

Chapter 10

Root-Associated Ectomycorrhizal Mycobionts as Forest Biofertilizers: Standardized Molecular Methods for Characterization of Ectomycorrhizal Wood Wide Web



Rezwana Assad, Zafar Ahmad Reshi, and Irfan Rashid

Abstract Ectomycorrhizal (ECM) fungi play a crucial role in nutrient mobilization and cycling, particularly in temperate forests dominated by coniferous species. The belowground ectomycorrhizal Wood Wide Web interconnects innumerable host plants and serves as a sustainable continuum for plant and soil health in forest ecosystems. Conifers, particularly conifer roots harbouring ectomycorrhizal fungi, are rich in phenolics and other secondary metabolites, which interfere and hamper their DNA extraction and inhibit all downstream processes like amplification and sequencing. The present study was projected for presenting the standardized molecular methodology for characterization of ectomycorrhizal fungi from conifer roots, starting from extraction of high-quality DNA and its PCR amplification, followed by DNA purification and loading, to final sequencing, all things reflected in a chronological manner. This chapter highlights the role of root-associated ectomycorrhizal fungi as biofertilizers in forest ecosystems and efficient molecular methods specially optimized for characterization of ectomycorrhizal fungi associated with conifers.

Keywords Biofertilizers · Ectomycorrhiza · Forestry · Microbiota · Molecular characterization · Soil health

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10.1 Introduction

Belowground microbiota is an imperative constituent of forest ecosystems. The rhizospheric microbiota comprises of diverse microorganisms including actinomycetes, algae, archaea, bacteria, fungi, protozoa, and viruses (Tarkka et al. 2018; Adeleke et al. 2019). Amongst these ecologically important communities, ectomycorrhizal (ECM) fungi are essential acquaintances of this intricate forest microbiota that function as biological linkages between diverse assemblage of forest organisms and serve as a sustainable continuum for plant and soil health in forest ecosystems (Molina 1994). Root-associated ECM fungi are a ubiquitous group of microorganisms that correlate plants through a huge belowground hyphal network, which facilitate interplant metabolite passage (Chatterjee et al. 2019; Domínguez-Núñez et al. 2019). This belowground diverse mycorrhizal network that interconnects rootlets of innumerable plants has been named as “common mycorrhizal networks (CMN)” or “Wood Wide Web” (Giovannetti et al. 2006; Simard 2012; Martin et al. 2016; Adeleke et al. 2019). ECM fungi through these hyphal networks enhance nutrient acquisition capability of host plants by extending their root surface area and facilitate interplant communications (Bücking et al. 2012; Adeleke et al. 2019).

The species-level identification of individual ECM mycobionts is a prerequisite for understanding the ecological significance of this symbiotic association (Gil-Martínez et al. 2018). However, these plant-fungal species interactions are poorly studied, primarily because of methodological limitations to the accurate identification of these mutualists. The study of taxonomy and structural and functional diversity of ectomycorrhizal fungi has proven reasonably exigent (Gil-Martínez et al. 2018). Isolation of DNA from ectomycorrhizal roots is intricate due to the presence of tough chitin cell wall, co-occurrence of host plant cells along with fungal cells, and co-precipitation of secondary metabolites of host plant which obstruct downstream processes like PCR and sequencing (Janowski et al. 2019).

Initially, ECM diversity studies were primarily based on sporocarp analysis (Horton and Bruns 2001; Domínguez-Núñez and Albanesi 2019). However, in view of the poor correspondence between the diversity of ECM based on survey of aboveground ECM fruiting bodies and ECM actually colonizing roots of host species, sampling and screening of conifer roots for associated ECM fungi employing morpho-anatomical and standard molecular methods was undertaken. Furthermore, recent advancements in molecular research and DNA sequencing technologies have made exceptional contribution to our understanding of ECM fungal diversity, ecology, and biogeography (Horton and Bruns 2001; Nilsson et al. 2011; Smith and Peay 2014; Domínguez-Núñez and Albanesi 2019; Janowski et al. 2019).

This chapter highlights the role of root-associated ectomycorrhizal fungi as bio-fertilizers in forest ecosystems and efficient molecular methods for characterization of ectomycorrhizal fungi associated with conifers. Moreover, this chapter also divulges a comprehensive deliberation of chemical concentrations, their preparations, and corporations from which these chemicals were procured.

10.2 Root-Associated Ectomycorrhizal Fungi as Forest Biofertilizers

Biofertilizers encompass a live formulation of beneficial microbes which, on application to soils, plant surfaces, or seeds, colonize rhizosphere or plant interior and elevate growth by escalating the supply or accessibility of crucial nutrients to the host plant (Vessey 2003; Malusá et al. 2012; Mahanty et al. 2017; Thomas and Singh 2019). *Amanita* spp., *Hebeloma* spp., *Laccaria* spp., *Pisolithus tinctorius*, *Piriformospora indica*, *Rhizopogon luteolus*, *Suillus luteus*, and *Tuber melanosporum* are some ECM fungi that have been used as forest biofertilizers (Marx and Cordell 1989; Domínguez et al. 2006; Schwartz et al. 2006; Chavez et al. 2014; Pal et al. 2015; Sharma 2017; Domínguez-Núñez and Albanesi 2019; Domínguez-Núñez et al. 2019). ECM fungal biofertilizers serve as a natural, effective, economic, non-bulky, productive, and eco-friendly substitute of synthetic chemical fertilizers and pesticides (Pal et al. 2015; Bhat et al. 2017; Mahanty et al. 2017; Thomas and Singh 2019).

In forest ecosystems, ectomycorrhizal fungi are directly involved in nutrient cycling (Domínguez-Núñez et al. 2019). One of the most effective forest management strategies for regeneration and reinstatement of degraded forest ecosystems is the use of ECM fungi as promising biofertilizers to improve survival, growth, health, and establishment of seedlings (McAfee and Fortin 1986; Adeleke et al. 2019).

Ectomycorrhizal biofertilizers boost plant health and concurrently improve sustainability and health of the soil (Bhardwaj et al. 2014; Nuti and Giovannetti 2015; Pal et al. 2015; Bhat et al. 2017; Vecstaudža et al. 2018; Chatterjee et al. 2019), through processes like solubilization/mobilization of soil nutrients (mainly phosphorus and nitrogen), escalation of long-term soil fertility and soil aeration, mounting efficient uptake of water and nutrients by increasing surface area of host plant roots, repression of soil borne diseases, and production of plant growth-promoting substances into the soil (Mridha 2003; Malusá et al. 2012; Nuti and Giovannetti 2015; Pal et al. 2015; Mahanty et al. 2017; Fraç et al. 2018; Chatterjee et al. 2019; Thomas and Singh 2019).

The common ECM species associated with different coniferous hosts can be utilized for mass production of inoculum for in vitro mycorrhization of conifer seedlings in forest nurseries. This in vitro mycorrhization of tree seedlings has become the essential ingredient of successful reforestation programme because anthropogenic activities, like deforestation, urbanization, altered land-use pattern, pollution, etc., severely impair the ECM diversity in soil, which results in reduced natural regeneration.

Main sources of ectomycorrhizal inoculum are forest soil, chopped ECM roots, spores of fruiting bodies, and pure mycelial inoculum (Sim and Eom 2006; Domínguez-Núñez et al. 2019). Several biofertilizers consist of single ECM strain; however, numerous strains in the form of consortium have been also employed, which promote plant growth through diverse mechanisms (Pal et al. 2015; Vecstaudža

et al. 2018). However, the appropriate choice of apposite host-mycobiont is indispensable for the success of mycorrhization (Olivier 2000).

Various polymicrobial formulations like mycorrhizal helper bacteria (MHB) and other beneficial rhizospheric microbes have the capability to facilitate ectomycorrhiza formation and act in synchrony with ectomycorrhizal symbionts (Saravanan and Natarajan 1996, 2000; Schrey et al. 2012; Chatterjee et al. 2019; Domínguez-Núñez et al. 2019). A better understanding of mycorrhizospheric microbiota is crucial for comprehending the functional aspect of these symbionts for their exploitation in sustainable forest management and environmental protection (Tarkka et al. 2018; Janowski et al. 2019).

10.3 Standardized Molecular Methods for Characterization of Ectomycorrhizal Wood Wide Web

10.3.1 Sampling of ECM Root Tips

ECM root samples of various conifer species like *Abies pindrow* (Royle ex D. Don) Royle, *Cedrus deodara* (Roxb. ex D. Don) G. Don, and *Picea smithiana* (Wall.) Boiss. were collected from Naranag, Ganderbal province (N34° 22' 16"; E74° 59' 45"), of Kashmir Himalaya, for molecular characterization of associated ECM diversity. A spade was used to collect soil cores, starting from the organic layer, after removal of litter layer. All the samples were collected in sealed polybags and were stored at 4 °C before being processed, but were not held longer than 2 weeks.

Prior to analysis, the soil cores were immersed in water and soaked carefully in water until saturated. Fine ECM-infected root tips (short lateral roots) were gently rinsed under a cold running water in a 1 mm sieve to limit damage to the ectomycorrhizas. Cleaned lateral roots were placed in a Petri dish containing double distilled water, to avoid drying up of delicate root tips (Fig. 10.1). Fine root tips were carefully sorted from the main roots and processed for isolation of fungal DNA.

10.3.2 Scanning Electron Microscope and Compound Microscope Study of Ectomycorrhizal Roots

To observe surface features of ectomycorrhizal roots with scanning electron microscope (SEM), tertiary roots of the studied species (showing a well-developed mycorrhizal sheath) were excised and prepared for SEM study by the method of Kinden and Brown (1975) and Chung et al. (2003).

The ectomycorrhizal roots were rinsed twice with 0.1 M sodium phosphate buffer solution (pH 7.3) and fixed in 2.5% glutaraldehyde solution at 4 °C for 2 h, then washed it off with 0.1 M sodium phosphate buffer solution (pH 7.3), and post fixed

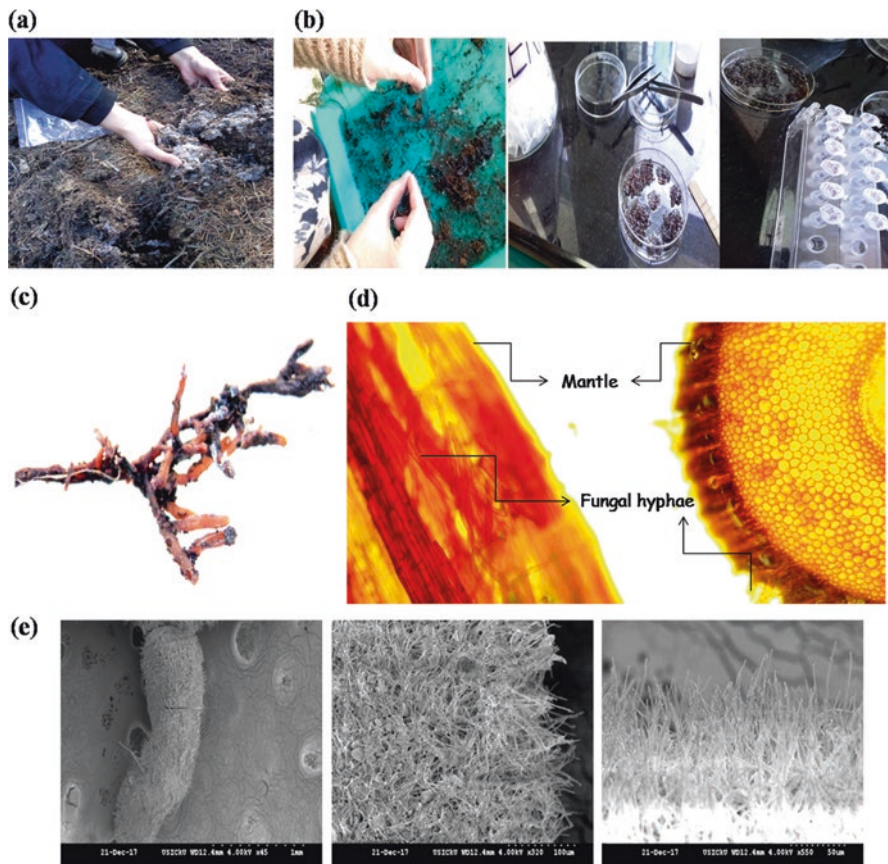


Fig. 10.1 (a) White-coloured ECM mycelium in forest soil, (b) processing conifer ECM root material in laboratory, (c) association of ectomycorrhizal fungi with conifer roots, (d) mantle peel with fungal hyphae and transverse section of ectomycorrhizal root showing distinct mantle and emanating hyphae, (e) scanning electron micrographs showing external surface features of ectomycorrhizal roots with projected fungal hyphae

the sample with 1% osmium tetroxide (1% OsO₄ in 0.1 M cacodylate buffer). Post-fixed specimens were rinsed thrice with 0.1 M sodium phosphate buffer solution (pH 7.3) for around 40 min. These samples were then dehydrated with 50%, 70%, 80%, 90%, 95%, and 100% ethanol for 20 min, followed by treatment with 100% isoamyl acetate for 40 min. Subsequently, these samples were dried up. After attaching the carbon tape on aluminium stub, these samples were pasted carefully on it and were coated with gold using a sputter coater and were then observed with SEM (Hitachi S-3000H).

The morpho-anatomical structural details of mantle, Hartig net, and emanating hyphae of ECM roots were observed via mantle peels and thin hand-cut transverse sections of ectomycorrhizal root. These sections were gently cleared in hot alkali

(10% KOH), stained overnight with Aniline blue (0.5%) or Trypan blue (0.5%), followed by destaining with 10% lactic acid. These were then examined at 4x, 10x, and 40x magnification under a compound microscope (Magnus MLX LED).

10.3.3 Molecular Methods for Characterization of Ectomycorrhizal Fungi

Molecular characterization of root-associated ECM was done by extracting genomic DNA from the root tips of the studied species, followed by the amplification of the ITS region using universal primers and universal fungal-specific primers. The amplified products were sent for sequencing. The sequences were then identified by performing BLAST searches on GenBank (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) and UNITE (<https://unite.ut.ee/>).

10.3.3.1 Protocol for ECM Root Tip DNA Extraction

Modified CTAB (cetyltrimethylammonium bromide) protocol was employed for DNA extraction of ectomycorrhizal roots of selected conifer species:

- (i) **Mechanical lysis:** 2–3 frozen root tips were taken in a 2 ml microcentrifuge tube (having a flat base). This material was crushed by a sterilized cold Micro Pestle (made of polypropylene, designed for crushing a material inside a microcentrifuge tube), without using liquid nitrogen.
- (ii) **Chemical lysis:** 1 ml prewarmed isolation buffer (freshly prepared) was poured immediately into each microcentrifuge tube and mixed well. The tissue was completely homogenized in buffer. (Optional step: Samples can be pulverized further after addition of isolation buffer.) The composition of isolation buffer is given in Table 10.1.

Table 10.1 Composition of isolation buffer

S. No.	Stock	Working (for five reactions)
1.	CTAB (10%)	1 ml
2.	NaCl (5 M)	1.4 ml
3.	EDTA (0.5 M) (pH = 8)	0.2 ml (200 μ l)
4.	Tris-HCl (1 M) (pH = 8)	0.5 ml (500 μ l)
5.	β -Mercaptoethanol (2-mercaptoethanol) (99.0%)	0.005 ml (5 μ l)
6.	Double distilled water	To make the volume up to 5 ml (like 1.9 ml was added)
7.	PVP (1%) or powdered – optional	–

All the reagents of isolation buffer and other requisite material like microcentrifuge tubes, tips, etc. were autoclaved, prior to use. Use of prewarmed and freshly prepared isolation buffer provided better results. Preparation and concentration details of CTAB reagents are stated in the supplementary material.

- (iii) **Incubation:** The samples were then incubated at 65 °C for 1 h (in a water bath) with occasional mixing by gentle inversion of the microcentrifuge tubes.
- (iv) **DNA purification:** For purification purposes, the tubes were centrifuged at 12,000 RPM for 10 min, and the supernatant (upper layer) was collected in a fresh microcentrifuge tube. The cell debris was discarded along with the tube.
- (iv-a) **Protein denaturation and removal step:** Equal amount of chloroform:isoamyl alcohol (CI-Mix) (24:1, v/v) was added to each tube (like 500 µl CI-Mix was added to the 500 µl collected supernatant). The mixture was emulsified by inversion of the tube. The mixture was then centrifuged at 12,000 RPM for 5 min, and the upper layer was pipetted out to a fresh tube. Repeat CI treatment if samples seem unclean. (The upper aqueous phase must be taken off carefully without touching the lower layer or else the DNA will get contaminated; furthermore, wide-bore tips should be used in this step; otherwise, the DNA will get sheared.)
[Optional step: Chloroform- or Tris-saturated phenol:chloroform:isoamyl alcohol (PCI-Mix) (25:24:1, v/v) can be used instead of CI-Mix.]
- (iv-b) **RNA removal step:** For removal of RNA, 3–4 µl of RNase A (Sigma or Qiagen) was added to each tube, followed by incubation at 37 °C for 1 h (in an incubator). The tubes were occasionally mixed by gentle inversion. To remove additional unused RNase, 500 µl of chloroform:isoamyl alcohol was added and mixed by inversion. Then the samples were centrifuged again at 12,000 RPM for 5 min. The supernatant (upper phase) was collected in a fresh tube.
- (v) **DNA precipitation:** The DNA was precipitated in these tubes by adding 750 µl (or two-third volume) of ice-cold isopropanol. These tubes were gently inverted for proper mixing and precipitation of DNA and were then kept overnight at –20 °C in a deep freezer. These samples were then centrifuged at 13,000 RPM for 20 min. The upper phase (most of the supernatant) was discarded by pouring, and the pellet was collected. Nucleic acid concentrations are minute and transparent in nature, so even if there is not any noticeable pellet in the tube, DNA can be still there. (The samples can be even kept for only 1 h in a deep freezer; increase the time according to the quantity of pellet required; however, keeping tubes for longer duration for DNA precipitation also co-precipitates impurities and inhibitors with it, which further hinder the downstream processes.)
- (vi) **DNA washing:** The pellet was washed with 200 µl of 70% ice-cold ethanol and was centrifuged at 8000 RPM for 5 min. The upper phase was discarded,

and the pellet was dried in an oven at 37 °C in an incubator (do not overdry the pellet; otherwise, it will not get dissolved).

- (vii) **DNA pellet solubilization:** The dried pellet was dissolved in 10–20 µl nuclease-free water. The samples were kept at 4 °C for an hour or so, for proper DNA pellet solubilization. These samples were then stored at –20 °C until further use.

10.3.3.2 Polymerase Chain Reaction (PCR) Protocol

The fungal ITS region of rDNA was amplified by polymerase chain reaction (PCR) by using gene-specific primers (ITS1/ITS2; ITS3/ITS4; ITS1/ITS4; ITS1F/ITS4) in a thermal cycler (Table 10.2). The amplified fragments include ITS1, 5.8S, and the ITS2 of rDNA.

The 20 µl reaction mixture for each PCR reaction contained 2 µl PCR buffer (with MgCl₂), 0.5 µl of 10 mM dNTPs, 0.4 µl of each primer (10 µM), 2 µl template DNA, 0.5 µl of 5% DMSO, 0.5 µl of 0.1% BSA, and 0.2 µl of 5 U/µl Taq polymerase (Table 10.3). Preparing master mix is more convenient and time-saving as compared to setting up each reaction independently.

Amplifications were performed in a thermal cycler (Applied Biosystems) with an initial denaturation step of 95 °C for 10 min, followed by 35 cycles of 95 °C for 1 min, 55 °C for 30 s, and 72 °C for 1 min, and a final extension of 72 °C for 10 min. A negative control reaction was performed with same reaction components and conditions except that nuclease-free water was added instead of DNA template. PCR amplification was confirmed on 1.5% agarose gel. Preparation of PCR reagents is reflected in the supplementary material.

10.3.3.3 Agarose Gel Electrophoresis

The integrity of DNA was checked on 0.8% agarose gels (for genomic DNA) or 1.5% agarose gels (for PCR amplicons). Electrophoresis was performed at room temperature in 1X TAE buffer with an applied voltage of 8–10 V cm⁻¹. The compo-

Table 10.2 Primers used for amplification of ITS region

Forward primer	Reverse primer	T _m (°C)	GC (%)
TCCGTAGGTGAACCTGCGG (ITS1)	GCTGCGTTCTTCATCGATGC (ITS2)	ITS1: 68.5 ITS2: 68.2	ITS1: 63.1 ITS2: 55
GCATCGATGAAGAACGCAGC (ITS3)	TCCTCCGCTTATTGATATGC (ITS4)	ITS3: 68.2 ITS4: 61.5	ITS3: 55 ITS4: 45
TCCGTAGGTGAACCTGCGG (ITS1)	TCCTCCGCTTATTGATATGC (ITS4)	ITS1: 68.5 ITS4: 61.5	ITS1: 63.1 ITS4: 45
CTTGGTCATTTAGAGGAAGTAA (ITS1F)	TCCTCCGCTTATTGATATGC (ITS4)	ITS1F: 56.6 ITS4: 61.5	ITS1F: 36.3 ITS4: 45

Table 10.3 Composition of 20 μ l PCR reaction

Component	Basic concentration	Volume
PCR buffer	10X	2 μ l
dNTPs	10 mM	0.5 μ l
Forward primer	10 μ M	0.4 μ l
Reverse primer	10 μ M	0.4 μ l
Taq polymerase	5 U/ μ l	0.2 μ l
Template DNA	10 μ g	2 μ l
DMSO	5%	0.5 μ l
BSA	0.1%	0.5 μ l
Nuclease-free water		To 20 μ l

Note: Using concentrated DNA template with high DNA content hindered the PCR. So, in order to overcome this, DNA template was diluted by nuclease-free water up to 1:10 (1 μ l DNA:9 μ l water); 1:100 (1 μ l DNA:99 μ l water), or even 1:1000 (1 μ l DNA:999 μ l water)

Table 10.4 Composition of loading dye and TAE buffer

Buffer	Composition
6X DNA loading dye	Glycerol
	Bromophenol blue
	Xylene cyanol
1X TAE buffer	Tris base
	Glacial acetic acid
	EDTA

sition of loading dye and TAE buffer are given in Table 10.4. The gels were visualized on a gel imager after ethidium bromide (0.5 μ g ml⁻¹) staining.

10.3.3.4 Purification of PCR Products

PCR products were purified prior to further downstream analysis using Gene-Jet Gel Extraction Kit (Thermo Fisher Scientific; Cat No. K0691), following manufacturer's instructions. PCR products were resolved on 1.5% agarose gel, and each of the separating bands was cut and placed in separate 1.5 ml Eppendorf tubes. The gel solubilization buffer was added to gel pieces and incubated at 50 °C for 10 min, till the gel dissolved completely. The mixture was then passed through a binding column after addition of 1 volume isopropanol. After proper washing, samples were eluted with elution buffer and stored at -20 °C until use.

10.3.3.5 Sequence Analysis and Identification of ECM Species

The purified PCR products were sent for sequencing (in both directions with respective primers) to Xcelris, Gujarat, India. Each sequence was then separately identified by performing individual nucleotide BLAST searches on GenBank (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) and UNITE (<https://unite.ut.ee/>).

10.4 Findings and Inferences

10.4.1 Morpho-Anatomical Characteristics of ECM Roots

ECM roots are characterized by the incidence of mantle, Hartig net, and extraradicular mycelium. The surface of these ECM root tips was found to be harbouring fine fungal hyphae. ECM fungi grow and extend their hyphal network (mycelial strands or rhizomorphs) into the rhizospheric soil, forming dense mycorrhizal mycelial mats with specialized conducting hyphae (Fig. 10.1). The major distinction between mycorrhizal and dead roots is that mycorrhizal roots are living, coloured, and swollen with a growing mycelium attached to the surface whereas the dead roots are shrivelled and black (Fig. 10.1).

10.4.2 Molecular Characterization of Ectomycorrhizal Fungi

10.4.2.1 Analysis of ECM Root Tip Genomic DNA

The purity and integrity of ECM root tip genomic DNA was checked on 0.8% agarose gel (Fig. 10.2). The DNA quality and quantity was determined by measuring the optical density (OD) at 260/280 nm on spectrophotometer, and samples with OD ranging between 1.7 and 1.9 were selected for PCR amplification of ITS region.

10.4.2.2 PCR Amplification Analysis

The purity and integrity of amplified DNA was checked on 1.5% agarose gel (Fig. 10.3). The amplicons ranged between 250 and 300 bp for ITS1/ITS2; 350 and 450 bp for ITS3/ITS4; 600 and 755 bp for ITS1/ITS4, and 700 and 800 bp for ITS1F/ITS4 primer combinations (Table 10.5). Negative control in which nuclease-

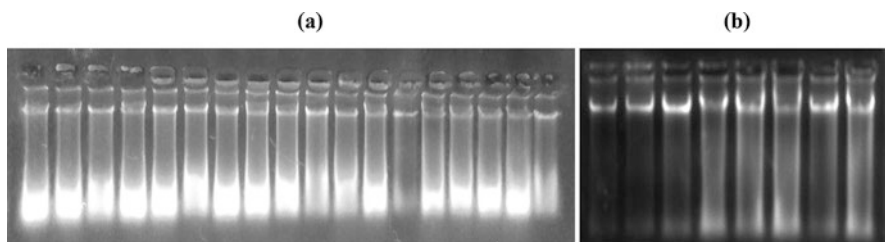


Fig. 10.2 Representative gel for ECM root tip genomic DNA loaded on 0.8% agarose gel. DNA extracted by (a) CTAB method (with RNA) and (b) CTAB method (without RNA after RNase treatment)

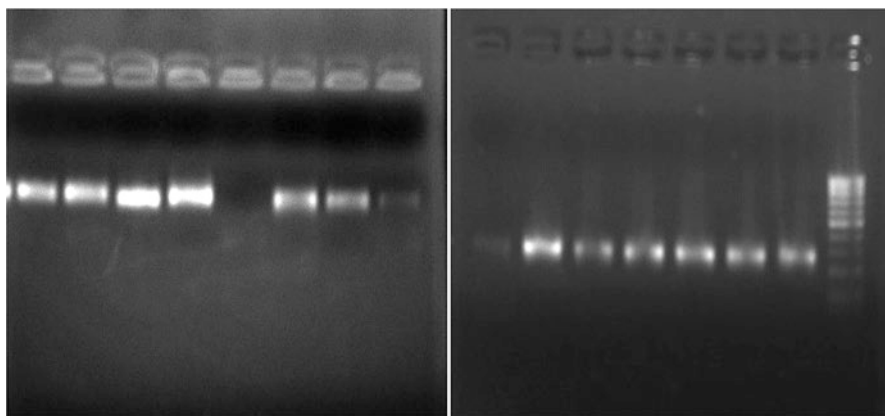


Fig. 10.3 Representative gel for PCR-amplified DNA loaded on 1.5% agarose gel with 100 bp ladder

Table 10.5 Amplicon size obtained using various primer combinations

S. No.	Primer combination	Amplicon size (in bp)	Annealing temperature (°C)
1.	ITS1/ITS2	250–300	55
2.	ITS3/ITS4	350–450	55
3.	ITS1/ITS4	600–755	55
4.	ITS1F/ITS4	700–800	53/55

free water was used instead of DNA template showed no band. Each band was then gel purified for further downstream analysis.

10.4.2.3 Role of Dimethyl Sulphoxide (DMSO)

Various enhancing agents can be employed in PCR reactions to increase specificity and yield. One of those enhancing agents is dimethyl sulphoxide (DMSO), which is used in PCR to interrupt the formation of secondary structures in DNA template or in DNA primers. DMSO formulates hydrogen bond with template and consequently distorts the double helix structure of DNA. This is predominantly useful in templates with high GC content because augmented hydrogen bond strength enhances intricacy of denaturing template and leads to the formation of intermolecular secondary structures, which then compete with primer annealing (Chakrabarti and Schutt 2001). Moreover, DMSO decreases melting temperature needed for separation of both strands of DNA. Thus, addition of DMSO can significantly improve specificities of PCR priming reactions (Frackman et al. 1998; Hardjasa et al. 2010; Jensen et al. 2010).

10.4.2.4 Role of Bovine Serum Albumin (BSA)

Bovine serum albumin (BSA) facilitates specific fragment amplification (Nagai et al. 1998) and enhances PCR amplification yields from low purity templates (Farell and Alexandre 2012). Addition of BSA improves yield of PCR product, by binding fatty acids and phenolic compounds that can inhibit PCR reaction. It also checks adhesion of various PCR reagents to tip and tube surfaces.

10.4.2.5 Why DNA Template Dilutions?

DNA template was diluted by nuclease-free water to 1:10 (1 μ l DNA:9 μ l water); 1:100 (1 μ l DNA:99 μ l water), or even 1:1000 (1 μ l DNA:999 μ l water). Using concentrated DNA template with high DNA content hindered PCR. One of the reasons of diluting DNA template before PCR is to counteract the effect of inhibitors (less DNA: less inhibitors). In addition to this, by limiting the quantity of template, it confines non-specific binding of primers and thus results in the formation of only one specific PCR band.

10.4.2.6 Sequence Analysis and Identification of ECM Species

The sequences were identified by performing BLAST searches on GenBank (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) and UNITE (<https://unite.ut.ee/>) (details in the supplementary material). The outputs from the BLAST searches were sorted on account of the maximum identity and were recorded according to their coverage. Sequence similarity with a cut-off of 90% or greater was considered significant, while others which showed similarity of 80–90% were considered as low confidence score species.

10.4.2.7 Advanced Techniques for the Study of Ectomycorrhizal Microbiome

The Sanger sequencing resulted in the identification of only one fungal species per sample (the most abundant one). The conifer root samples are categorized as environmental samples (contain a mixture of fungi).

Amongst different techniques like cloning, terminal restriction fragment length polymorphisms (T-RFLP), single-strand conformation polymorphism (SSCP), denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), and next-generation sequencing (NGS) for sequencing of environmental samples, NGS is the most advanced, accurate, and reliable technique. Globally, metagenomic NGS techniques like Illumina sequencing technology are

apposite techniques for the study of samples taken directly from diverse environments and plant-associated ectomycorrhizal communities.

10.5 Conclusions

Root-associated ECM fungi, informally named as “Wood Wide Web”, are a ubiquitous group of microorganisms that correlate plants through a huge belowground hyphal network and serve as sustainable continuum for plant and soil health in forest ecosystems. Ectomycorrhizal fungi like *Amanita* spp., *Hebeloma* spp., *Laccaria* spp., *Pisolithus tinctorius*, *Piriformospora indica*, *Rhizopogon luteolus*, *Suillus luteus*, and *Tuber melanosporum* serve as a natural, effective, economic, and eco-friendly substitute of synthetic chemical fertilizers and pesticides. One of the most effective forest management strategies for regeneration and reinstatement of degraded forest ecosystems is the use of ECM fungi as promising biofertilizers, to improve survival, growth, health, and establishment of seedlings. Ectomycorrhizal biofertilizers boost plant and soil health through processes like mobilization of soil nutrients, escalation of soil fertility and aeration, and mounting efficient acquisition of water and nutrients by increasing the surface area of host plant roots. However, the appropriate choice of apposite host-mycobiont is indispensable for the mycorrhization success.

The species-level identification of individual ECM mycobionts is a prerequisite for understanding the ecological significance of this symbiotic association. However, the study of taxonomy and structural and functional diversity of ectomycorrhizal fungi has proven reasonably exigent. Subsequently, sampling and screening of conifer roots for associated ECM fungi employing morpho-anatomical and standard molecular methods was undertaken in the present study.

This chapter highlights the role of root-associated ectomycorrhizal fungi as biofertilizers in forest ecosystems and efficient molecular methods specially optimized for characterization of ectomycorrhizal fungi associated with conifers. A better understanding of mycorrhizospheric microbiota is crucial for comprehending the functional aspect of these symbionts for their exploitation in sustainable forest management and environmental protection.

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Conflict of Interest The authors declare that they have no conflict of interest.

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