Chapter 18
Morphotyping and Molecular Methods to Characterize Ectomycorrhizal Roots and Hyphae in Soil

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18.1 Introduction

At the interface between plants and soils, ectomycorrhizal (ECM) fungi explore soils, acquire resources, transfer resources to plants, and acquire carbon from plants. Mycorrhizas enhance plant survival, nutrition and growth and play key roles in ecosystems processes such as decomposition, nutrient cycling, soil carbon storage, productivity and sustainability. Mycorrhizas are critical for plant colonization of new soils (e.g. mine spoils, volcanic deposits, glacial moraines). ECM diversity ensures plant reestablishment after disturbance and can enhance survival and growth of trees in reforestation. ECM fungi can promote fine root development as well as produce antibiotics, hormones and vitamins. Mycorrhizal associations may help protect roots from pathogens and moderate effects of heavy metals and toxins. Many environmental problems may be alleviated by mycorrhizas – problems such as pollution, erosion, soil degradation, climate change, degradation of natural resources, and poor land use management.

Mycorrhizal abilities to carry out important functions are linked to diversity. ECM diversity is large and documented in many ecosystems, particularly coniferous ecosystems (Gehring et al. 1998; Goodman and Trofymow 1998; Kranabetter and Wylie 1998; van der Heijden et al. 1998; Stendell et al. 1999; Bidartondo et al. 2000). This ECM diversity has been based on surveys of fruiting bodies, but is now based on more recent methods – morphotyping (microscopic observations) and phylotyping (molecular characterization). An advantage of fruiting body surveys is ease of collection and identification based on morphology; a disadvantage is the assumption that fungi fruiting in an area also form ectomycorrhizas on nearby roots. Clearly identification of ectomycorrhizas on roots is preferable. However there are difficulties in ECM identification – complex sampling design and

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extraction of roots from soils. We describe two complementary methods – morphotyping and phylotyping. This chapter focuses on these two methods, because they are effective and allow ECM fungi to be identified to genus and species. Use of both methods capitalizes on the benefits of each, while minimizing the disadvantages. Both methods are time consuming, although morphotyping requires time at a microscope while phylotyping requires time at a lab bench. Morphotyping is relatively inexpensive, but requires training to recognize key microscopic features. Morphotyping may not allow identification to genus or species in some cases. Phylotyping requires more expensive molecular reagents and materials but often allows identification of ECM root tips to genus and species.

18.2 Background

18.2.1 Taxa Forming ECM

ECM fungi form ectomycorrhizas with many woody plants such as plants in the Pinaceae, Fagaceae, Betulaceae, Myrtaceae and Ericaceae. There are about 5000–6000 ECM fungal species in the Ascomycotina (Ascomycetes) and Basidiomycotina (Hymenomycetes). Ascomycetous ECM belong almost exclusively to the orders Elaphomycetales, Leotiales, Pezizales and Pleosporales (Molina et al. 1992; de Roman et al. 2005) while basidiomycetous ECM include the class Hymenomycetes, subclass Hymenomycetidae (Agerer 1987–2006; Molina et al. 1992) which comprise the orders Boletales, Gomphales, Thelephorales, Agaricales (families Amanitaceae, Cortinariaceae and Tricholomataceae), Russulales (family Russulaceae) and Cantharellales (family Cantharellaceae) (Agerer 1987–2006). ECM fungal species can have narrow, intermediate and broad host ranges (Molina et al. 1992).

18.2.2 Description of ECM Structures in Roots and in Soils

Anatomical characteristics of ECM structures are conserved at the species and genus level (Agerer 1987–2006; Agerer and Rambold 2004–2007). Among the anatomical features, there are four key complexes that distinguish the ECM structure and allow recognition of the fungus involved in the symbiosis: (a) outer mantle layers in plan view, (b) rhizomorphs, (c) shape of cystidia, and (d) emanating hyphae (Agerer 2006). Other anatomical features that may be used as diagnostic features are the Hartig net, sclerotia, chlamydospores, laticifers and contents (Agerer 1987–2006).

Mantles are hyphal sheaths around roots and can be divided into two main groups according to hyphal distribution and organization: (1) plectenchymatous and (2) pseudoparenchymatous (Agerer 1987–2006, 1991, 1995; Agerer and Rambold 2004–2007). Plectenchymatous mantles have discernible hyphae, frequently loosely

woven. Pseudoparenchymatous mantles do not have discernible individual hyphae; they have short-celled, inflated, densely packed hyphae, resembling a true parenchyma. Both mantle types include standard organizational patterns (Agerer 2006). In Fig. 18.1 the letters a–f correspond to different types of plectenchymatous mantles and the letters g–j correspond to pseudoparenchymatous mantles.

Among plectenchymatous mantles, mantle type B is considered the most primitive (Agerer 2006) and has hyphae randomly arranged with no discernible pattern (Fig. 18.1a). Mantle type A is ring-like and hyphae commonly grow together for a short distance and ramify at places where other hyphae join, forming loops (Fig. 18.1b). When loops of the joining hyphae become very massive and the connecting hyphae between the loops less distinct, mantle type A is called star-like. The ring-like mantle is well represented in the order Boletales while the star-like mantle is present in the family Bankeraceae (Agerer 1987–2006; Agerer and Rambold 2004–2007) and in some Thelephoraceae (Azul et al. 2006d) (Fig. 18.1c). Mantle type C is distinguished by the presence of a gelatinous matrix between hyphae (Fig. 18.1d), apparently as a water reservoir; hyphae are randomly distributed with no discernible pattern. Mantle type D is characterized by the occurrence of cystidia. This mantle type is used only when cystidia are present on the surface of plectenchymatous mantles, since they can be present on pseudoparenchymatous mantles surface (see Fig. 18.1i). Mantle type E is differentiated by the presence of multiramified hyphae with short, frequently y-shaped or almost rectangular branches, so-called squarrosely branched (Fig. 18.1e). Mantle type F is identified by the occurrence of inflated cells, globular terminal cells or other cells, above an undifferentiated mantle type. Mantle type G is typified by its star-like pattern, with hyphae compactly distributed and with no space between them, e.g. *Cenococcum geophilum* Fr. and *Quercirhiza flavocystidiata* (Fig. 18.1f) (Azul et al. 2006c). Mantle type H is rather similar to mantle type E and is characterized by the presence of hyphae somewhat inflated and loosely distributed, with interspaces between hyphae. Mantle type I is characterized by the presence of quite short, often slightly tortuous or irregularly bent perpendicular cells, forming a velvet-like structure on the mantle surface. These hyphal ends can be regarded as cystidia, since they are all stainable with sulfo-vanillin (Agerer 1986).

Among pseudoparenchymatous mantles there are mantles that exhibit angular to roundish cells: types K, L, O, and P (Fig. 18.1i,g) and mantles that show epidermoid, puzzle-like structures: type M and Q (Fig. 18.1h–j). Mantle type K is differentiated by the presence of angular to roundish cells sometimes arranged in rosettes. Mantle type O is differentiated by the presence of heaps of flattened cells. The majority of pseudoparenchymatous mantles bear a hyphal net, or small groups of globular or flattened cells, on the surface. Mantle type P corresponds to a pseudoparenchyma with angular cells bearing a hyphal net, and mantle type Q corresponds to a pseudoparenchyma with epidermoid cells bearing (Fig. 18.1j). Mantle type N, that has some cells containing oil droplets that stain in sulfo-vanillin (Agerer 1995), is now considered as an additional feature of mantle types O, P, and Q and not as a distinct mantle pattern (Agerer 2006). Although these standard mantle patterns can be used for recognition, there are transitions between some mantle types, making definitive attribution of a

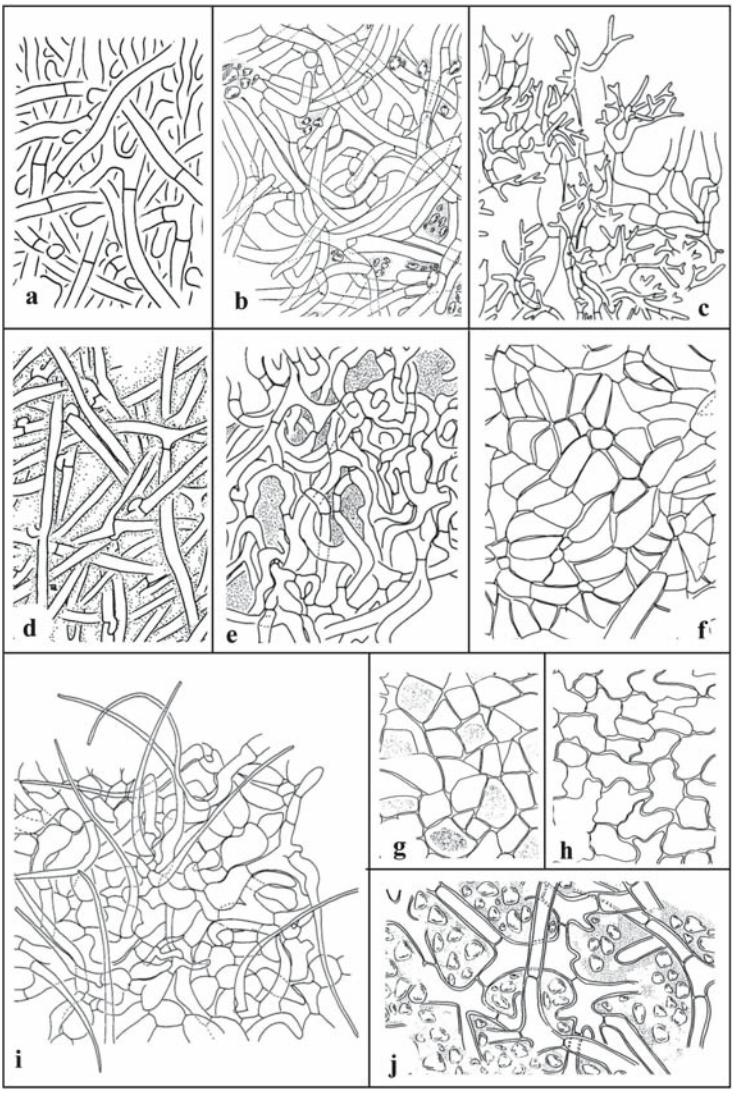

Fig. 18.1 Mantle types according Agerer 1987–2006. **a** Mantle type B: plectenchymatous, random hyphal arrangement, no discernible pattern (*Hysterangium stoloniferum*, Raidl and Agerer 1998). **b** Mantle type A: plectenchymatous, ring-like pattern (*Quercirhiza sclerotiigera*, Azul et al. 2001d). **c** Mantle type A: plectenchymatous, star-like arrangement, in this example bearing a loose, delicate hyphal net pattern (*Quercirhiza tomentellofuniculosa*, Azul et al. 2006d). **d** Mantle type C: plectenchymatous, no discernible pattern in a gelatinous matrix (*Gomphus clavatus*, Agerer et al. 1998). **e** Mantle type E: plectenchymatous, squarrosely branched hyphae (*Quercirhiza dendrohyphidiomorpha*, Azul et al. 2006a). **f** Mantle type G: plectenchymatous, star-like pattern (*Quercirhiza flavocystidiata*, Azul et al. 2006c). **g** Mantle type L: pseudoparenchymatous, angular to roundish cells (*Quercirhiza internangularins*, Azul et al. 2001b). **h** Mantle type M: pseudoparenchymatous, epidermoid cells (*Quercirhiza ectendotrophica*, Azul et al. 2001a). **i** Mantle type P: pseudoparenchymatous, angular to roundish cells with a hyphal net (*Quercirhiza aurateroocystidiata*, Azul et al. 2006b). **j** Mantle type Q: pseudoparenchymatous, epidermoid cells with a hyphal net (*Quercirhiza ectendotrophica*, Azul et al. 2001a). All ECM mantle pictures have been utilized with author's permission

pattern difficult. From a phylogenetic point of view, hyphal organization in pseudoparenchymatous mantles is considered more advanced (Agerer 1995).

Rhizomorphs are extramatrical mycelia that may grow either as simple scattered hyphae from the mantle into the soil or may form 'multi-hyphal linear aggregates', the so-called rhizomorphs (Agerer 1999). Rhizomorphs may have evolved as efficient structures for water and nutrient transport. Rhizomorphs may be characterized according to their structure and ontogeny into seven types according to their internal organization (Agerer 1999). Type A corresponds to uniform-loose rhizomorphs (Fig. 18.2a) with normal vegetative hyphae loosely distributed. Type B has uniform-compact rhizomorphs, i.e., densely agglutinated hyphae of uniform shape. Type C, thelephoroid rhizomorphs, is slightly differentiated and has one peripheral hypha, rather different in diameter and in structure (Fig. 18.2b,c). Type D, ramarioid rhizomorphs, are internally differentiated and distinct due to ampullate inflations at the hyphal septum of the internal cells (Agerer 2006). Type E, russuloid rhizomorphs, are characterized by irregularly distributed thickened hyphae, frequently with incomplete septa, accompanied by thick-walled hyphae with several septa separated by short distance. Type F, phlegmacioid rhizomorphs, have thicker hyphae, sometimes with a large septal pore, with a random distribution and often embedded in a matrix. Type G, agaricoid rhizomorphs, are highly differentiated and possesses vessel-like central hyphae with partially or even completely dissolved septa. This type has not been observed on ECM fungi. Type H, boletoid rhizomorphs (Fig. 18.2d,e), corresponds to highly differentiated rhizomorphs with central vessel-like hyphae. The internal organization of highly differentiated rhizomorphs is key to recognition and delimitation of fungal relationships (Agerer 1999). The main difference between agaricoid and boletoid rhizomorphs is related to their ontogeny. Agaricoid rhizomorphs have simple, vessel-like hyphae, while boletoid rhizomorphs have vessel-like hyphae that fork close to their origin, one branch growing toward the rhizomorph base, the other, towards the tip. For both types, vessel-like hyphae originate in early ontogenetical stages. Node-like structures and split-type hyphal ramification can be observed in the Thelephoraceae, Amanitaceae and Tricholomataceae.

Some genera have distinctive rhizomorphs that allow easy identification – *Cortinarius*, *Dermocybe*, *Sarcodon*, *Tomentella*, *Tricholoma* and *Xerocomus*. For example, *Cortinarius* and *Dermocybe* have abundant, distinctive rhizomorphs, while *Tricholoma* has diverse rhizomorphs. In the Boletales, highly differentiated "boletoid rhizomorphs" are observed in the Boletaceae, Gyroporaceae, Melanogastraceae, Paxillaceae, Rhizopogonaceae, Sclerodermataceae and Suillaceae (Agerer 2001). Unlike the Boletales, the Cortinariaceae exhibit undifferentiated or differentiated rhizomorphs, or no rhizomorphs at all. Rhizomorphs are particularly difficult to observe in some genera – *Russula*, *Lactarius*, *Hygrophorus*, or not seen in *Inocybe*, *Rozites* and *Tuber* (Agerer 1987–2006). Dimorphic rhizomorphs are less frequent in *Dermocybe crocea* (Schaeff.) M. M. Moser, *D. palustris* (M. M. Moser) M. M. Moser and *D. semisanguinea* (Fr.) M. M. Moser (Agerer 1995).

Organization and emanating hyphal structures of rhizomorphs allow categorization into different ECM exploration types: (a) contact, (b) short-distance, (c) medium-distance and (d) long-distance (Agerer 2001). The contact type, commonly

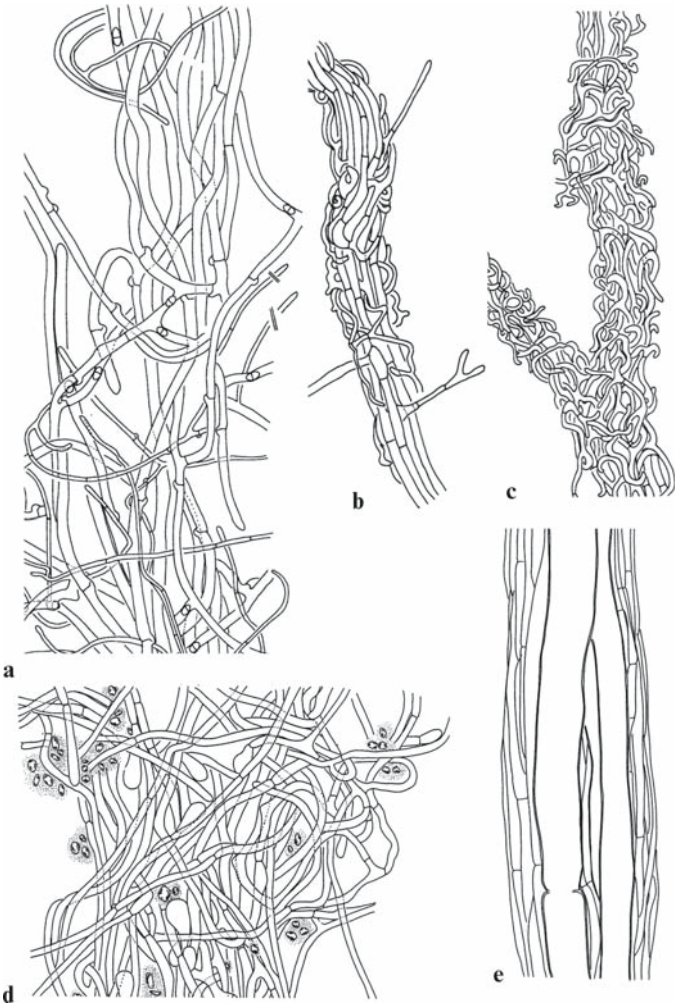

Fig. 18.2 Rhizomorph types according Agerer 1987–2006. **a** Mantle type A: uniform and loose hyphae (from *Quercirhiza aurateroc ystidiata*, Azul et al. 2006b). **b,c** Mantle type C: thelephoroid type, with peripheral hyphae thinner than central ones, (from *Quercirhiza tomentellofuniculosa*, Azul et al. 2006d). **d** Mantle type H: boletoid with vessel-like hyphae – surface (from *Quercirhiza pedicae*, Azul et al. 2001c). **e** Mantle type H: boletoid with vessel-like hyphae – inner layers with vessel-like hyphae with septa partially or completely dissolved (from *Quercirhiza sclerotiigera*, Azul et al. 2001d). All figures have been utilized with author's permission. All ECM rhizomorph pictures have been utilized with author's permission

hydrophilic, includes typical smooth mantles with infrequent emanating hyphae, which are frequently lost during removal of ECM from soil cores. The short-distance type has ectomycorrhizas with dense emanating hyphae that grows widely into surrounding soil. The medium-distance type matches with ECM fungi that develop rhizomorphs uniform-loose, uniform-compact, thelephoroid, or phlegma-

cioid (Agerer 2006) and grows often more than 30–50 mm. The long-distance type includes the highly differentiated rhizomorphs with vessel-like hyphae and may reach a length of several decimeters. Usually the long-distance exploration types include hydrophobic rhizomorphs.

Cystidia, very important diagnostic features, may be present on the ECM mantle and rhizomorph surfaces. They may also be observed on the cap skin, gills, and stipe of fruit bodies of Hymenomycetes. Cystidia may be diverse in structure and size (Agerer 1987–2006). Some are similar to normal hyphae, straight, bent, or hook-like; others present a distinct structure. They may be unramified or ramified at the proximal part with a monopodial ramification, or with dichotomous, tritomous or quadritomous ramifications.

Emanating hyphae may be present on mantles and rhizomorph surfaces. The main features of emanating hyphae are diameter, distance between septa, shape, color, cell wall thickness, surface, presence of crystals, presence of contents or drops of pigments, type of ramification, presence and type of anastomoses and presence and shape of clamps (Agerer 1987–2006). The type of anastomoses, the presence of intrahyphal hyphae and the shape of clamps are the most important features (Agerer 1995). Features of emanating hyphae are not as informative about fungal relationships as are mantles in plan view or rhizomorph organization.

The Hartig net, the zone of contact between plant and fungus, is produced by hyphae that penetrate intercellular spaces connecting outer cells of the root axis. This zone of contact is of central importance since it corresponds to the interface between symbionts. The Hartig net is usually formed from the inner mantle layers to the epidermal layers (in Angiosperms) or it can intrude into the cortical layers more deeply and toward the endodermis (in Gymnosperms). In most Angiosperms, the Hartig net may be limited to the anticlinal walls of the cortical cells – paraepidermal Hartig net – or may develop completely around the cortical cells – pariepidermal Hartig net (Godbout and Fortin 1983). Sometimes, very occasionally, haustoria-like intrusions may occur (Azul et al. 2001a).

Laticifers are typical of the genera *Lactarius* and *Russula* and correspond to latex-containing hyphae. They can be rather long, thick and/or scarcely branched, and are present in both plectenchymatous and pseudoparenchymatous mantles. Sclerotia are rather infrequent among the ectomycorrhizas. They have been observed in *Cenococcum geophilum* Fr. and in some members of the Boletaceae, Cortinariaceae, Paxillaceae and Pisolithaceae (Agerer and Rambold 2004–2007).

Chlamydospores are quite frequent in the Bankeraceae but also observed in the Thelephoraceae (Agerer 1987–2006). Mantle hyphae, rhizomorphs, cystidia and emanating hyphae may contain oil droplets, brownish contents, bluish contents, crystals or other appositions. For example, some members of the Thelephoraceae have blue granules on the surface of mantle hyphae and cystidia.

Chemical reactions are useful in identification of fruiting bodies and ectomycorrhizas (Table 18.1) (Agerer 1991). Reagents may be applied to whole ectomycorrhizas or to preparations of mantles and rhizomorphs. One of the most important chemicals is Melzer's reagent; the amyloid reaction is well represented in members of the Gomphidiaceae, Albatrellaceae and *Rozites*. Another distinctive chemical reaction is the

Table 18.1 Reagents and chemical reactions used to identify ectomycorrhizas

	Reagent	Preparation	Positive reaction
Very important	Melzer's reagent	0.5 g iodine, 1.5 g KI, 20 mL distilled water and 20 mL chloral hydrate	Amyloid reaction: stain blue Dextrinoid reaction: stain slightly brownish
	Lactic acid	90%	Mantle colors become brighter. Reagent used to make permanent mantle preparations
	KOH	10% aqueous solution (w/v)	Three distinct reactions have been recognized: (a) cell walls with blue or brown color change to green, (b) blue granules change to green and (c) cell walls with brownish or brown color become darker or lighter
	Iron(II) sulfate	1 g Iron(II) sulfate in 10 mL distilled water and some drops of H_2SO_4	Mantle color changes to greenish, bluish or grayish. Care is needed to avoid misinterpretation of a similar reaction with root cells remnants on mantle preparation
	Guaiac	Solution of 1 g guaiac resin in 6 mL ethanol 70%	Patchy cells of bluish color, particularly in inner mantle layers. Very important to displace the reagent by water or lactic acid
	Brilliant cresyl blue		Two different reactions: (a) cell walls become bluish; (b) cell walls turn to reddish (called metachromatic reaction)
	Sulfo-vanillin	Crystals of vanillin, H_2SO_4	Cell contents turn to pink or black and hyphal walls turn to red. On *Lactarius* species, laticifers become all staining of the, and/or some cells of the mantle and all hyphal ends on a mantle
Important	Formol	40% aqueous solution (w/v)	Mantle color changes to grey or greenish
	Toluidine blue	1% (w/v) aqueous solution	Differently intense blue color of cell walls
	Cotton-blue-lactic-acid	0.05 g cotton blue solution in 30 mL lactic acid (90%)	Intense bluish color of cell walls after displacing the reagent by water or lactic acid
Optional	Ethanol	70% aqueous solution (w/v)	Elution of wall colors, contents or of pigment granules on the hyphal wall
	Phenol-aniline		Mantle cell changes to grayish color
	Sudan III	Dissolve 1 g of Sudan III in 500 mL ethanol 96% (in water bath) and add 500 mL glicerin	Lipids become reddish

sulfo-vanillin test. To a water-mantle preparation vanillin crystals and a drop of H_2SO_4 are added to one side of the coverslip and observed immediately (due to rapid reaction, laticifers may dissolve quickly). The sulfo-vanillin reaction is typical of *Lactarius* and *Russula*.

18.3 Study Design

We cannot overemphasize the importance of clear research hypotheses and related study design. While there are many research questions concerning ECM diversity and function, not all are testable. It is essential to form a question that is testable. Once a hypothesis or research question is refined and focused, the sampling design may be developed. A key element in sampling design is an understanding of the spatial and temporal distribution of ectomycorrhizas and roots in soil. Ectomycorrhizas are associated with plant roots that are not evenly distributed in soils but are aggregated based on patterns of soil fertility, soil texture, competing roots, etc. Since one often does not know the spatial and temporal distribution of roots and their associated mycorrhizas, preliminary sampling may be necessary. For example, one may collect soil cores to sample ECM diversity and determine "species effort curves". Detailed discussion of sampling design is beyond the scope of this chapter, so the reader is referred to Johnson et al. (1999). See also Sect. 18.5.2 in this chapter.

18.4 Morphotyping of ECM Roots

18.4.1 Introduction

The ability of ECM fungi to benefit their hosts is closely related to their structures. ECM fungi that colonize roots, and modify root color, shape and function, are often characterized by extensive external hyphal development. Few ECM species are well known and described in detail, particularly field-grown ECM fungi. Advances in molecular biology and morphotyping have demonstrated the poor correspondence between the species composition of fruiting bodies and of fungi colonizing roots. Identification of inconspicuous fruiting bodies (e.g. resupinates) highlights the overall diversity among ECM communities. These advances make it possible to study functional properties in nature by monitoring and by spatiotemporal analysis of below-ground ECM fungal communities. Studies on ectomycorrhizal structures are equally important to clarify functional properties in the relationship between different mycosymbionts (Agerer 2006).

ECM morphology is useful but not considered as diagnostic because color, ramification and mycorrhizal system size depend on growth conditions and plant host (Agerer 1991). Since the first studies on ectomycorrhiza structure on *Castanea sativa* by Gibelli (1883) and on *Fagus sylvatica* and *Carpinus betulus* by Frank

(1885), over 343 species have been described in detail (de Román et al. 2005) and only about 6–7% of all presumably ECM fungi. Morphotyping has limitations for ECM identification, particularly in *Russula*, *Lactarius* and *Cortinarius*. Many fungal groups can be distinguished by morphological and anatomical characterization, a relatively inexpensive method allowing examination of large numbers of root tips. Studies of ECM structures allow better understanding of their spatial distribution (Tedersoo et al. 2003; Lilleskov et al. 2004; Baier et al. 2006).

18.4.2 Extraction of Roots from Soils and Sample Storage

To study ECM structure, morphological and anatomical characteristics must be intact as samples are removed from soil. Ectomycorrhizas can be sampled from roots or by tracing hyphal or rhizomorphs connections to fruiting bodies. In both situations samples should be taken with soil cores to ensure that connections between ECM roots, hyphae and rhizomorphs are not damaged or disrupted. If the research focuses on investigation of ECM fungi associated with fruiting bodies, the stipe of the fruiting body should be carefully cut and marked, especially in species with dark stipes. It is necessary to balance the number of samples and the size of the soil core to enhance retention of ECM natural features. Unfortunately, many ecological studies involve large numbers of samples with low numbers of ECM tips and no voucher specimens. Soil core samples must have unique collection numbers and can be stored at 4°C in plastic until processing within two weeks if at all possible.

18.4.2.1 Protocols: Extraction and Assessment of ECM

Procedure

List of Materials Dissecting microscope, large Petri dishes, needles, pipettes, fine forceps.

Cleaning Procedure and ECM Extraction from Soil (1) Immerse the soil core in water and soak carefully in water until saturated; (2) Wash roots gently with pipettes to limit damage to the ectomycorrhizas; (3) Place cleaned roots (<1 mm diameter) in a Petri dish with filter paper soaked with water.

Notes (a) Petri dishes with filter paper prevent color changes of ectomycorrhizas and hyphal growth; (b) ECM tips can be stored in the refrigerator up to seven days after sampling.

ECM Abundance and Richness To evaluate changes in ECM abundance and richness in soil horizons or under different soil conditions, root tips must be counted.

ECM Abundance Total ECM abundance corresponds to the total number of living ectomycorrhizas per 100 cm^3 soil volume. Relative abundance can be expressed as either (a) number of living ectomycorrhizas per meter of fine roots, or

(b) number of tips of a given genus or species divided by total number of living ECM root tips in the same soil core.

ECM Richness ECM diversity within soil horizons and/or study areas may be estimated using distinct diversity descriptors: (a) species richness, i.e., number of ECM species observed (*S*); (b) Shannon-Wiener (*H*) and Simpson (λ) diversity indexes; (c) Pielou evenness (*H'*); (d) Margalef (D), Log α (*S*) and Jack-knife richness indexes; and (e) Whittaker β-diversity index (Magurran 1988).

Notes (a) The effects of soil horizons, soil types, or land use systems on ECM abundance may be determined by univariate analyses – ANOVA (Zar 1996); (b) The relationship between ECM diversity descriptors and study areas conditions can be further determined upon multivariate analyses, e.g. by using CANOCO 4.5 software (Ter Braak and Smilauer 2002).

18.4.2.2 Protocols: Description of ECM

Procedure

List of Materials Dissecting microscope, microscope (NIC), pipettes, needles, fine forceps, Petri dishes, chemicals (see Table 18.1).

ECM Morphology (1) Observe ECM root tips in water under a dissecting microscope (6×, 12×, 25×) using a black background and lamps of daylight quality; (2) Isolate morphotypes by morphology, i.e., color, ramification type, systems, size and texture, presence of emanating hyphae, cystidia, rhizomorphs, and/or sclerotia (see Agerer and Rambold 2004–2007); (3) Take photos of mycorrhizal systems, maintaining the black background and lamps of daylight quality (Agerer 1991); (4) Use some living ectomycorrhizas to check the chemical reactions (see Table 18.1).

Notes (a) Ectomycorrhizas may exhibit high diversity of colors (Agerer 1987–2006). The color of the ectomycorrhizas is very useful for the first isolation after extraction from soil and can be an important feature for identifying some species, since ectomycorrhizas color often mirrors the color of the fruiting body cap; (b) Dimensions of ECM systems and unramified ends should be taken into account despite not being a distinctive character. The dimensions are affected by the host species and by the physical properties of the substrate in which the ectomycorrhizas have grown. The values always should be presented considering the different range of measurements.

ECM Anatomy (1) Prepare slides by using mantle squashes and mantle peels from fresh material; (2) Use remaining root tissue of the ectomycorrhizas to prepare slides by squashing for confirming presence of and structure of the Hartig net; (3) Observe slides prepared with water at 400× and 1000× magnification, with a Normarski interference contrast microscope, to register the presence of cytoplasmic contents, such as oil droplets; (4) Add lactic acid to the slide after observing mantle peels in water to study anatomical features: mantle plan view, rhizomorphs, cystidia, emanating hyphae, Hartig net, chlamydospores, sclerotia (Agerer 1991, 1995; 1987–2006; Agerer and Rambold 2004–2007); (5) Take photos and drawings of anatomical features (Agerer 1991; Agerer and Rambold 2004–2007); (6) Store

ECM samples and reference vouchers in ethanol 50%, FAA, or 2.5% CTAB. Preserve slides with lactic acid as reference vouchers as well.

Other Considerations

See Deemy (an information system for characterization and DEterminatin of EctoMYcorrhizae): http://www.deemy.de/

18.5 Molecular Identification of ECM Roots and Hyphae in Soil

18.5.1 Introduction

In recent years, use of molecular methods to identify fungal species has provided new insights into the below-ground fungal community and a more precise approach to fungal diversity studies. In ECM fungi, molecular identification is especially important for root tips and hyphae whose morphological identification can be difficult or impossible. In this section, we provide DNA-based protocols to identify ECM fungi in different fungal and environmental samples. Different molecular techniques can be chosen depending on the aims of the study, research hypotheses and the fungal material. Following DNA extraction and PCR, various techniques such as RFLP, T-RFLP, cloning and sequencing resolve identification of individual fungi or develop fungal community profiles. When the aim is to quantify the amount of DNA from a certain fungus in a pure or complex DNA sample, one can use a quantitative PCR method (Real-time PCR).

18.5.2 Guidelines for Sampling Design and Collection of Samples

ECM fungi can be present in the environment in four different stages: spores, ectomycorrhizas or root tips, mycelium and fruiting bodies. This section focuses on procedures to detect and identify ectomycorrhizas (both single and pooled root tip samples) and mycelia (hyphae) in soil. However, these procedures can also be used for identification of fruiting bodies and spores. Sampling area, sample size, distances among samples, spatial and vertical distribution of fungi and complexity of samples (single vs pooled) are factors that should be considered in sampling design. Presence or absence of species in soil can be influenced by sample size, since diversity can change within 1 cm in soil. It is recommended to keep distances between samples greater than size of samples (or soil cores) collected (Taylor 2002). Some ECM species seem to develop preferentially in the organic layer while other species grow in mineral layers of the soil profile (Stendell et al. 1999; Taylor and Bruns 1999). Moreover, the apparent non-random distribution of species complicates sampling of ECM communities to assess ECM richness (Landeweert et al. 2005). When studying root tip diversity, it is also important to be cautious in the interpretation of diversity data, especially when comparing two ECM communities (Taylor 2002). Single root tip DNA extractions are not realistic when analysing thousands

of root tips and therefore extracted root tips are sometimes pooled prior to DNA extraction (Zhou and Hogetsu 2002). Before DNA isolation, soil and root tip samples can be stored at 4 °C for a few days. However, samples should be processed as soon as possible to reduce contamination from other fungi and DNA degradation. For long-term storage, samples should be frozen (at −20 °C or −80 °C for longer periods) or freeze-dried and then frozen. Samples can also be ground into a fine powder after freeze-drying. Root tip samples can be stored in CTAB buffer.

18.5.3 DNA Extraction

18.5.3.1 Introduction

Efficient isolation of DNA is essential in order to perform techniques to identify ECM fungi. DNA extraction protocols are subject to modifications depending on the origin of the samples and the quality and length of DNA needed for posterior analyses. These modifications usually consist of improving homogenization (e.g. in soil samples or mummified root tips), including additional cleaning steps or using extra reagents (e.g. polyvinylpyrrolidone (PVP) or Proteinase K) to remove substances that could inhibit the PCR reaction such as polyphenols and humic acids in soils and tannins in root tips. Apart from DNA, RNA can also be isolated and in higher amounts than DNA, since it is more abundant in cells. RNA requires special procedures to be extracted and cannot be stored for long periods of time because it is much more susceptible to degradation and contamination than DNA. This chapter includes only descriptions of DNA-based techniques.

18.5.3.2 Protocol: Root Tip DNA Extraction

This CTAB-based protocol of DNA extraction from single to several root tips is based on protocols previously developed by Rogers and Bendich (1985), Doyle and Doyle (1990) and Henrion et al. (1992). It can also be use to isolate DNA from fruiting bodies and mycelium from pure culture of ECM fungi.

Procedure

Equipment and Plasticware Water bath, 1.5-mL microcentrifuge tubes, pellet pestles, micropipettes, microcentrifuge.
Reagents 2% CTAB lysis buffer, chloroform, isopropanol, ice-cold ethanol (70%), sterile deionized water (or TE buffer).

Mechanical and Chemical Lysis (1) Place one or several root tips in a 1.5-mL microcentrifuge tube; (2) Add 600 µl of 2% CTAB lysis buffer to each sample and grind with a pellet pestle.

Incubation (3) Place microcentrifuge tubes in a water bath at 65 °C for 40 min to 1 h.

DNA Purification (4) Centrifuge the tube at 13,000 rpm for 5 min; (5) Transfer the upper phase into a new microcentrifuge-tube; (6) Add 600 µl of chloroform. Mix well by hand until the suspension is colloid; (7) Centrifuge at 13,000 rpm for 15 min; (8) Transfer the upper phase to a new 1.5-mL microcentrifuge-tube.

DNA Precipitation (9) Precipitate the DNA with 1.25 volumes of isopropanol (≈750 µl). Mix well by agitating the microcentrifuge-tube and keep it at −20 °C for 30 min. Samples can remain in the freezer overnight. (10) Centrifuge samples at 13,000 rpm for 30 min; (11) Discard the upper phase (by pipette or pouring).

DNA Washing (12) Add 200 µl of 70% ice-cold ethanol (−20 °C) to wash the DNA; (13) Centrifuge samples at 7,000 rpm for 5 min; (14) Discard the upper phase and let the DNA-pellet dry (about 5 min on the heating block).

DNA-Pellet Solubilization (15) Solubilize the DNA-pellet in 50 µl of sterile double deionized water or TE buffer.

Notes (a) Before incubation, 5–7 µl of Proteinase K (20 mg/mL) can be added to the lysis buffer; (b) To inhibit polyphenol oxidisation processes, 0.2% of β-mercaptoethanol can be added after incubation (step 3) followed by incubation for another 30 min after the purification steps.

Other Considerations

Troubleshooting Low DNA yields may result from different reasons: (a) Incomplete tissue homogenization: use liquid nitrogen prior to grinding to get fine powder from each sample before incubation in lysis buffer; (b) Insufficient lysis buffer: increase its volume in homogenization step; (c) Old material: add proteinase K during incubation as described above; (d) Incomplete solubilization of the final DNA pellet: warm the tubes at 65 °C for a few minutes, vortex briefly if needed; (e) DNA degradation: root tips were not correctly stored or frozen before DNA isolation; (f) Presence of proteins, salts, etc. DNA was not sufficiently washed; pellet the DNA again and repeat the protocol from washing step.

DNA Quantification The quality and purity of the extracted DNA is essential for PCR amplification of the target DNA. Once crude DNA is obtained and solubilized, there are two procedures to quantify the DNA: (1) with electrophoresis: run an aliquot of DNA in an agarose gel (usually 0.8%) and measure fluorescence emitted by an added nucleic acid gel stain (ethidium bromide or SYBR® Green I dye) against serial dilutions of a known amount of DNA such as calf thymus DNA (Ranjard et al. 1998, 2003); (2) with a spectrophotometer, measure absorbance of the DNA sample at 260 nm and apply the formula: 1 O.D.=50 µg DNA/mL to estimate the quantity of DNA (include the dilution factor). The first procedure has the advantage that it provides together with the quantification of DNA an estimation of the presence of contamination by RNA and an estimation of the quality of DNA. However, DNA in very low quantities may not be visible in a gel but can be amplified by PCR.

Other Protocols Henrion et al. (1994) developed one of the most frequently used protocols for DNA isolation in fungi, based on modifications of the protocols of Henrion et al. (1992) and of Gardes and Bruns (1993). Another SDS protocol developed by Edwards et al. (1991) for DNA isolation from plants has been exten-

sively used for ECM fungi (Paolocci et al. 1999; Baciarelli-Falini et al. 2006). Lee and Taylor (1990) also developed an SDS protocol for fungal mycelia and single spores, using β-mercaptoethanol.

Commercial Kits To isolate DNA from fungi, the following fungal DNA commercial kits are often used: DNA E.Z.N.A.® Fungal DNA miniprep kit (Omega Bio-Tek; Martín and García-Figueres 1999; Aguín-Casal et al. 2004) and Ultra Clean Microbe DNA Isolation Kit (Mo Bio Laboratories; Koide et al. 2005b). The DNeasy® Plant Mini Kit (Qiagen) for plants has been used by Genney et al. (2006), Hortal et al. (2006) and Gagné et al. (2006).

18.5.3.3 Protocol: Soil (Hyphal) DNA Extraction

When working with soil samples, one may use either direct or indirect methods to extract DNA. With the direct method, cells are lysed while they remain in the soil matrix (Ogram et al. 1987). With the indirect method, cells are recovered from the soil matrix before lysis (Balkwill et al. 1975). The direct method is more commonly used because it yields more DNA and a less biased sample of the microbial community diversity (Holben et al. 1988; Miller et al. 1999). The quantity and quality of DNA extracted per gram of soil will depend on the method used and on the properties of the substrate sampled (Martin-Laurent et al. 2001). When the objective is to detect hyphae in soil, it is essential to examine soil samples with a dissecting microscope for the presence of spores or ECM mantle tissues before DNA extraction. Soil samples can be sieved to remove small roots, stones and other debris. This CTAB-based direct DNA extraction protocol is based on the root tip DNA extraction protocol described above and adapted to soil conditions by Suz et al. (2006). Only modifications are described.

Procedure

Equipment and Plasticware Micropipettes, 50-mL polypropylene tubes, 1.5-mL microcentrifuge tubes, mortar/mortar pestle, pellet pestle, water-bath or thermoblock, microcentrifuge, centrifuge for 50-mL tubes.

Reagents 2% CTAB–1%PVP, lysis buffer, chloroform-isoamyl alcohol, isopropanol, ice-cold ethanol (70%).

Mechanical and Chemical Lysis (1) Weigh 7 g of soil and place it in a porcelain mortar; (2) Add 40 mL of 2% CTAB containing 1% PVP and grind the sample with a mortar pestle.

Incubation (3) Transfer sample to a 50-mL propylene tube and incubate for 1 h at 65 °C in a water bath.

DNA Purification (4) Centrifuge the tubes at 9800 g for 5 min at 10 °C; (5) Transfer 600 µl of the supernatant to a 1.5-mL.microcentrifuge tube; (6) Add 600 µl of chloroform-isoamyl alcohol (24:1). Mix well by hand until the suspension is colloid; (7) to (11) Follow protocol for root tip DNA extraction.

DNA Washing (12) to (14) Follow Protocol for root tips. Repeat once.

DNA-Pellet Solubilization (15) Solubilize the DNA-pellet as in protocol for root tips. Warm the 1.5-mL microcentrifuge tubes in a heating block at 65 °C for few minutes if pellet is hard to solubilize.

Other Considerations

DNA Purity To estimate DNA purity, ratios of absorbance at 260/280 should be measured. Ratios up to 1.8 indicate that the DNA extract is relatively free of proteins. When this ratio is lower, commercial kits are available to purify DNA extracts, e.g. Wizard DNA clean-up system (Promega; Smalla et al. 1993; Landeweert et al. 2003a,b) and Gene Clean® II kit (Bio 101; Bertini et al. 1998; Amicucci et al. 1998).

Other DNA Isolation Protocols DNA extraction protocols for soil samples were initially designed to extract DNA from bacterial communities; more recently they have been used for fungal communities. Some protocols involve grinding samples with liquid nitrogen (Volossiouk et al. 1995; Zhou et al. 1996). Other protocols include homogenization with beads (Yeates et al. 1998; Landeweert et al. 2003a,b [based on Smalla et al. 1993]) or freeze-thaw lysis (Miller et al. 1999). Some protocols use SDS as a detergent (Martin-Laurent et al. 2001; Ranjard et al. 2003) while others use CTAB (Anderson et al. 2003, from Griffiths et al. 2000).

Commercial Kits Several commercial kits are available to isolate DNA from soil: Ultraclean™ Soil and PowerSoil™ DNA Isolation kits (MoBio; Dickie et al. 2002; Chen and Cairney 2002; Koide et al. 2005a; Hortal et al. 2006; Parladé et al. 2007) and Fast DNA Spin kit for soil (Bio101; Griffiths et al. 2000; Guidot et al. 2003). These kits are often used for complex samples of root tips (Bergemann and Garbelotto 2006) or even for individual root tips (Tedersoo et al. 2006).

18.5.4 Polymerase Chain Reaction (PCR)

18.5.4.1 Introduction

Nucleic acid extraction coupled with PCR (Polymerase Chain Reaction) (Mullis and Faloona 1987) has significantly improved DNA-based detection and identification of ECM fungi. This technique allows in vitro amplification of a specific DNA fragment (target DNA) through successive temperature cycles (25–40). Thus the PCR method involves repeated cycles of three phases: (1) DNA denaturation, (2) annealing or primer hybridisation, and (3) DNA extension (Fig. 18.3). This results in exponential amplification of a target DNA sequence. A standard PCR reaction contains the DNA target sequence (10–100 ng), two primers (0.1–0.5 μM) – forward and reverse – that are complementary to the sequence of target DNA, nucleotides (dNTPs, 20–200 μM

each), a thermal-stable DNA polymerase (0.5–2.5 units) with its corresponding buffer (1/10 of the final volume) and cofactor ($MgCl_2$, 0.5–5 mM, typically 1.5 mM) and sterile distilled water to a final volume of 25–100 µL (Edel 1998). In theory 'n' PCR cycles will correspond to 2^{n-1} copies of the target DNA.

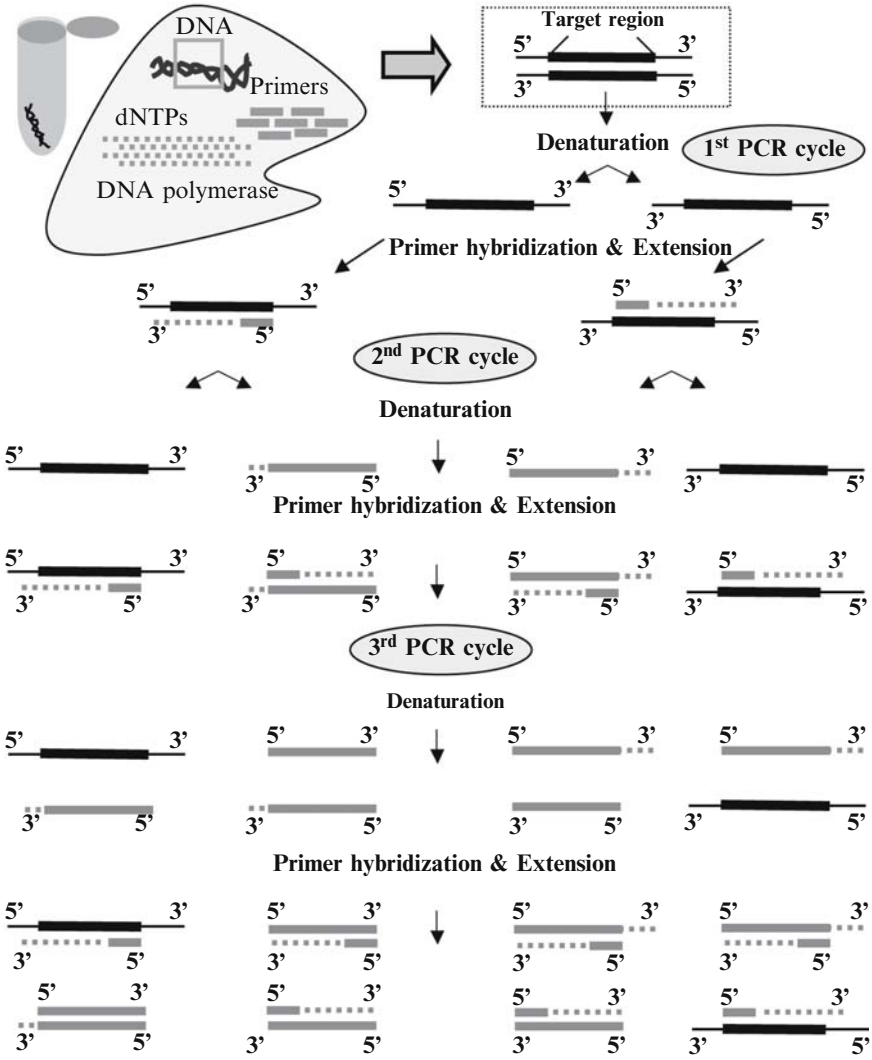

Fig. 18.3 Diagram showing three consecutive cycles of DNA amplification by PCR in three steps: DNA denaturation, Primer hybridization and DNA extension (based on Martín, 2000)

18.5.4.2 Selection of the Target Region

Since ECM fungi are not monophyletic, there are no primers designed to amplify this group. The ribosomal RNA operon (rRNA), present both in nuclei and mitochondria, is the most widely targeted region for PCR in ECM fungi. The number of copies repeated in tandem varies among fungal species (Hibbett 1992). Each rRNA gene unit repeat contains highly conserved rRNA genes 18 S, 5.8 S, 28 S and 5 S (in some taxa) and variable spacer or non-coding regions (ITS: Internal transcribed spacer region, and IGS: Intergenic spacer regions) allowing the comparison and discrimination of ECM fungi at different taxonomic levels. The 18 S (small subunit, SSU) and 28 S (large subunit, LSU) rRNA gene sequences allow identification to genus and family level (Bruns et al. 1992). Both ITS and IGS regions are more variable than other regions of the rRNA gene, presenting few or no homologies between divergent genomes. Polymorphisms in the ITS region allow identification between ECM species (Kårén et al. 1997; Pritsch et al. 1997). The largest database sequence information for molecular identification of fungi corresponds to ITS regions. In some species, the IGS region is more variable than the ITS region and is used to identify intraspecific polymorphisms. However, it may not be useful for isolates of the same species (Erland et al. 1994).

18.5.4.3 Selection of the Primer Set

Primers are short DNA sequences 15–30 bp in length. Efficiency and specificity should be considered when selecting primers. Primers should have ~50% CG content. Complementarity between forward and reverse primers and within each primer should be avoided to minimize formation of primer-dimers because they decrease the product yield. There are specific software programs to design and confirm the availability of primers. The earliest set of primers designed for ITS region amplification was ITS1/ITS4 (White et el. 1990) but they also amplify DNA from plant and algae. The fungal-specific primer ITS1F (Gardes and Bruns 1993) and the universal reverse primer ITS4 (White et al. 1990) have been extensively used in ECM fungi studies (Dickie et al. 2002, 2004; Chen and Cairney 2002; Genney et al. 2006). Since the majority of ECM fungi are basidiomycetes, the basidiomycete-specific reverse primer ITS4B has been coupled with ITS1F to amplify this group (Landeweert et al. 2003a,b; Gagné et al. 2006). Larena et al. (1999) developed a reverse primer specific to ascomycetes called ITS4A. Mitchell and Zuccaro (2006) reviewed published primers that amplify fungal sequences from nuclear SSU, LSU and ITS regions including 5.8 S rRNA. Primers designed to hybridize in complementary sequences of the DNA regions described above are detailed in several web sources: http://www.biology.duke.edu/fungi/mycolab/primers.htm; http://www.lutzonilab.net/pages/primer.shtml.

Primers to amplify the IGS region have also been designed (Henrion et al. 1992; Gardes and Bruns 1993). Martin and Rygiewicz (2005) designed a set of primers to

discriminate between plant and fungal sequences, very useful in root tip identification. Primers specific to a certain ECM species have been designed for genera such as *Tuber* (Amicucci et al. 1998), *Armillaria* (Sicoli et al. 2003), *Lactarius* (Hortal et al. 2006) and *Rhizopogon* (Kennedy et al. 2007).

18.5.4.4 Cycling Conditions

In DNA denaturation, temperature to separate both strands is usually 90–95 °C. Annealing temperature is typically 40–60 °C. In DNA extension temperature is typically 72 °C but duration will depend on the length of the PCR products. PCR products <500 bp require ~30 s in this phase. Each 500-bp increase in length corresponds to an additional 30 s in the extension phase (1 min per each 1 Kb).

18.5.4.5 Tips

To avoid nucleic acid contamination, use sterile pipette tips with filters, autoclave all buffers, clean work surfaces with bleach followed by sterile water and prepare PCR reactions in a separate place in the laboratory. Keeping the components of the PCR in small aliquots helps avoid contamination from continuously pipetting from the same tube and degradation by continuous cycles of freeze-thawing.

18.5.4.6 Protocol: PCR

Procedure

Equipment and Plasticware 0.2-mL PCR tubes, 1.5-mL microcentrifuge tubes, thermocycler, micropipettes, transilluminator, vortex.
Reagents Sterile deionized water, *Taq*-polymerase/appropriate buffer, $MgCl_2$, dNTPs, direct and reverse primers, template DNA, agarose, ethidium bromide or SYBR® Green I dye, molecular weight marker (DNA ladder), TAE1X or TBE1X, load buffer.

(1) Calculate the total amount of each reagent needed for the total number of samples in the PCR-mix (see note (b) below).Concentration and volumes of the different components of the PCR reaction will depend on the thermo-stable polymerase chosen (check manufacturer's instructions). See example in Table 18.2 below; (2) Turn on thermocycler (lid needs to be 10 °C over the highest temperature of the PCR program before starting the PCR reaction); (3) Place on ice all components for PCR reaction except *Taq*-polymerase; (4) When reagents have thawed, prepare in a 1.5-mL microcentrifuge tube the PCR-mix for a final volume per sample of 20 µl in this order: sterile deionized water, enzyme buffer,

$MgCl_2$ (in the example, it is included in the Phusion buffer), dNTPs and primers; (5) Remove *Taq*-polymerase from the freezer and add the units per reaction recommended by the manufacturer to the PCR-mix (e.g. 0.4 units/μL of Phusion™ High-Fidelity DNA Polymerase in the example); (6) Vortex the mixture for 5 s to homogenize the reagents; (7) Distribute equal volumes of PCR-mix in each 0.2-mL microcentrifuge tube (in the example, 17 μl); (8) Vortex each DNA extraction briefly and add the DNA template to each PCR-tube; (9) Cover the PCR-tubes and place them in the thermocycler. Program cycling conditions (e.g. for ITS1F/LR3 amplicon: initial denaturation at 98 °C for 30 s, followed by 35 cycles of denaturation at 98 °C for 10 s, annealing at 56 °C for 30 s and extension at 72 °C for 1 min, with a final extension at 72 °C for 7 min). Variations on time and length of each step will depend on the *Taq*-polymerase chosen. (10) Resolve PCR products by gel electrophoresis in agarose 1.5% stained with ethidium bromide or SYBR® Green I dye (use gloves) and run samples in the gel with an appropriate molecular weight marker; (11) Visualize PCR products under UV light.

Notes (a) If thermocycler lacks a thermal lid, it is necessary to add oil to the tubes to avoid evaporation; (b) Make enough PCR-mix for three extra reactions (one negative control, one positive control and one for any pipetting errors); (c) Changes in volume of the different PCR-components will determine the volume of water added.

18.5.4.7 Protocol: Nested PCR

One challenge of working with root tips and hyphae in soil is that low amounts of DNA or DNA of low quality are obtained after DNA extraction, leading to weak or non-amplifications. Furthermore, plant DNA is co-extracted with fungal DNA in root tip samples. In soil samples, DNA from different organisms may be co-extracted with fungal DNA, resulting in low quality and low amounts of target DNA. To solve these problems it is possible to use a technique called "Nested-

Table 18.2 Concentrations of components needed for PCR amplification of the ITS regions and part of the 28 S of rRNA gene with primers ITS1F and LR3 (Gardes and Bruns 1993; Hopple and Vilgalys 1994). DNA extraction was performed with the Ultraclean™ DNA Isolation kit (MoBio) using pooled samples of ~100 lyophilized roots tips

Reagent	Initial concentration	μL/sample (1X)	Master mix (μL) (10X)
Milli-Q water		8.4	84.0
Phusion Buffer	5X	4.0	40.0
dNTPs	200 μM	0.4	4.0
ITS1F	5 μM	2.0	20.0
LR3	5 μM	2.0	20.0
Phusion *Taq*[a]	2 units/μl	0.2	2.0
Template DNA	Variable	3.0	
Total volume		20.0	

[a]Phusion™ High-Fidelity DNA Polymerase (New England, BioLabs)

PCR", that consists of two consecutive PCR reactions. The first PCR reaction uses an external primer pair, while the second PCR reaction uses an internal (nested) primer pair, or one of the external primers and an internal one (semi-nested PCR). Thus, the product of the first PCR is used as a template for the second PCR, increasing the sensitivity of the technique and allowing the detection of the target DNA, that would be undetectable or in very low amounts with only one PCR reaction. In soil DNA extractions, nested-PCR provides a lower threshold of sensitivity and permits much higher levels of dilutions of the DNA template (Volossiouk et al. 1995; Anderson and Parkin 2007).

Procedure

Choose two pairs of primers. Primers for the first PCR reaction should hybridize in external sequence parts of the sequence of interest. The product obtained from this PCR will be the template in the second PCR. After following procedure described in the previous PCR protocol: (12) Depending on the amount of product obtained by the first PCR using the first primer set, dilute the PCR product 10- to 1000-fold with sterile deionized water; (13) Follow the PCR process described in the previous protocol, using the second pair of primers (see note below) and the dilution series of the DNA template. The negative control (no DNA template) of the first amplification should be used as template in the second one. Thus, two negative controls are set up in the second amplification; (14) Place tubes in the thermocycler and run the appropriate program; (15) Resolve PCR products by gel electrophoresis in agarose 2% stained with ethidium bromide or SYBR® Green I dye (use gloves) using an appropriate molecular weight marker; (16) Visualize PCR products under UV light.

Note When first PCR is carried out with ITS1F and LR3 primers, primers that hybridize on 5.8 S ribosomal subunit, such as the forward primer ITS3 and the reverse one ITS2 (White et al. 1990) can be used for seminested PCR reactions coupled with the former primers. Also ITS1F coupled with ITS4 or ITS4B can be useful for ECM fungal identification.

Other Considerations

Use of 1–10% (w/v) dimethylsulfoxide (DMSO) decreases the melting temperature needed for separation of both strands of DNA. Addition of bovine serum albumin (BSA) (10–100 µg/mL) improves yield of the PCR product, binding fatty acids and phenolic compounds that can inhibit the PCR reaction.

PCR-Beads Use of PCR kits such as puRe Taq Ready-To-Go PCR beads (GE Healthcare) significantly reduces the number of pipetting steps, decreases handling errors and increases reproducibility. This kit has been used in several studies about ECM fungi (Martín and Calonge 2000; Tedersoo et al. 2006; Suz et al. 2006).

Troubleshooting Weak or no amplification could be due to: (1) Reagents are not in the required concentration or one of them is missing: check the protocol; (2) Low amount of DNA template: increase the volume of DNA template and correspondingly decrease the volume of deionized water, if improvement is not shown: try to perform a nested-PCR with inner primers to the DNA target; (3) Presence of PCR

inhibitors: dilute DNA template or purify the DNA extraction. Add BSA or DMSO in the PCR-mix, to increase DNA yield. (4) Too short extension times: Increase 30 s for each 500 bp of PCR product. You can also increase $MgCl_2$ concentration: yield of amplified products will increase but specificity will decrease. When excessive background amplification is present, possible reasons could be: (1) Very high amount of DNA template: dilute it for PCR reaction; (2) Too many PCR cycles can increase the amount of non-specific background products; (3) High concentration of primers; (4) Poor quality of DNA template; (5) Too low annealing temperature. When non-specific amplification is shown, reasons could be: (1) High concentration of $MgCl_2$: decrease $MgCl_2$ concentration; (2) Contamination of reagents with amplifiable DNA: discard old reagents and prepare a new stock; (3) Primers hybridize to a secondary site in DNA template: think about designing new primers; (4) Annealing temperature is low; (5) Excessive number of cycles.

18.5.5 PCR-Based Techniques

18.5.5.1 Introduction

PCR-based techniques permit the study of environmental samples without the need to culture organisms, leading to a better understanding of the role of ECM fungi in complex environments. They also provide methods to identify ECM fungi at different life stages when morphological characters are ambiguous or missing. These PCR-based techniques allow investigation of relationships between closely related species and populations of single species. In direct PCR-based techniques such as RFLP, T-RFLP, sequencing and cloning, the primers hybridize to known sequences of the fungal genome (ITS regions, LSU and SSU of the rRNA gene, etc.). Selection of the appropriate PCR-based technique depends on the research objectives, the fungi of interest and the type of data analyses planned (Anderson and Cairney 2004).

18.5.5.2 PCR – Restriction Fragment Length Polymorphism (RFLP)

After checking by gel electrophoresis that single intense bands were obtained from each PCR reaction, the product can be analyzed by Restriction Fragment Length Polymorphisms (RFLPs). This analysis involves digestion of PCR products with a number of restriction enzymes that cleave DNA molecules at specific nucleotide sequences. Restriction fragments are resolved by gel electrophoresis; smaller DNA fragments travel greater distances towards the positive electrode than larger fragments. After a successful restriction digestion, a series of DNA fragments will appear in the gel. Pattern similarity can be used to differentiate species, strains and other taxonomic categories. To identify ECM and to carry out population studies, it is important to compare patterns obtained with those from local PCR-RFLP databases of fruiting bodies or pure cultures. In most projects, 100–500 root tips are sampled and 3–6 enzymes are

used in RFLP analysis. With such large number of samples, automatic processing of data is required (see below: Taxotron® software package). One of the best tools for identification of those RFLP patterns that are not associated with a known pattern, is the direct sequencing of ITS regions followed by BLAST search (Altschul et al. 1997).

18.5.5.2.1 Protocol: RFLP

Procedure

Equipment and Plasticware Micropipettes, 1.5-mL microcentrifuge tubes, water rakes, vortex, microcentrifuge, water bath, camera.

Reagents Enzymes/buffer for each enzyme, sterile deionized water, molecular weight marker (DNA ladder).

Distribute 5 µl PCR-product to 1.5-mL microcentrifuge tubes, according to the number of restriction enzymes. Keep on ice.

Master Mix (15 µl/Sample/Enzyme) (2) Prepare a digestion mix for each enzyme according to manufacturer's instructions. Include an extra volume to each 10 samples. For each sample, mix the reactants in the following order: (a) Two microliters of buffer (1/10 of final volume). Be careful, each enzyme has its own buffer, which should be completely defrosted before use; (b) Sterile deionized water to final volume of 15 µl. Mix gently (vortex); (c) Two units of restriction enzyme (volume will vary depending on enzyme concentration). Mix gently (vortex) and spin down.

Incubation (3) Add 15 µl of master mix to each sample. Mix gently (vortex) and spin down; (4) Place tubes in a water bath at the temperature require for each enzyme, for at least 1 h/enzyme unit. After incubation, tubes should be stored at −20 °C.

Analysis of RFLP Patterns (5) Visualize DNA fragments by 2–2.5% agarose gel electrophoresis. According to the range of length of the analyzed fragments, different DNA size standards can be used. Record results with polaroid or digital pictures. Tip: run a DNA ladder with each two or three digested samples to obtain a more accurate measure of fragment lengths; (6) Determine fragment size using the software chosen (e.g. Taxotron® software system, see below).

Other Considerations

All recognition sequences are palindromes: both strands read the same in both directions. If the PCR buffer is not compatible with the restriction enzyme, purify the PCR product by the standard procedure (phenol-chloroform and ethanol) or by a commercial kit. The Taxotron® software system, especially developed for RFLP data processing (Institute Pasteur, Paris) includes: RestrictoScan® that allows detection of lanes, band and migration values in a digitalized picture and RestrictorType® that calculates molecular size, using the function of Schaffer and Sederoff (1981) to estimate fragment lengths. Authors have published RFLP data from different species of *Rhizopogon* (Martín et al. 1998, 2000b), *Macowanites* (Martín et al.

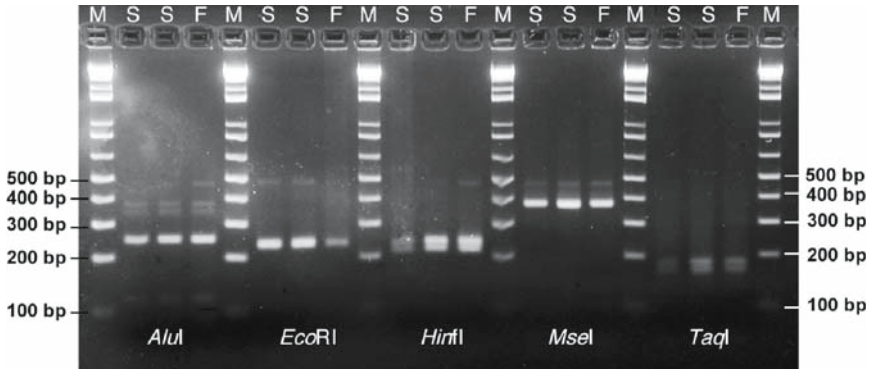

Fig. 18.4 RFLP patterns obtained alter digestion with five restriction enzymes (*Alu*I, *Eco*RI, *Hin*fI, *Mse*I and *Taq*I) of a 465-bp PCR product corresponding to *Tuber melanosporum* from soil mycelia (S), and fruiting bodies (F). M: 1 Kb plus DNA Ladder. Fragments were resolved in 2% agarose gel electrophoresis for 40 min at 100 V

1999), *Russula* (Martín et al. 1999), *Tuber* (Suz et al. 2006, see Fig. (18.4), *Terfezia* and *Tirmania* (Martín et al. 2000a).

18.5.5.3 Terminal-RFLP (T-RFLP)

Terminal-Restriction Fragment Length Polymorphism (T-RFLP) is a molecular fingerprinting method commonly used to characterize ECM fungal communities (Burke et al. 2005; Koide et al. 2005a,b). This method facilitates increased throughput compared with gel-based fingerprinting techniques because it uses fluorescence electrophoresis and automated DNA sequencing technologies (Anderson and Cairney 2004). T-RFLP utilizes fluorescently labeled forward and/or reverse primers resulting in terminal restriction fragments (TRFs) containing labeled primers. Separation and size detection of fluorescently labeled TRFs is performed with a DNA sequencer where labeled fragments are recognized by the fluorescence detector.

18.5.5.3.1 Protocol: T-RFLP

Procedure

Equipment and Plasticware Thermocycler, incubator, capillary DNA sequencer.
Reagents Fluorescently labeled primers, dNTPs, *Taq*-polymerase, PCR buffer (sterile), Milli-Q filtered water, restriction enzymes and buffers, sizing standard, formamide, PCR purification method (e.g. QIAquick PCR purification kit). (1) Perform PCR (50-μL reaction volumes) using fluorescently labeled forward and reverse primers (0.2 μM of each primer); (2) Run PCR products on agarose gel; (3) Purify PCR product using a commercial kit such as QIAquick PCR purification kit (Qiagen) or ChargeSwitch®

PCR clean-up kit (Invitrogen); (4) Digest PCR product with restriction enzymes and recommended buffers according to manufacturer's protocol (see RFLP protocol above); (5) Incubate at recommended temperature depending on enzymes used; (6) Add 1.0 μL digestion product (diluted to optimum concentration) to 9 μL formamide and 0.5 μL of GS-500 ROX size standard (Applied Biosystems); (7) Denature at 95 °C for 5 min; immediately chill on ice; (8) Perform capillary gel electrophoresis on an ABI 3100 genetic analyzer (Applied Biosystems) or equivalent system.

Other Considerations

Amplification efficiency of fluorescently labeled primers tends to be low compared to unlabeled primers, which frequently leads to lower yields of PCR product. Since the output of T-RFLP is digital, specialized software is needed for determining TRF sizes and intensities. Peak Scanner™ and GeneMapper® are software programs available from Applied Biosystems that can be used with Applied Biosystems Genetic Analyzers. One major limitation of T-RFLP analysis is that sequence data cannot be produced from T-RFLP peaks, thus making it difficult to identify unknown taxa. In addition, in order to match unknown T-RFLP peaks to previously identified T-RFLP peaks, a robust local database must be created. As with RFLPs, enzyme selection is critical since closely related species can generate similar TRFs. In mixed-template environmental samples, rare taxa may be overlooked by T-RFLP because they are represented by relatively low amounts of DNA (Burke et al. 2005). Despite some limitations, T-RFLP is a relatively efficient and effective method for characterization of ECM communities.

18.5.5.4 DNA Sequencing

DNA sequencing allows determination of the nucleotide sequence of a given DNA segment. Sequencing is the most accurate technique to identify fungal species. Sequencing is applied to PCR products either directly or after cloning. The most popular sequencing method, 'chain termination' method, was developed by Sanger et al. (1977) and is based on use of labeled-dideoxinucleotides (ddNTPs) as DNA chain terminators, since they lack an OH group on the 3 carbon atom, necessary to form the linkage with other nucleotide. These fluorescently labeled ddNTPs are added together with non-labeled dNTPs to the sequencing reactions. Each time the chain incorporates a labeled ddNTP, this nucleotide constitutes the end of the chain. Thus, the reaction results in fragments of DNA with different lengths that only differ by one base from each other (Fig. 18.5). These fragments are heat denatured and separated by polyacrylamide gels. A laser within an automated DNA sequencing machine is used to analyze DNA fragments produced. Therefore, the composition and order of the whole sequence can be obtained, reading all single-stranded sequences from the fixed point to the last specific base. It is now possible to perform the reaction using a commercial kit that provides the required reagent compo-

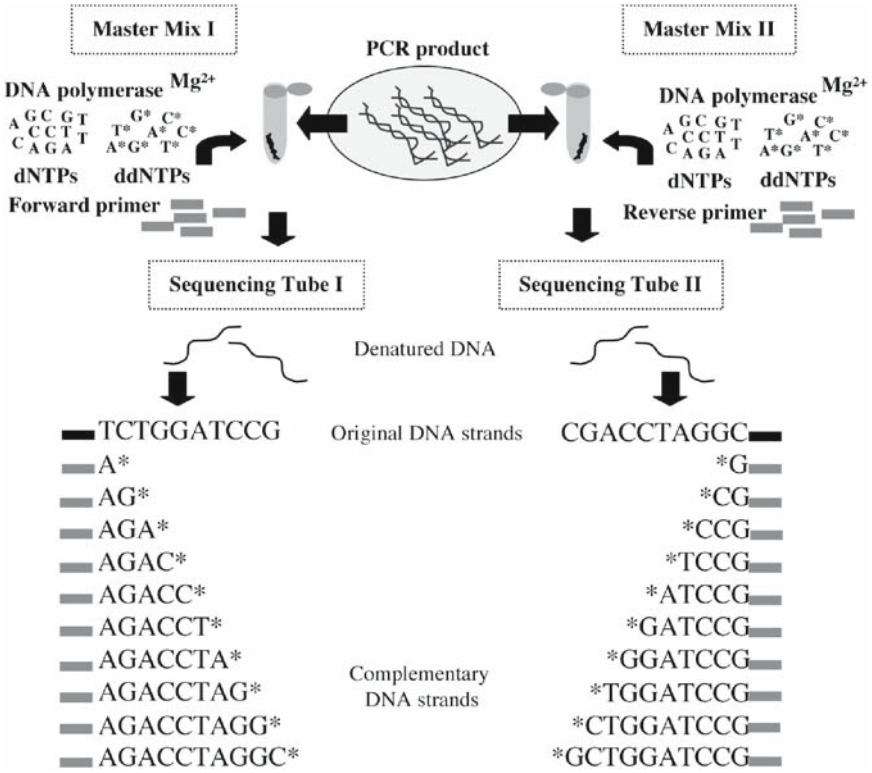

Fig. 18.5 Schematic diagram of the 'Chain Termination' sequencing method (Sanger et al. 1977). Two reactions per sample corresponding to both strands of the DNA PCR product are detailed

nents for the sequencing reaction (cycle sequencing) in a pre-mixed format. The user only provides the DNA template and the template-specific primer/s.

18.5.5.4.1 Protocol: DNA Sequencing from a PCR Product Using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems)

Procedure

Equipment and Plastic ware Instrument Platform (ABI PRISM® 3700 DNA Analyzer; ABI PRISM® 3100 Genetic Analyzer; ABI PRISM® 3100-Avant Genetic Analyzer ABI PRISM® 310 Genetic Analyzer or all models of ABI PRISM® 377 DNA Sequencer), thermocycler (GeneAmp® PCR Systems 9700, 9600, 2700 or 2400), 1.5-mL microcentrifuge tubes/96-well plates (depending on the thermocycler used), micropipettes, vortex.

Reagents DNA template, primers (forward-reverse) complementary to the sequence of the DNA template, BigDye Terminator v3.1, Cycle Sequencing Kit.

Purification of the PCR Product (1) Purify PCR product to sequence using a commercial kit such as QIAquick PCR purification kit (Qiagen) or EZNA® Cycle Pure kit (Omega Bio-Tek). If nonspecific PCR products are detected when the PCR product is run out on agarose gel, the PCR product purification can be performed by excising the band from the gel and using a gel purification kit such as QIAquick Gel PCR purification kit (Qiagen). Single PCR products can be purified using Exo-Sap enzymes (Sigma).

DNA-Quantification of the PCR Product (2) Quantify purified DNA by gel electrophoresis or by spectrophotometry at 260 nm (Sect. 5.3; DNA quantification). The amount of DNA necessary depends on the length of the PCR product to sequence. It should range around 2 ng/μL per 100 bp of PCR product.

Sequencing Reaction (3) Distribute the DNA template volume into 1.5-mL microcentrifuge tubes. Keep on ice; (4) Take out from the freezer the components of the kit. Prepare the master mix as indicated in manufacturer's instructions (4 μL of Ready reaction Premix 2.5X, 2 μL of BigDye Sequencing Buffer 5X, 3.2 pmol of primer, 2 ng/μL each 100 bp of PCR product and water to a final volume of 20 μL); (5) Mix well and spin briefly; (6) Add the required volume of master mix to each DNA simple. Mix gently; (7) Place tubes in the thermocycler and follow the corresponded cycling conditions. E.g.: cycling conditions for ITS primers: 25 cycles of denaturation at 96 °C for 10 s, annealing at 50 °C for 5 s and extension at 60 °C for 4 min.

Purification of Extension Products Incorporated dye terminators should be removed before electrophoresis. There are some recommended purification protocols such as ethanol/EDTA, ethanol/EDTA sodium acetate precipitation and plate and spin column purification; (8) Choose the purification protocol supplied by the manufacturers depending on the desired particular application; (9) Send the resultant reactions to an automated sequencer. Each sample will result in a chromatogram of peaks of four colors, each corresponding to one labeled-ddNTP.

Other Considerations

Sequencing options after cloning are described in Sect 5.5 (Cloning technique). The reagents provided by the kit described above are suitable for performing fluorescence-based cycle sequencing reactions on single- or double-stranded DNA templates, on PCR fragments and on large templates (e.g. BAC clones). Users can adjust the quantities of reagents to a final volume < 20 μL.

Troubleshooting When the chromatogram shows peaks on top of peaks or peaks with a weak signal, it is probably due to the low quality of the DNA template or to the presence of contaminants: include a control DNA template in each run of sequencing to determine whether failed results are due to DNA quality or to a failure in the sequencing reaction. High concentrations of EDTA from the TE buffer used to resuspend the DNA in its isolation inhibit the BigDye reaction. Do not use

too much EDTA in the DNA samples and follow recommended procedures detailed in the user manual for this kit. If the thermocycler used is not from Applied Biosystems you may need to optimize cycling conditions. DNA sequencing protocols will vary depending on the sequence systems and equipment used. Sequencing results can be compared to a number of publicly available fungal databases, including NCBI GenBank (http://www.ncbi.nlm.nih.gov/), UNITE (http://unite.ut.ee/) and mor (http://mor.clarku.edu/).

18.5.5.5 Cloning

While direct sequencing can be used for single-species PCR products, cloning or creation of clone libraries is a technique that can be used for environmental samples that produce mixed PCR products. In samples with multiple species of ECM fungi, the mixed product generated by PCR requires separation by cloning into a suitable vector. Several methods exist for cloning PCR-derived DNA fragments. The T/A cloning method is used because of convenience and efficiency. T/A cloning depends on the terminal transferase activity of *Taq*-polymerase, which adds a single deoxyadenosine (A) to the 3´ ends of PCR products. Individual PCR products are then inserted into linearized vectors with single overhanging 3´ deoxythymidine (T) residues. Next vectors are integrated into bacterial cells resulting in bacterial colonies that contain different fragments of fungal DNA. Since an efficient cloning reaction produces hundreds of colonies, profiling techniques (e.g. RFLP or DGGE/TGGE) can be used to screen clones and reduce the total amount of sequencing needed. Two cloning systems commonly used for construction of clone libraries in ECM studies are the TOPO TA Cloning® Kit (Invitrogen) and the pGEM®-T vector systems (Promega).

18.5.5.5.1 Protocol: Cloning (This Modified Protocol Uses Invitrogen's TOPO TA Cloning® Kit for Sequencing)

Procedure

Equipment and Plasticware Sterile toothpicks, 96-well microtiter plates, 0.5-mL microcentrifuge tubes, micropipettes, microcentrifuge, thermoblock 42 °C, 37 °C shaking and non-shaking incubator, thermocycler.
Reagents PCR Product, plasmid vector, *E. coli* competent cells, S.O.C. media (2% Tryptone, 0.5% Yeast Extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose), LB (Luria-Bertani) agar plates with 50 µg/mL of ampicillin, LB broth with 50 µg/mL of ampicillin (add ampicillin just before use), sterile Milli-Q filtered water.

PCR (1) Add a 10-min extension time at 72 °C at the end of PCR cycling to facilitate addition of 3´A-overhangs on the PCR products.

Ligation (Day 1) (2) Incubate LB plates at 37 °C; (3) Set up 6-µL cloning reaction: (a) add 4.0 µL of fresh (see note below) PCR product to a 0.5-mL-microcentrifuge tube, (b) add 1.0 µL of sterile Mili-Q filtered water, (c) Add 1.0 µL of plasmid

vector (pCR®4-TOPO®); (4) Mix gently and centrifuge briefly; (5) Incubate reaction for 20 min at room temperature and then put on ice.

Transformation (Day 1) (6) Thaw One Shot® TOP10 chemically competent cells on ice; (7) Add 2 μL of cloning reaction to each tube with TOP10 cells; (8) Mix gently and incubate on ice for 30 min; (9) Heat shock at 42 °C for 30 s; then transfer immediately to ice; (10) Add 260 μL of room temperature S.O.C. medium to each tube; 11) Place in incubator at 37 °C on shaker (200 rpm) for 1–1.5 h; (12) Spread 100 μL and 150 μL of each transformation onto prewarmed LB plates; (13) Invert plates (to prevent condensation) and place in a 37 °C incubator overnight (~12 h).

Picking Colonies (Day 2) (14) Dispense 100 μL of LB broth with ampicillin into each well of a 96-well microtiter plate; (15) Gently touch tip of sterile toothpick to a single colony (without dipping into agar); (16) Tap or swirl toothpick into well with LB broth and then carefully remove toothpick; (17) Incubate plate at 37 °C overnight.

Analyzing Clones (Day 3) (18) Perform PCR using <0.5 μL of LB culture mixture as template; (19) Run PCR products on 1.5% agarose gel; (20) Use profiling techniques to screen PCR products and then sequence representative clones.

Note Fresh PCR products should be used for cloning because terminal deoxyadenosines are susceptible to cleavage from repeated freezing and thawing.

Other Considerations

PCR amplification of environmental samples with complex DNA mixtures can result in generation of chimeric sequences (O'Brien et al. 2005; Jumpponen 2003). Chimeric sequences occur when a DNA fragment of one gene anneals with a homologous template to prime the next cycle of DNA synthesis. Formation of chimeric sequences can result in the identification of non-existent fungal species and overestimates of species diversity. Reducing the number of PCR cycles (fewer than 20) and increasing PCR extension times can minimize formation of chimeras (Suzuki and Giovannoni 1996; Qiu et al. 2001; Acinas et al. 2005). Computer programs such as Recombination Detection Program (Martin and Rybicki 2000) and Chimera Check from the Ribosomal Database Project II (Maidak et al. 2001) can be used to detect possible chimeric sequences. PCR bias and cloning bias can also influence the relative frequencies of DNA fragments recovered from cloning of mixed-template reactions. Cloning bias results from preferential cloning of certain DNA fragments while PCR bias is a result of unequal amplification of certain templates. The following modifications are recommended in order to minimize PCR bias in construction of environmental clone libraries: (1) combine several independent replicate PCR amplifications; (2) use low numbers of PCR cycles and (3) use low annealing temperatures (Suzuki and Giovannoni 1996; Qiu et al. 2001; Acinas et al. 2005). The number of clones to analyze per sample depends on the objectives of the study, the diversity of the sample, the importance of detecting

rare species and the availability of time and financial resources. Landeweert et al. (2003a) determined that analyzing 30 clones per soil sample was sufficient for detecting the most common ECM species. Sequencing 50 clones per sample resulted in reasonable detection of fungal diversity in soil samples from a natural grassland (Anderson et al. 2003). Screening of clones with a profiling technique such as RFLP or denaturing gradient gel electrophoresis (DGGE) (Middleton et al. 2004) can greatly reduce the total amount of sequencing needed. For example, Smith et al. (2007) and Morris (2006) used RFLPs to screen 48 clones from samples of pooled EM root tips. Once it has been determined which clones will be sequenced there are two options for sequencing. Plasmid DNA can be extracted from the *E. coli* cells and sequenced directly or PCR can be performed with the clone culture mixture and then the PCR products purified and sequenced. Sequencing can be performed using primers that flank the vector cloning sites or primers used in the original PCR.

18.5.5.6 Real-Time PCR – Quantitative PCR

Real-time PCR measures the quantity of nucleic acid target in a DNA sample. Using this technique it is possible to estimate copy number or the amount of DNA target in the exponential phase of the PCR reaction rather than at the end, when increment of fluorescence truly correlates to the amount of DNA target. DNA synthesis is monitored using DNA-binding dyes such as fluorophore SYBR® green I (Invitrogen, Life Technologies). This dye binds to double-stranded DNA, but not to single stranded. As more PCR product is produced, more fluorescence is registered from SYBR® green. Another way to screen formation of copies of the target DNA through the reaction is by registering the fluorescence generated by fluorogenic target-specific probes, such as Real-time TaqMan™ PCR system (also known as fluorogenic 5´ nuclease chemistry). Copies of the DNA target emit fluorescence that is recorded in each cycle, and this fluorescent signal increases with number of copies. To determine the starting amount of the DNA target in an unknown sample, it is necessary to measure its Ct (cycle number at which fluorescence crosses the threshold) and interpolate this value in a determined standard curve. Confirmation of the identity of the PCR product is performed by examining the thermal denaturation plots or melting curves (Fig. 18.6), since the melting temperature of the PCR product mainly depends on the nucleotide composition. Formation of the PCR product can be monitored throughout the reaction, thus, it is possible to adjust the exact number of PCR cycles needed. Moreover, Real-time PCR does not need post-PCR processing, saving time. The availability of a technique such as Real-time PCR has opened a door for quantification of specific ECM fungi in environmental samples, allowing evaluation of their ecological and functional importance in different ecosystems. This technique is able to detect twofold changes in concentration of the target DNA, being very useful to estimate changes in fungal biomass.

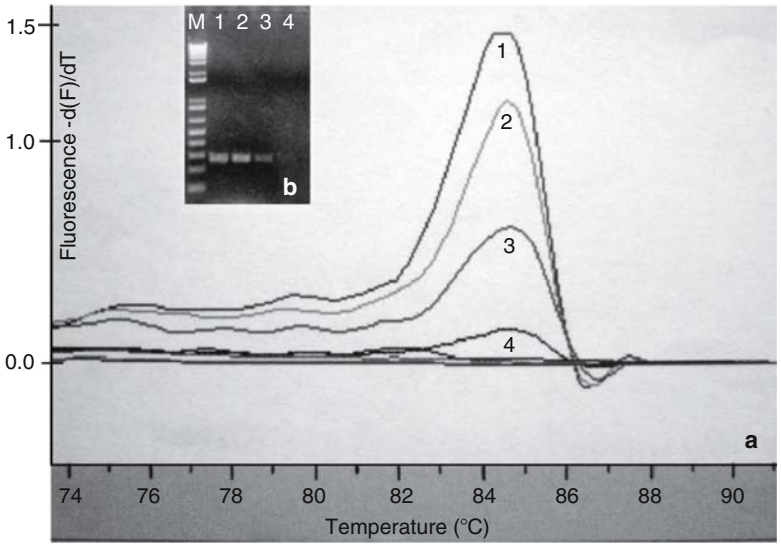

Fig. 18.6 Diagrams of **a** melting curves of tenfold dilution series of DNA from *T. melanosporum* obtained after Real-Time PCR with the LightCycler (Roche) system using SYBR® Green dye I, and **b** gel analysis after the PCR reaction: (1): undiluted simple; (2) to (4) dilutions 10^1, 10^2 and 10^3; (M) 1 kb plus DNA ladder. The lower the DNA concentration, the lower melting peaks (fluorescence emitted) and band intensities detected

18.5.5.6.1 Protocol: Real-Time PCR

Procedure (Using ABI Prism 7700 Equipment; Applied Biosystems)

Equipment and Plasticware ABI Prism® 7700 equipment, 96-wells plates, Sequence Detection System (Applied Biosystems), software version SDS 1.9.1 and Dissociation Curves 1.0, 1.5-mL microcentrifuge tubes, micropipettes, vortex.
Reagents Sterile Milli-Q water, primers, template DNA, known and unknown samples, agarose, molecular weight marker (DNA ladder), TAE1X or TBE1X, load buffer.

Establishment of a Calibration Curve (1) Establish a calibration curve with serial dilutions of a known amount of DNA template, a known number of copies of target DNA (standard) or plasmid DNA. The system determines the calibration curve and plots the Ct of these serial dilutions obtained after a certain number of PCR cycles, vs $\log_{10}$ of the quantity of target DNA ($\log_{10}$ (quantity)). Each calibration standard should be tested in duplicate or triplicate in each different run. The amount of target DNA in each unknown sample is calculated by interpolating the Ct value in the standard curve. The r^2 of the standard curve should be >0.99. Specificity of the designed primers, linearity of the reactions and sensitivity of the Real-time PCR should be checked.

Tips Serial dilutions of the standard should contain the correct amount of DNA target. Check the absolute quantity of DNA in each standard. Prepare aliquots of each dilution and freeze them at −80 °C, thaw only once before use.

Real-Time PCR Reaction (2) Prepare the PCR cocktail, preferably one cocktail per group of sample replicates. Calculate the quantity of each PCR component for all samples, containing: 0.2 µM of each primer, 12.5 µL of SYBR® Green PCR Master Mix and 10.5 µL of sterile Milli-Q water. Include DNA-free, negative and positive controls for each cocktail in each plate, vortex to homogenize the mixture; (3) Distribute 24 µL of the PCR cocktail in each well; (4) Add 1 µL of DNA template previously vortexed; (5) Cover the plate, place in thermocycler and program, e.g. as follows: (a) incubation step at 95 °C for 10 min; (b) DNA amplification for 20–40 cycles of 95 °C for 30 s, 58 °C for 30 s and 72 °C for 1 min. Annealing temperature will depend on the primers used and extension time will depend on the length of the PCR product. Number of cycles can be adjusted after monitoring the formation of the PCR product all over the reaction; (6) Program the melting curve temperature profile by, e.g. 95 °C for 1 min, 60 °C for 1 min and heating to 95 °C in 20 min; (7) Analyze data with the software version SDS 1.9.1 and Dissociation Curves 1.0.

Additional Confirmation of PCR Product (8) Confirm product identity by electrophoresis in a 2% agarose gel; (9) Visualize the gel on a transilluminator.

Notes Recommended primer concentration in SYBR® Green reactions is 50 mM, products should be ~150 bp on length (the shorter, the better) and the primers should not include more than two G or C bases in their last five bases. The PCR product obtained can be used for sequencing.

Other Considerations

Application of Real-Time PCR to ECM Studies Real-time PCR has recently been applied to ECM fungal community studies not only for fungal quantification (Landeweert et al. 2003b; Anderson and Parkin 2007) but also for ecological and functional studies such as the examination of gene expression (Miozzi et al. 2005), investigation of spatial distribution and temporal persistence of mycelia (Kennedy et al. 2007) and determination of ECM fungal competition (Kennedy et al. 2007; Parladé et al. 2007).

Optional Real-Time PCR Systems Other Real Time systems can be used such as the LigthCycler™ (Roche Molecular Biochemicals) (Landeweert et al. 2003b), RotorGene 3000 centrifugal amplification system (Corbett Research) (Martin and Rygiewicz 2005) or Bio-Rad iCycler iQ Multi-Color Real Time PCR Detection System (Guescini et al. 2003).

Troubleshooting if amplification does not appear, dilute or purify the DNA extracts. Losses of DNA occur after purification. If primer-dimmers are formed, raise the annealing temperature or the temperature when primers are not in a double strand. If unsuccessful, add lower concentrations of primers or design a new pair. The fluorophore SYBR® Green I dye does not distinguish target DNA from non-target DNA, thus, it is essential to use a good specific primer pair and to examine

the melting curves to confirm the PCR product. When working with ITS regions, it is necessary to perform a calibration for each species. Quantification of a biomarker gene present in a known number of copies is another possibility (Raidl et al. 2005).

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