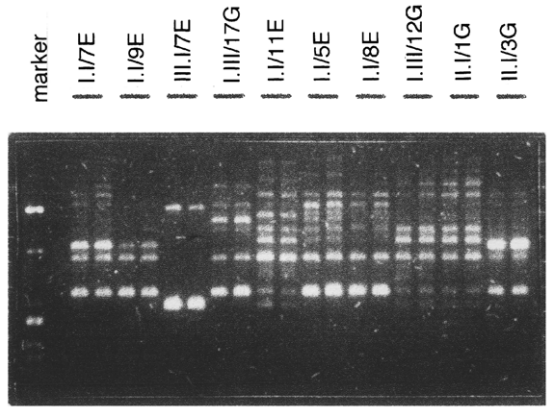

Fig. 6.4. DNA fingerprints of *Oidiodendron maius* isolates after PCR–RAPD amplification using short arbitrary primer OPA-14. Isolates may be divided into several groups on the basis of their DNA banding.

Group I introns have four conserved regions involved in the formation of secondary structures that are important for splicing. These elements are quite rare in the nuclear ribosomal genes of eukaryotes. They occur sporadically in a few fungal species and in algae, but their role has not been elucidated (Johansen *et al.*, 1996). Interestingly, they are abundant in the polyphyletic group of lichen-forming fungi (Gargas *et al.*, 1995).

The distribution and sequence of group I introns are highly variable in ericoid fungi. One intron element inserted in the region towards the 3′ end of the small ribosomal subunit in *H. ericae* is well characterized (Egger *et al.*, 1995) and a number of additional insertions have been found in this and other ericoid fungal isolates (Perotto, unpublished work), suggesting that this may be a common feature. Although their role is not known, introns certainly increase the genetic diversity of ericoid fungi.

In conclusion, PCR-based techniques are a powerful tool for the study of ericoid fungi: the identification of isolates through specific fingerprinting methods is quickly overcoming the lack of morphological characters often encountered with these fungi.

6.6 The Interactions between Mycorrhizal Fungi and Endosymbiotic Bacteria

External hyphae of mycorrhizal fungi encounter many other soil micro-organisms in the rhizosphere, and a network of interactions is created (Azcon-Aguilar and Barea, 1992). PCR has also been of great assistance in this field, particularly in the investigation of the interactions between

mycorrhizal fungi and bacteria. The surface of both ECM and AM fungi can be colonized by soil bacteria that use the hyphae as a specific substrate (Bianciotto *et al.*, 1996a; Schelkle *et al.*, 1996). Furthermore, the cytoplasm of AM fungi harbours structures called bacteria-like organisms (BLO). Identification of these bacterial endosymbionts has been hampered because they cannot be grown on cell-free media. Amplification and partial sequencing of the 16S rDNA region of endosymbiotic bacteria, followed by database searches for sequence similarity and phylogenetic analyses, have now unambiguously placed the symbiont of *Gigaspora margarita* among the pseudomonads of rRNA group II (Bianciotto *et al.*, 1996b). The endosymbiont was identified as a member of the genus *Burkholderia*. The sequence of the gene coding for the small subunit rRNA has been used to design two primers (BLOf and BLOr) specific for the *Burkholderia* endosymbiont of *Gi. margarita*. These primers were tested for their specificity on a number of free-living bacteria, and then used on resting spores, external mycelium and clover roots mycorrhized with *Gi. margarita* in order to follow the fate of BLOs during the fungal life cycle. Spores of different origins belonging to *Glomaceae*, *Gigasporaceae* and *Acaulosporaceae*, were analysed by confocal microscopy using a fluorescent dye specific for the visualization of bacteria to determine whether other AM fungi possess bacterial endosymbionts. In addition, PCR amplifications with eubacterial primers (704f/1495r) and with BLOf/BLOr were performed on DNA preparations from spores of different isolates. All spores, with one exception, contained intracellular bacteria, although their number and shape greatly differed among the different species. In addition, the BLOf/BLOr primers were found to amplify only a DNA fragment from *Gigasporaceae* spores (Bianciotto *et al.*, 1996c).

In conclusion, these experiments suggest that intracellular bacteria are not a sporadic phenomenon but may be a general feature of AM spores. Their presence raises many questions about their role in mycorrhizal functions and about evolutionary processes and it has been suggested that they may be organelle precursors (Holzman, 1996).

6.7 Areas that Need Further Developments

Despite the huge progress of recent years, many problems remain unresolved; most of these are of a technical nature. For example, it is important to improve the level of amplification for samples collected in the field; at present this level is unsatisfactory (Harris, 1996). This is probably caused by the presence of inhibitory substances.

Another important point that has rarely been tackled is the quantification of DNA in the samples. Simon *et al.* (1992) were the first to propose a protocol for estimation of the amount of AM fungi living inside the plant roots by amplifying the sample with different concentrations of an internal standard.

Edwards *et al.* have developed an alternative protocol and tested the possibility of evaluating the effect of rhizosphere bacteria on the development of AM fungi (S.G. Edwards, A.H. Fitter and J.P.W. Young, 1997, personal communication). A more precise scenario of the competitive colonization of roots by mycorrhizal fungi will only be available when it is possible to quantify the presence of one fungal genome with respect to another.

6.8 Conclusions

The development of PCR-based techniques has allowed us to better define the presence and role of mycorrhizal fungi in the rhizosphere, which is a complex niche where microorganisms develop and interact with each other. Because of PCR-based techniques (Fig. 6.5), many crucial questions about phylogenesis, identification and polymorphisms of mycorrhizal fungi have begun to be answered. For example, the extent of diversity in natural populations has been probed and shown to be greater than previously thought. Several symbiotic fungi are often present, not only in the same environment, but on the same root irrespective of its size. The thin roots of *Ericales* are a good illustration of this phenomenon. The dynamics of the resident fungal population have also been directly evaluated, as well as the fate of introduced strains selected for their physiological properties. It is clear that the ability of these strains to survive depends on the environment, as well as on their interactions with other soil microorganisms.

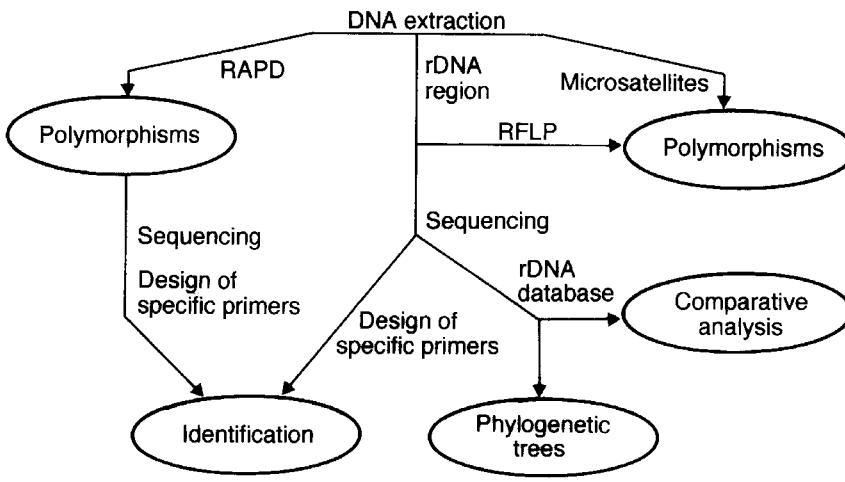

Fig. 6.5. A flow chart showing the most important PCR-based strategies that have been used to investigate the genome of mycorrhizal fungi.

The genetics of mycorrhizal fungi is still in its infancy, particularly in the case of AM fungi, which are intractable organisms due to their obligate biotrophic nature. PCR-based techniques have allowed us to investigate these organisms inside the roots, to elaborate a phylogenetic framework and to give an insight into their genetics for the first time. Molecular tools have revealed an unexpected level of variability in the ribosomal genes in individual isolates. The discovery of several introns in the ribosomal genes of ericoid mycorrhizal fungi suggests similar conclusions, as such features are also a source of genetic variability.

Further technical developments are still needed to solve specific problems in mycorrhizal research. However, despite these current limitations PCR-based techniques have so far provided an invaluable contribution to the definition of the mycorrhizal fungal dimension in the rhizosphere.

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