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Genetic variability within n-rDNA region of ectomycorrhizal isolates originating from temperate ecosystems

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Identification of Ectomycorrhiza Fungi (ECM) based on morphological characters has been laborious and time consuming, especially samples collected from the environment. Additionally, due to its microscopic nature and limited morphological characters, intraspecies variation is difficult to detect. In view of this, this study aimed at confirming the earlier identification of these fungi, which was based on morphological characters, and also to find suitable molecular restriction fragment length polymorphism (RFLP) markers for the identification of ECM fungi up to the level of species or isolates as part of an expandable database of RFLP patterns of the internal transcribed spacers (ITS) region of ECM fungi. Mycelia of 14 species of ectomycorrhizal fungi representing five genera were isolated in pure culture and characterized by morphological and molecular methods. Molecular identification was performed by analysis of the internal transcribed spacers of the nuclear encoded ribosomal RNA (n-RNA) gene region using restriction fragment length polymorphism (RFLP). The region was first amplified by polymerase chain reaction with specific primers and then cleaved with different restriction enzymes. The degree of polymorphism, although extensive, proved inadequate for proper identification of most of the isolates. Depending on the restriction enzymes used, the genera or species could be grouped on the basis of common fragment patterns, thereby confirming the potential of the small subunit (SSU)-ITS region in PCR-RFLP in molecular characterization and identification of ectomycorrhizal fungi. Based on the results of this study, congruent of morphology and molecular RFLP analysis is recommended for characterization of species/strain of ectomycorrhizal fungi.

Key words: Classification, diversity, ectomycorrhizal fungi, ecosystem, ribosomal DNA.

INTRODUCTION

Ectomycorrhizal (ECM) fungi are the most important components of the soil fungal community in most boreal and temperate forests and play a major role in the ecosystem: the symbiotic relationship between the fungi and different plant species helps the plant by increasing its ability to absorb nutrients and its resistance to pathogens and abiotic forms of stress (Smith and Read, 1997). A wide range of ECM fungi forming mycelial networks between plants of the same or different species may be crucial to plant community dynamics and functioning ecosystem (Simard et al., 1997a).

Previously identification and characterization of ectomycorrhizal isolate mainly based on the morphological analysis of EM isolates in pure culture

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originated from environmental soil and also from fruit bodies grown under pure culture (Lotti et al., 2005). However, many taxa of EM fungi have never been cultivable in pure culture, because it is very difficult to manipulate their nutrition (Tedersoo et al. 2010).

More report by Gravesen et al. (1994) and Brundrett (2004) suggested that identification of ectomycorrhizal fungi based on phenotypic characters required expert taxonomist and time consuming process whereas identification of sporulating ectomycorrhizal fungi in pure culture has been comparatively easy. DNA-based molecular marker therefore combines PCR with RFLP for specific identification of ECM fungi at any stage of the life cycle (Iotti et al., 2005; Keisuke et al., 2007; Yakhlef et al., 2009). However, efficient molecular methods have so far been applied for the identification of EM fungi but only very few numbers of ectomycorrhizal fungi multiply in *in vitro* condition (Lotti et al., 2002). For example, Tuber species, *Boletus* species, and *Hebeloma* species originated from temperate ecosystems with limited morphological and molecular information (Rossi et al., 1999; Lotti et al., 2005). Therefore, the aim of the study was to determine the extend to which the molecular and morphological characterization of ecotmycorrhizal mycelia collected from pure culture will help better the understanding of the diversity, structure and dynamics of selected ectomycorrhizal fungi that originated from different host plant of temperate ecosystem.

The present study examined the extent of interspecific and intraspecific variation in SSU-ITS region of rRNA genes of some common ECM fungi collected from temperate zone to validate the earlier identification of these fungi, which was based on morphological data sets, and also to find suitable molecular RFLP markers for the identification of ECM fungi; up to the level of isolates as part of an expandable database of RFLP patterns of the ITS region of ECM fungi.

MATERIALS AND METHODS

Fungal isolates, culture conditions and morphological characterization

Sporocarps of 20 isolates of ectomycorrhizal isolates were collected from the woodland forest all located in different region of the France. EM isolates associated with different host plants shown in Table (1). Pure cultures of the isolates were obtained by aseptically transferring pieces of fungal fruiting bodies on modified MelinNorkrans medium (Marx1969); the composition of which was as follows: (NH4)2 HPO4 (250 mg/L), KH2PO4 (500 mg/L), $MgSO_4.7H_2O$ (150 mg/L), NaCl (25 mg/L), $CaCl2.2H_2O$ (50 mg/L), thiamine HCl (100 µg/L), $FeCl_3$ (12 mg/L), glucose (10 g/L), malt extract (3 g/L), and agar (8 g/L). Three mycelial discs from the cultures initiated with fruiting bodies were placed equidistant from each other on each Petri dish. The inoculated plates were incubated at 22°C in the dark for 1 month. Pieces of mycelium taken from the edge of actively growing colonies were used for morphological characterization. Morphological features of cultures were examined under Zeiss compound microscope equipped with a digital imaging system; and photographed using a Zeiss Axiacam RTC 99 (Germany). The Hyphal diameter and septal distance were measured in the peripheral growth zone of the mycelium using Axio Vision (ver. 4.7) attached to the compound 100 microscope (Zeiss Axicam, Germany).

DNA extraction from ECM fungal isolates grown *in vitro*

DNA was extracted from 0.5 to 1.0 mg of fresh mycelium taken from liquid broth and homogenized in liquid nitrogen using a plastic mortar and pestle. 300 to 500 µL hexacetyltrimethylammonium bromide (CTAB) lysis buffer (final concentration 0.1 M) containing 100 mMTris-Cl (pH 6.8), 0.02 M EDTA (pH 8.0), 1.4 M NaCl, 2% CTAB, and 0.2 % mercaptoethanol was added and solution was kept for 1 h at 65°C. Proteins were denatured and removed by sequential extraction with 500 µL Trissaturated phenol/chloroform/ isoamyl alcohol and chloroform. The solution was then gently mixed with 1 ml of isopropanol and the DNA was pelleted by centrifuging the solution. The pellet was washed with 70% ethanol, the solution centrifuged again, and the pellet dried at room temperature. Finally, the DNA was dissolved in 30 to 50 µL Tris- EDTA buffer and stored at -20°C until required.

PCR amplification of SSU-ITS region of rRNA gene

The SSU-ITS region of nuclear rDNA of all ECM fungal isolates used in the present study was amplified by the methods described by Gardes and Bruns (1993) using the primer pair NS5 (5'-AACTTAAAGGAATTGACGGAAG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). The thermal cycle consisted of initial denaturation at 95°C for 5 min; 5 cycles of denaturation at 95°C for 30 s, annealing at 52°C for 30 s, and extension at 72°C for 30 s; 30 cycles of denaturation at 95°C for 30 s, annealing at 51°C for 30 s, and extension 72°C for 1.5 min; and final extension at 72°C for 10 min. Controls with no DNA were maintained for every series of amplifications to check for contamination with reagents and reaction buffers. The amplification reactions were performed in a thermocycler (M.J. Mini PCR, Bio-Rad). The composition of the samples (25 µL each) used for the PCR was as follows: 10 ng of the DNA template, 50 µM each of dNTPs (dGTP, dCTP, dATP, and dTTP), 1.5 mM $MgCl_2$, 0.2 µM each of the primers NS5 and ITS4, 1× reaction buffer, and 0.1U of *Taq* polymerase. Amplified DNA fragments (1.1 to 1.4 kb) were made visible on 1.5% agarose gel.

RFLP and electrophoresis

The amplified DNA (samples of 8 µL each) was digested with 5 to 10 U of the restriction enzyme (*Hinf*1*, Taq*1, or *EcoR*1) according to the manufacturer's instructions. The reaction mixture (50 µL) was prepared for each restriction digestion with a particular restriction enzyme containing 5 µL of 10 × restriction enzyme buffers, 0.7 µL of 10 U/µL restriction enzyme buffer, 8 µL of DNA, and 36.30 µL of Milli-Q water. The reaction mixture was incubated as recommended by the manufacturer. The restricted DNA fragments were resolved on 2% agarose gel (Invitrogen, USA) prepared in 1× TAE buffer. Electrophoresis was performed in 1× TAE buffer for 80 min at a constant voltage of 60 V at 25°C in Bio-Rad gel casting apparatus (Bio-Rad, USA). The DNA samples were made visible by staining with 0.6 µg/ml of ethidium bromide. The agarose gel was observed and photographed using UV gel documentation (Elechrom Scientific, Switzerland).

Data analysis

The restriction patterns obtained were entered into a database

Table 1. Twenty isolates of ectomycorrhizal fungi used in the present study and their hosts.

EM Specie	EM[1] Isolate	Host
Tricholoma albobruneum	EM- 1248	*Pinus monticola*
Hebeleoma crustuliniforme	EM-1008	*Pinus monticola*
Hebeleoma edurum	EM-1174	*Picea*
Hebeleoma edurum	EM-1175	*Hetre pourpre*
Laccaria deliciosus	EM-1261	*Pinus sylvestris*
Laccaria refus	EM-1052	*Larix larcina*
Tricholoma scalparatum	EM -1250	*Populus euramericana*
Tricholoma populinum	EM-1249	*Populus euramericana*
Laccaria amethystina	EM-1091	Sousdes pins
Laccaria laccatta	EM- 1191	*Eucalyptus sp.*
Laccaria laccatta	EM- 1104	*Eucalyptus sp.*
Laccaria laccatta	EM -1190	*Hetre pourpre*
Suillus brevipes	EM -1121	*Pinus contorta*
Suillus brevipes	EM- 1124	*Pinus contorta*
Suillus granulatus	EM-1120	*Pinus contorta*
Laccaria biocolor	EM -1187	*Betula*
Laccaria biocolor	EM -1102	*Picea mariana*
Suillus bovinus	EM-1239	*Sous resineux*
Suillus bovinus	EM-1074	*Sous resineux*
Suillus bellini	EM-1237	*Pinus petula*

[1]All the isolates originated in France.

created in Microsoft Excel, scored as 1 (for the presence of a restriction site) or 0 (for the absence of a restriction site) and used for determining genetic distances between the isolates (Nei and Li, 1979). Genetic distance was calculated using Jaccard similarity coefficient of UPGMA analysis of NTSYS-pc version 2.02 software package (Rohlf, 1993).

RESULTS

Morphological description

Anatomical characteristics of each EM isolate were observed. Detailed records of mantle morphology, characteristics of hypha and structure of the Harting net in terms of Hyphal arrangements are shown in Table 2. Mantles morphology revealed eight distinct ECM types (Table 2 and Figure 1): Type 1, net like arrangements; 2, loosely arrangement; 3, plecttenchymatous mantles; 4, absence of gelatinous matrix; 5: ring like; 6, centrally arranged hype.

PCR amplification SSU-ITS region of ribosomal rRNA gene

Fragments of SSU-ITS from rDNA measured 1.1 to 1.4 kb in all the ECM fungal species except *Tricholoma scalpturatum*, which showed 1400 bp fragment (Figure 3). No polymorphism was observed within the SSU-ITS rDNA fragment of two *S. brevipes* isolates (EM -1121 and EM-1124); however, differences at the species level were detected between the isolates of *S. brevipes* and *Suillus granulatus* (Figure 2).

Restriction digestion and gel electrophoresis

Polymorphism within the SSU-ITS fragments of rDNA was observed when the amplicon was cleaved with different restriction enzymes, namely *Hinf*1, *EcoR*1, and *Taq*1 (Figure 3). Two isolates of *S. brevipes* did not show any polymorphism with the all restriction enzyme tested; however, two species of genera *Suillus* showed differences in their RFLP profiles with *Taq*1 R.E. Similarly three isolates of *Laccaria laccatta* did not show a different restriction pattern with all three enzyme tested. Moreover, two isolates of *Laccaria bicolor* (EM-1102 and EM-1187) did not show any polymorphism with *Hinf*1 and *EcoR*1 enzyme however, variability was observed with *Taq*1. To analyses the variation in ITS restriction pattern within the four closely related species of *Suillus* from ecosystems in the temperate region, the species were screened using restriction enzymes (*EcoR*1, *Taq*1, and *Hinf*1). Two species of genera *Suillus* (*S. bovinus* EM-1239 and *S. brevipes* EM-1124) had similar RFLP patterns with *EcoR1* and *Taq1,* and different one with *Hinf*1. Two species of genus*Tricholoma* (*T. albobruneum* EM- 1248 and *T. scalparatum* EM -1250) showed variability with

Table 2. Hyphal morphology and macroscopic characteristics of selected EM fungal isolates used in present study.

CMCC* EM Strain	Appearance	Appearance in Melzer's (M) reagent	Texture of mat	Mantles morphology	Hyphal diameter (µm)	Septal length (µm)	EM Species
EM-1248	Variable	White to light brown	Cottony	Hypha arranged net-like without bundles	2	49.22	*Tricholoma albobruneum*
EM-1008	White	Very pale to brown	Cottony	Loosely arranged hypha	1-1.5	46.19	*Hebeleoma crustuliniforme*
EM-1174	White rumpled culture	Very pale	Cottony	Loosely arranged hypha	1-1.5	32.5	*Hebeleoma edurum*
EM-1175	White rumpled culture	Very pale	Cottony	Loosely arranged hypha	1-1.5	31.1	*Hebeleoma edurum*
EM-1261	White mycelium with clamps	Dark reddish	Smoothy to shiny	Plectenchymatous Mantles	1.5	41.2	*Laccaria deliciosus*
EM-1052	White mycelium with clamps	Light reddish to light brown	Smoothy to shiny	Absence of gelatinous matrix	1.8	25.88	*Laccaria refus*
EM -1250	Variable	Light reddish to light brown	Smoothy to shiny	Loosely organized mantle surface	3.24	34.29	*Tricholoma scalparatum*
EM-1249	Variable	Light reddish to light brown	Spiny	Loosely organized mantle surface	2	46.19	*Tricholoma populinum*
EM-1091	Variable	Dark reddish brown to black	Velvety	No pattern recognized ,Absence of gelatinous matrix	2.2	85.25	*Laccaria amethystina*
EM- 1191	White mycelium	Dark reddish to brown	Smoothy	Plectenchymatous Mantles, No pattern recognized ,Absence of gelatinous matrix	1	81.2	*Laccaria laccatta*
EM- 1104	White mycelium	Dark reddish brown	Smoothy	Plectenchymatous Mantles, No pattern recognized ,Absence of gelatinous matrix	2.35	85.2	*Laccaria laccatta*
EM -1190	White mycelium	Dark reddish brown	Smoothy	Plectenchymatous Mantles, No pattern recognized ,Absence of gelatinous matrix	2.5	80.1	*Laccaria laccatta*
EM -1121	light-coloured aerial mycelium, darker when submerged	Dark brown	Loosely wooly	Plectenchymatous Mantles, Net like arrangement of hyphal bundle	3	84.82	*Suillus brevipes*
EM- 1124	light-coloured aerial mycelium	White	Smoothy	Ring like	2.8	82.3	*Suillus brevipes*
EM-1120	light-coloured aerial mycelium	Pale yellow	Loosely wooly	No pattern recognized ,Absence of gelatinous matrix	5	81.1	*Suillus granulatus*
EM -1187	White mycelium	Dark reddish brown	Velvety	No pattern recognized , Absence of gelatinous matrix	4	72.1	*Laccaria biocolor*
EM -1102	White mycelium	Reddish brown	Velvety	Undifferentiated hypha of equal diameter, Absence of gelatinous matrix	4.1	58.1	*Laccaria biocolor*
EM-1239	light-coloured aerial mycelium	Pale yellow to light brown	Smoothy	Highly differentiated with centrally arranged thicker hypha	3.1	86.1	*Suillus bovinus*
EM-1074	light-coloured aerial mycelium	Light brown	Spiny	Highly differentiated with centrally arranged thicker hypha, septa dissolved	3.05	65.2	*Suillus bovinus*
EM-1237	light-coloured aerial mycelium	Dark yellow	Shiny	No pattern recognizable	2.8	66	*Sulllus bellini*

both restriction enzyme *Taq*1 and *EcoR1* whereas had similar pattern with *Hif1* (Figure 3).

Restriction fragments obtained with all endonuclease enzymes tested were used for determining genetic distances between the isolates so as to cluster them into specific groups (Figure 2). Topology obtained using the presence or absence of restricted digested products as a criterion generated two major clusters (Cluster I and Cluster II), and all the species of ECM fungi. Mostly, isolates from the same genus/species clustered together except those of *T. scalpturatum* EM-1250 and *T. albobruneum* EM-1248 which fell into two sub clusters

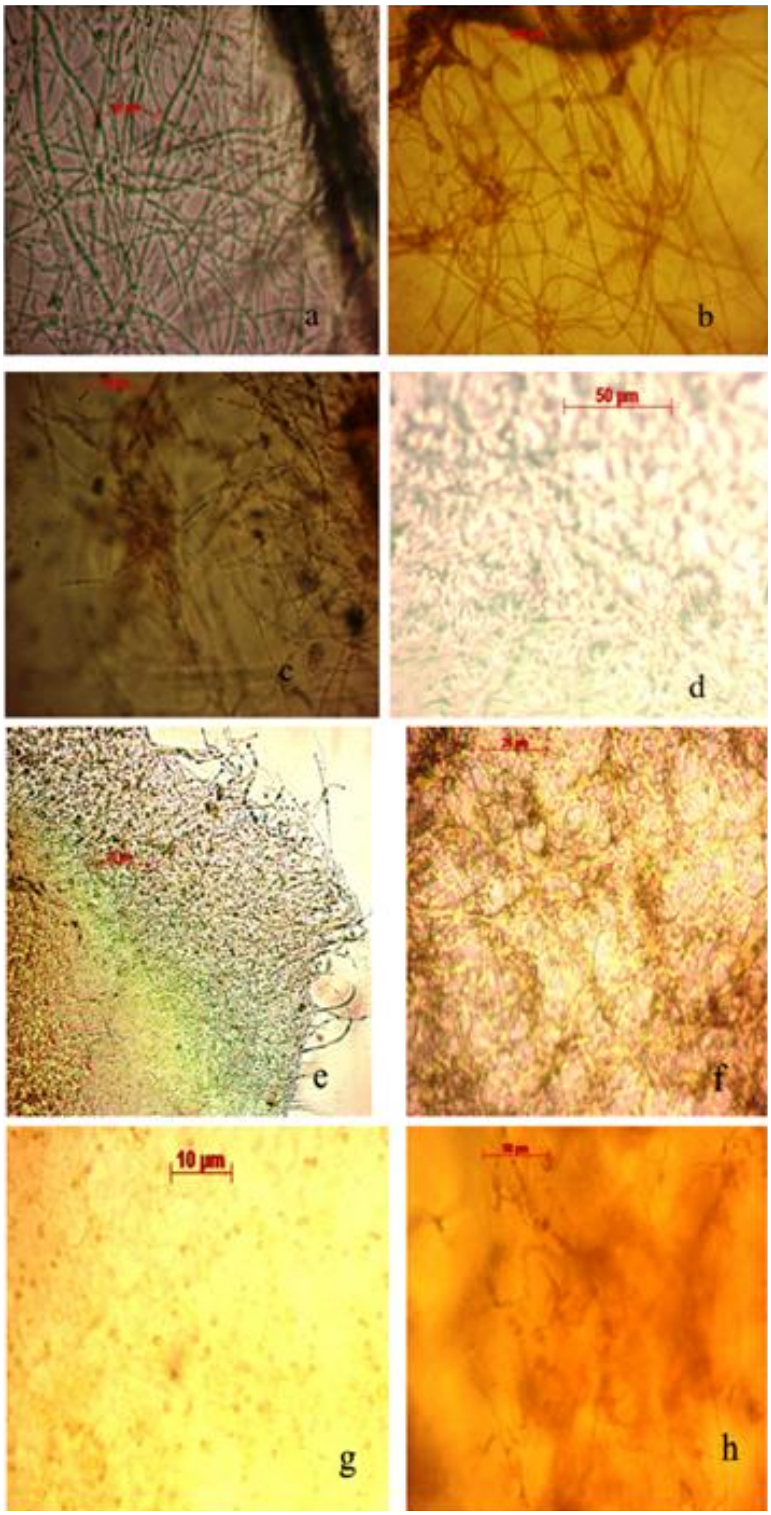

Figure 1. Morphological and anatomical features of culture were observed under a compound microscope. Microscopic structures of selected mycelia of ectomycorrhizal isolate used for study. A-h: Type 1-8 distinct ECM types.

(Figure 2). Isolates of same species generally cluster together ex. isolates of *Laccaria bicolor;* fell in major cluster of Cluster group II. In general, isolates with similar RFLP profiles could be grouped by their genus or species: for example, *Laccaria* and *Suillus* species clustered into different groups except species of genera

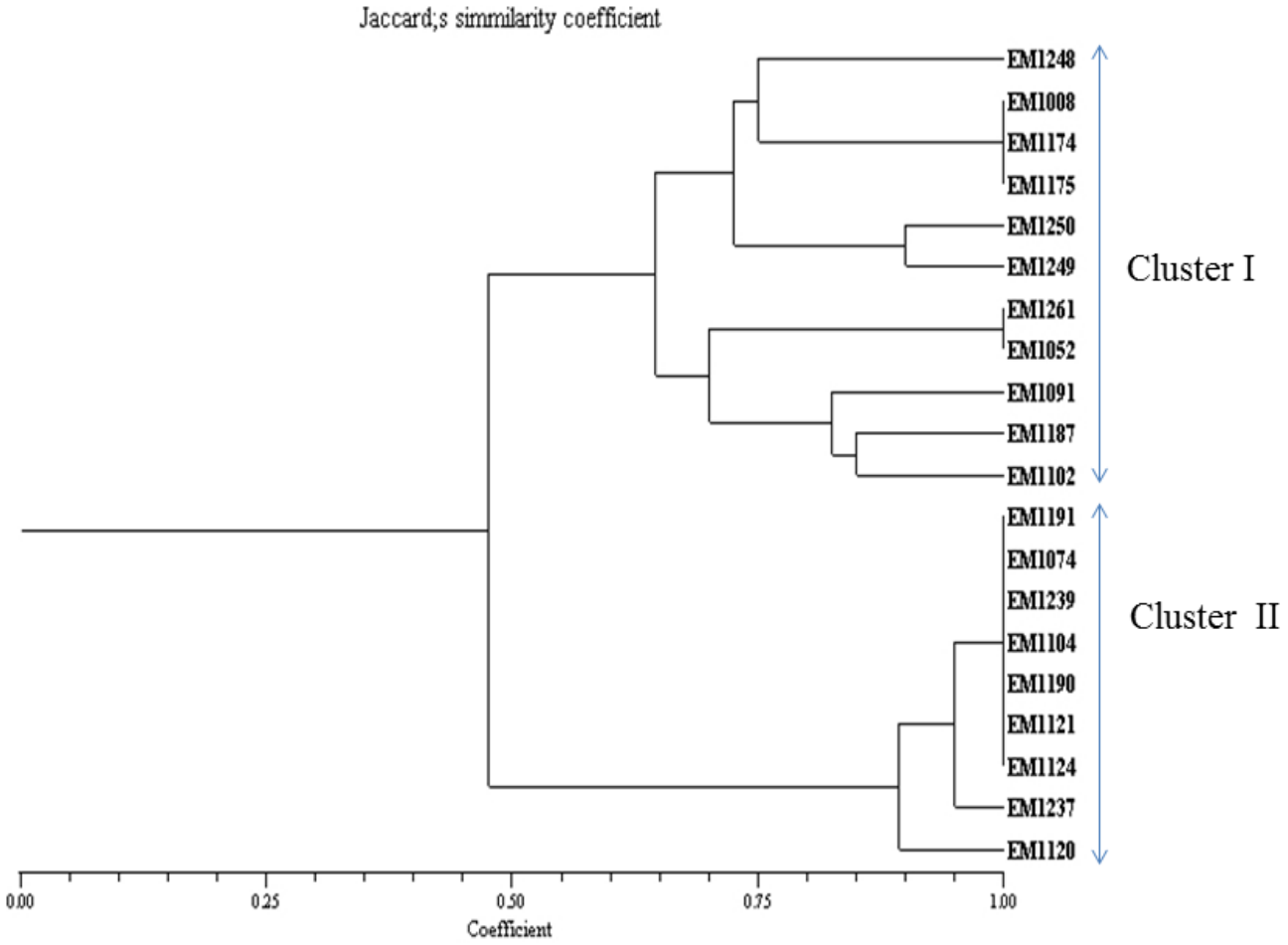

Figure 2. Cluster diagram of the PCR-RFLP pattern from digestion of the SSU-ITS region of rDNA amplified from mycelium of EM fungi.

Tricholoma (ex. *T*. *albobruneum* EM-1248) which was not clustered in similar group.

DISCUSSION

In the present study, PCR-RFLP analysis was used not only to support morphological classification but also to find genetic variations among species or isolates of ECM fungi. We observed that higher genetic and phenotypic variation of ectomycorrhizal isolates originated from temperate soil. Similar study by Tedersoo (2012) showed that greater EM fungal community and genetic variation among EM isolates originated from temperate soil as compared with tropical soil. Relatively low richness of EM fungal isolates in tropical soil may be due to rapid loss of organic materials and also weak soil stratification.

Polymorphism detected by PCR-RFLP of the SSU-ITS region of rRNA gene has been successfully used in identifying several species of fungi (Amicucciet al., 1996; Kårén et al., 1997). In the present investigation, 20 isolates of ECM fungi identified by morphological means were subjected to molecular analysis. Molecular analysis based on PCR-RFLP mainly uses the ITS region of ribosomal DNA to study polymorphism in fungi at the species level (Amicucci et al., 1996). Such analysis requires minute quantities of DNA for PCR amplification of multi copy rRNA gene with two specific primers flanking the SSU and the ITS region. The ITS region, like the intergenic spacer region, has evolved much faster, and the sequence is suitable to differentiate between different populations of a species. However, the coding regions of small ribosomal subunits are considered more useful in understanding more distant relationships among ECM fungi.

Walker et al. (2007) reported that the conserved regions in SrRNA and LrRNA of ribosomal DNA along with the highly variable ITS region clearly describe closely related mycorrhizal species. This present study was based on conserved and variable regions of rRNA genes clearly describing ECM fungi upto genera as well as species level. Moreover, the present study used a

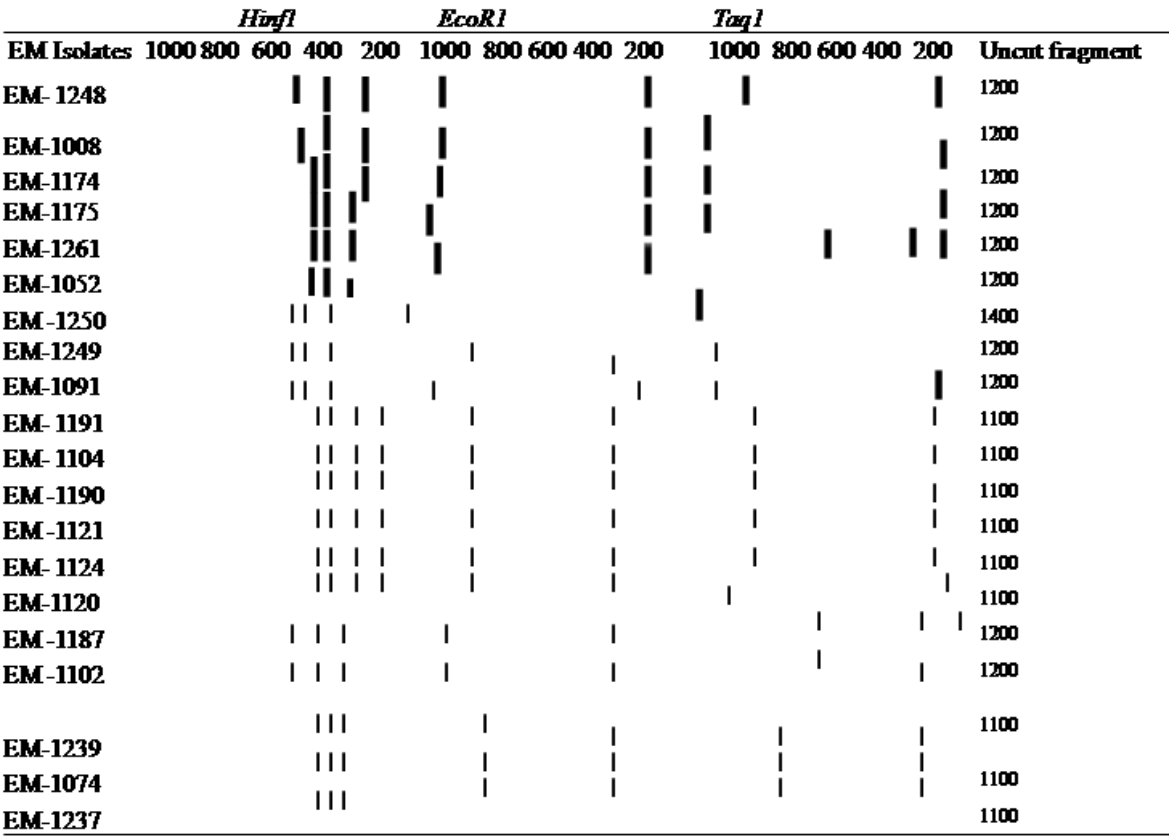

Figure 3. Restriction fragment sizes of SSU-ITS regions after digesting with *EcoR*1, *Hinf*1, and *Taq*1.

≈1200 bp amplicon for restriction analysis, which provide greater polymorphism than shorter fragments for resolving species or isolates of ECM fungi as reported earlier by Matsuda and Hijii (1999), Horton (2002) and Pritsch et al. (1997). We observed that 20 isolates of EM fungi used in this study separated into five genera by use of any of the restriction enzyme tested however, for a species level, resolution combination of restriction enzyme were used as similar investigation reported by Gomes et al. (2002).

Amplification products of the SSU-ITS region from all the ECM fungal isolates were 1.1 to 1.4 kb though, this variation in length did not separate the ECM fungal genotypes tested in the present investigation. Several authors have recommended RFLP analysis of the ITS region to distinguish ectomycorrhizal fungal isolates at the interspecific and intraspecific levels (Grades et al., 1990; Bruns et al., 1991; Manassila et al., 2005; Yakhlef et al., 2009). The present study shows that the length of an amplification product of ribosomal DNA does not distinguish clearly between different isolates or species of ECM fungi and suggest that the ITS-RFLP pattern, using at least three endonucleases, is a superior technique to study polymorphism in ECM fungi. We found that *Tricholoma scalpturatum* EM-1250 formed a morphologically distinct group due to mantle morphology, texture of mat and the isolate did not cluster with *T. albobruneum*EM-1248 (Table 2).

This result shows that interspecific variation in the ITS region of ECM fungi in *T. scalpturatum* is higher than that in *T. albobruneum* EM-1248. This greater variation could be due to the complex and unique genetic structure of *T. scalpturatum*, as reported by Carriconde et al. (2008) and Jargeat et al. (2010). Carriconde et al. (2008) describe genetic diversity among *T. scalpturatum* using three different sets of molecular markers and found that morphotypes of this species showed considerable genetic variation and resolved it into two distinct groups. Our investigation showed that the considerable variation in the ITS region in some isolates of ECM fungi used for the present study is potentially useful in developing markers at the species or isolates level for many, but not necessarily all, species of ECM fungi.

S. brevipes EM-1121 and *S. granulatus* EM-1240 with similar ITS-RFLP patterns clustered together with all three enzymes tested whereas, *S. bovinus* EM-1239 showed a similar ITS-RFLP pattern only with two restriction enzymes (*EcoR*1 and *Hinf*1), showing *S. bovinus* to be phylogenetically diverse from the other two *Suillus species* used in present investigation. Subsequently, dif-

ferentiated centrally arranged thicker hypha was observed in *S. bovinus* whereas net like arrangement of hyphal bundles found in isolate of *Suillus brevipes* (Table 2). Kretzer et al. (1996) described 38 species of *Suillus* using sequencing analysis of the ITS region of rDNA and suggest that *S. brevipes* and *S. granulatus* are closely related species whereas *S. bovinus* falls into a separate group.

The obtained data indicates that *L. bicolor* EM-1102 and *L. bicolor* EM-1187 had similar ITS-RFLP profiles with *EcoR*1 and *Hinf*1 but different ones with *Taq*1. This is in agreement with earlier studies by Grades et al. (1991b) using mitochondrion rDNA marker and they observed high level of variability within *L. bicolor* isolates. Our study on nuclear ribosomal DNA suggested that no major differences were observed between the trends indicated by RFLP results on mtDNA. Clustering analysis showed that *L. bicolor* and *L.amethystina* apparently were diverse (Figure 2) in the ITS regions of nuclear-encoded rDNA originating from the same continent. In contrast, studies by Gardes et al. (1990) found variation in nuclear rDNA repeat of *L. bicolor* and *L. amethystine;* originated from two different contiments. Furthermore, Kretzer et al. (1996) also found genetically diverse profiles of *L. bicolor* and *L. amethystina* collected from different continents. Our data also confirmed that sequence variation within the ITS region supports morphological difference in texture of mat between isolates EM-1091 and EM-1187 (Table.2).

Present data indicated some closely related species; *L. bicolor* and *L. laccata* grouped together because of similar pattern by three enzyme match criteria. Bruns et al. (1991) suggested that RFLP fingerprinting should not be single parameters for comparing restriction patterns with reference RFLP types. Use of integrated approach including morphology, biochemistry and molecular sequencing analysis of highly conserved protein sequences (Chitin synthases reported by Lanfranco et al. (1995) and glyceraldehyde 3-phosphate dehydrogenase by Kreuzinger et al. (1996) may overcome this problem.

We suggested that extraction and sequencing analysis of a unique fragment of genotype from agarose gel can be used for strain specific molecular marker development in EM fungi. We also suggest that the development of ribosomal DNA ITS array (PhyloChip) from unique EM genotype be used for monitoring isolates of ECM fungi in a mixed sample representing different environment. Present investigation also suggested the potential of not only ITS region PCR-RFLP for the molecular characterization of ectomycorrhizal fungi but also SSU region and their identification and monitoring in artificial inoculation programs.

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