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RESEARCH ARTICLE

Molecular characterization using rRNA genes: A simple and direct tool for identification of ectomycorrhizae from pooled root-tip samples.

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Abstract

Identifying ectomycorrhizal species associated with roots of higher plants had always been a laborious work mostly relying upon generation of pure cultures and morphotying. No doubt these techniques are invariable unmatched, yet these limit to address the diversity associated with soil or root samples. Here we describe a quick and reliable method for identification of ectomycorrhizae directly from root tips without compromising diversity associated with the sample. This method is speedy as there is no need of morphotyping or any anatomical study of root tips. Fine root-tips harboring ectomycorrhizae are pooled and then directly processed for molecular characterization under highly stringent conditions using overlapped rRNA gene specific primers to generate multiple bands, each band being representative of an individual fungal species. This is followed by band elution and RFLP analysis to generate individual clades. Representatives of each clade are then sequenced, assembled and subjected to global BLAST analysis for identification of ectomycorrhizal specie(s).

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Introduction

Taxonomic and ecological extrapolations by Brundrett (2009) showed that 86% of terrestrial plant species acquire mineral nutrients via mycorrhizal root symbionts. Ectomycorrhizae are the dominating mycorrhizal type in temperate and boreal forests as 80 to 90% trees in temperate and boreal forest ecosystems live in symbiosis with ectomycorrhizal (EM) fungi (Read 1992; Valtanen, 2012; Deckmyn et al., 2014). As per the estimations described by some workers (Rinaldi et al. 2008; Brundrett 2009) the number of plant and fungal species currently involved is 6,000 and 20,000–25,000, respectively. Identification to species level is an important component of many research efforts to study the diversity of ectomycorrhizal fungi associated with various plant species in temperate regions. In last two decades a revolution of molecular tools tremendously facilitated the identification and characterization of these ectomycorrhizae by overcoming the various problems and drawbacks associated with various traditional methods of identification (Horton and Bruns, 2001, Landeweert et al., 2003 and Lanfranco et al., 1998). Various PCR based molecular methods have been described from time to time for the identification of ectomycorrhizas using pure culture (Iotti et al., 2005), sporocarp (Brunner et al., 1992) or below ground root tips after morphotyping (Iotti and Zambonelli, 2006). Community analyses of ectomycorrhizal (ECM) fungi rely on the characterization of ectomycorrhizal root tips. Bent and Taylor (2010) analyzed the multiple tips of a given plant or a soil core. They compared the direct amplification of ectomycorrhizal root tip DNA using different methods. In the present study multiple root tips from sampling sites were pooled irrespective of their morpho-anatomical features and processed for molecular characterization.

Material and methods

1. Origin of ectomycorrhizal root tips:

The study was conducted on the root tips of *Pinus wallichiana* growing in a pure stand in temperate forests of Kashmir Himalaya. A total of 200 root tip samples were collected from 20 different locations covering Gulmarg forest region of Kashmir Himalaya. The root tips were packed in sealed polythene bags along with soil and stored at 4ºC. Fine root tips harboring ectomycorrhizae were carefully chosen and then pooled for DNA extraction without the laborious process of morphoanatomical analysis. So it is expected that the DNA extracted is a community DNA and may contain more than one species of ectomycorrhizae.

2. DNA extraction:

Fungal DNA was isolated and purified from the fine root-tips of *Pinus wallichiana* harboring ectomychorrhizae. The pooled root-tips were crushed in liquid nitrogen in mortar-pestle into a fine powder. From each sample DNA was extracted using a commercial DNA extraction kit (HipurA fungal DNA purification Kit, Himedia India) following manufacturer's instructions. Once isolated, the DNA samples were reconstituted in TE buffer and stored at -20º C. Integrity of the fungal DNA was checked on 0.8% agarose gel and quantity of DNA was measured on UV- visible spectrophotometer prior to PCR.

3. PCR amplification:

PCR amplification of ITS-1, ITS-2 and 5.8S rRNA genes was carried out using gene specific primers (Table 1). PCR was performed in a 50 µL reaction volumes containing 3.0 mM MgCl2, 0.2 µM of each primer, 0.2 mM dNTP, 1 U Taq DNA polymerase (Sigma Inc) with 10µg fungal DNA. In addition, DMSO was also added to PCR reaction in order to avoid the non specific bands. An initial denaturation step of 10 minutes at 94°C was followed by 40 PCR cycles each of 30 seconds denaturation at 94°C, 30 seconds annealing at 55°C and 60 seconds extension at 72°C. A final 7-minute extension at 72°C completed the protocol. PCR amplification was confirmed on 1.5% agarose gel visualized by staining with ethidium bromide using LAS 4000 (Image Quant, GE, USA) gel documentation system. Control reaction was performed with same reaction components and conditions except that Mili Q was added instead of DNA template in negative control. Positive control was performed by using the DNA of pure fungal culture.

4. Elution and purification of bands:

PCR amplicons generated from amplification of the community DNA were gel purified using HipurA Mini spin DNA elution Kit (Himedia). After re-amplification and re-purification, the PCR products were used for further downstream analysis.

5. RFLP of amplified ITS region:

The gel eluted amplicons after successful reamplification with specific primers were subjected to restriction digestion by commercially available restriction enzymes Hinf1 and Alu 1 which are considered to be sufficient for identifying most ectomycorrhizal fungal taxa (Cullings et al., 2000; 2001; Douglas et al., 2005).

6. Sequencing and species identification.

The samples showing variation in RFLP pattern were selected and directly sequenced using ABi 3730Xl (Sanger sequencing methodology). The sequences were assembled in BioEdit Sequence Alignment Editor (v 7.2.3). Each of the sequences was separately used to perform individual nucleotide-nucleotide searches with the BLASTn algorithm (Altschul et al. 1997) at the NCBI website http://BLAST.ncbi.nlm.nih.gov/BLAST.cgi against fungi taxon (taxid: 4751). The outputs from the BLAST searches were sorted on the basis of the maximum identity and were recorded according to their coverage. Sequence- similarity with a cutoff of 90% or greater was considered significant, and the best hit was defined as the sequence with maximum coverage/ identity to the query sequence.

Results:

1. ECM evaluation by molecular analysis

The pooled ECM root tips were washed gently and mixed root tips were subjected to molecular analysis within 10 days of sampling.

DNA extraction and PCR amplification

Internal Transcribed Spacer (ITS) is composed of three conserved domains-ITS1 region, ITS2 region and a short 5.8SrRNA segment separating the two regions. For identification of fungal species 3 primer combinations were used to specifically amplify –a full length ITS, ITS1 and ITS2 regions (Figure1, Table 1). As expected PCR amplification produced single or multiple bands depending on the number of species present in community DNA used as template. The amplicons ranged between 200-450bp for ITS-1; 520-550bp for ITS-2 and 600-700bp for full length ITS (Figure 2). The possibility of non-specific amplification was tested using temperature gradient (54-60ºC), DMSO gradient (0.5 to 3.0 µl) and $MgCl_2$ gradient (1.5 to 6.0mM) amplifications.

RFLP analysis

The re-amplified products were digested with restriction enzymes Hinf I and Alu I. As depicted in Figure 3, RFLP produced variable band pattern of varying molecular weight. A total of eight groups were identified. Samples showing identical RFLP band pattern were grouped together. Representatives from each group were further purified and prepared for Sanger sequencing.

2. Species evaluation:

All the samples were analyzed by double-pass sequencing using gene specific primers as described in previous sections. Sequences results obtained were then subjected to global BLAST analysis against fungal taxon (taxid: 4715). Based upon sequencing results and BLAST analysis, a total of 4 ectomycorrizal species were identified namely *Chalara holubovae* (KM999127), *Tuber maculatum* (KM999129), *Infundichalara microchona* (KM999128) and *Helotiales spp.* (KM999130) having maximum score for all the 3 rDNA regions amplified. Table 2 represents the BLAST results depicting the nearest possible match of the species identified.

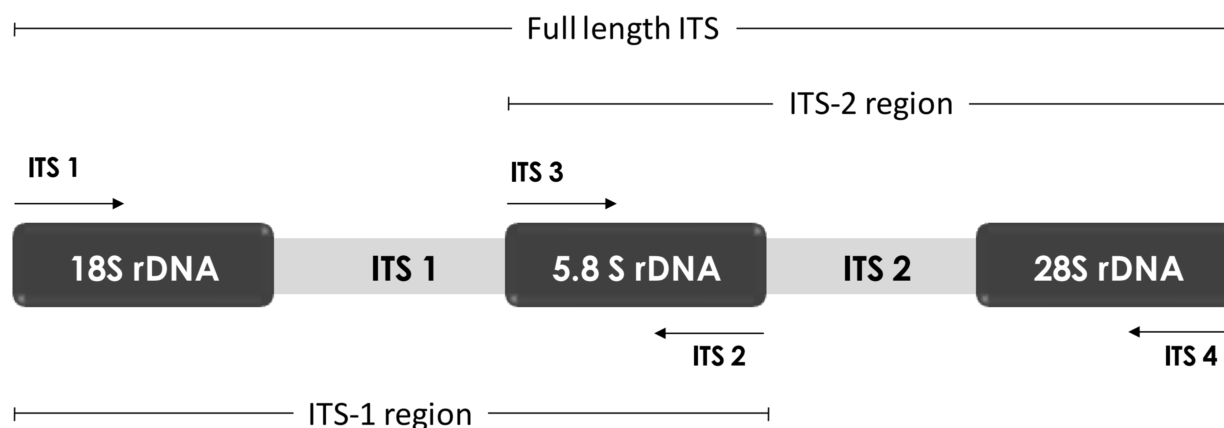

Figure 1. *ITS map showing the relative locations of primers used for amplification of the ITS regions of the Fungal DNA.*

Table-1: Primer characteristics used for amplification of ITS regions

S.NO	Gene segment	Forward Primer	Reverse Primer	Tm (^{0}C)	Amplicon (bp)
1.	ITS-1 region	TCCGTAGGTGAACCTGCGG	GCTGCGTTCTTCATCGATGC	54	262-324
2.	ITS-2 region	GCATCGATGAAGAACGCAGC	TCCTCCGCTTATTGATATGC	54	354-462
3.	Full length ITS	TCCGTAGGTGAACCTGCGG	TCCTCCGCTTATTGATATGC	54	600-755

Table 2. Summary of BLAST results showing sequence similarity of the identified species among fungal taxon.

Description	Gene Bank Accession ID	Query cover	%age Identity	Score
Seq 1 *Tuber maculatum* (KM999129)				
Tuber maculatum	AJ969627.1	98	95	1051
Tuber maculatum	EU753269.1	95	95	1018
Tuber maculatum	AF106889.1	95	95	1018
Tuber maculatum l	EU784428.1	93	95	983
Tuber borchii	EU784422.1	93	95	983
Tuber borchii	EU784423.1	92	95	981
Tuber sp.	AM900421.1	90	95	974
Tuber sp.	FN393383.1	90	95	974
Tuber sp.	FN393386.1	90	95	972
Seq *2 Chalara holubovae* KM999127				
Chalara holubovae	FR667223.1	99	92	760
Chalara holubovae	FR667222.1	96	92	754
Chalara microchona	HM036588.1	98	91	737
Uncultured Helotiales	FJ553817.1	97	91	717
Helotiales sp.	HG796907.1	96	91	710
Discocistella grevillei	GU727554.1	97	90	701
Hamatocanthoscypha laricionis	|JN033441.1	97	90	686
Infundichalara microchona	KF359590.1	90	91	678
Infundichalara microchona	KF156300.1	91	91	673
Cadophora sp.	DQ132821.1	92	90	671
Seq 3 *Infundichalara microchona* KM999128				
Infundichalara microchona	KF359590.1	98	91	782
Hamatocanthoscypha laricionis	JN033441.1	95	89	682
Discocistella grevillei	GU727554.1	86	88	678
Chalara holubovae	FR667223.1	84	88	673
Cadophora sp.	DQ132821.1	84	89	658
Chalara microchona	HM036588.1	89	89	645
Chalara microchona	FR799510.1	82	89	643
Chalara microchona	AY590782.1	80	91	630
Seq 4 *Helotiales spp.* KM999130				
Uncultured Helotiales	FJ553817.1	89	91	749
Helotiales sp.	HG796907.1	86	92	743
Uncultured Helotiales	DQ182458.1	80	91	723
Discocistella grevillei	GU727554.1	77	91	708
Chalara holubovae	FR667222.1	80	90	704
Infundichalara microchona	KF359590.1	79	90	684
Chalara microchona	HM036588.1	80	89	680

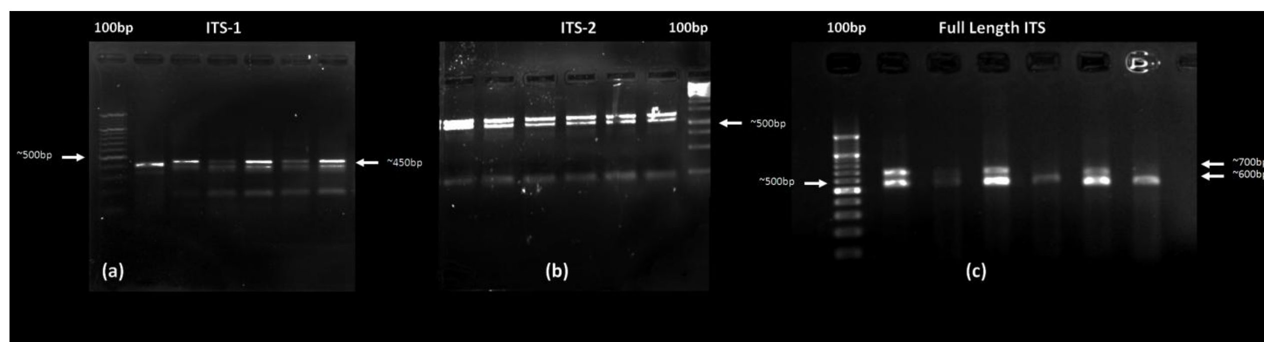

Figure 2. *Representative PCR amplification of internal transcribed spacer (ITS) regions showing multiple amplifications of a) ITS-1; b) ITS-2 and c) Full-length ITS. Non-specific amplification were ruled out using gradient PCR (DMSO, $MgCl_2$ and temp. gradient).*

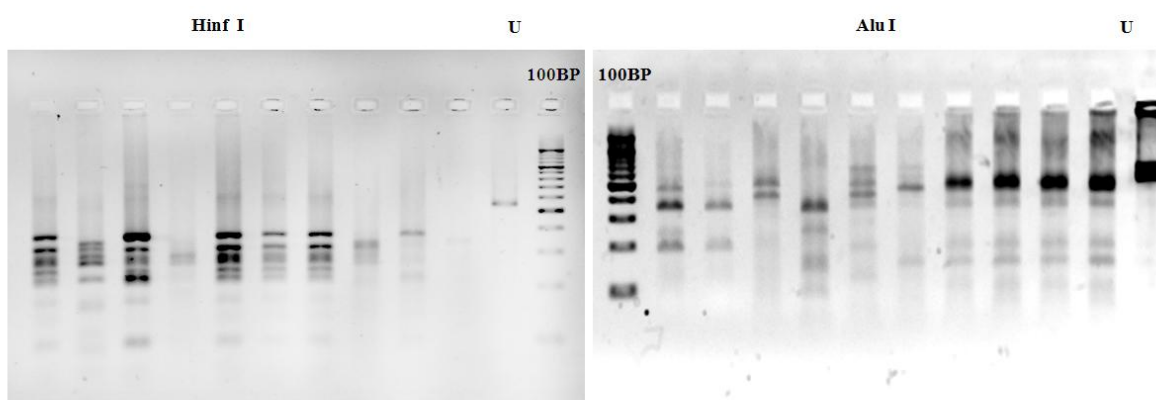

Figure 3. *Representative RFLP map using enzymes a) Hinf I and b) Alu I.*

Discussion:

Earlier, the ECM fungi were characterized by the presence of the sporocarps, however this method had a major limitation that sporocarps poorly represented the ECM taxa attached to the root tips (Egger 1995; Gardes and Bruns 1996a) as fruiting species merely constitute 20–30% of the mycorrhizas (e.g. Gardes & Bruns, 1996; Jonsson et al., 1999a). Then ECM morphotyping from the root tips was found to be more effective (Agerer 1991) as it evaluated the ECM fungal diversity based on vegetative structures instead of sporocarps (Goodman and Trofymow 1998; Kranabetter et al. 1999; Massicotte et al. 1999). Morphotyping can be performed relatively rapidly; however, it is a limiting technique, because the morphology of a particular fungal taxon can change with different hosts and environments (Egger 1995). During last two decades molecular techniques revolutionized the characterization of ectomycorrhizal fungi.Since DNA sequences can be amplified directly PCR can be applied to any tissue including fruit bodies, pure cultures or mycorrhizal roots. White et al., (1990) designed the universal primers for identification of fungi. Pioneer workers in this regard were Gardes et al., 1991; Gardes et al., 1990; Egger, 1995) who used molecular techniques to identify ECM. Some workers (Egger, 1995; Mardones- Hidalgo and Iturriaga, 2011; Nouhra et al., 2012) used the sporocarps as material for molecular characterization of the ECM. Since sporocarps did not represent the entire diversity of ECM below ground studies were initiated. A large number of ECM fungi have been identified after molecular characterization of roots which was followed by grouping the roots after morphotyping (Ding et al., 2011; Douglas et al., 2005). The present method is an attempt to directly identify the ectomycorrhizae without any morphological or anatomical analysis of root tips. By following highly stringent methodology using 3 tier ITS- analysis of 200 root tip-samples we were able to identify 4 predominant ectomycorrhizal species associated with root-tips of the conifer.

The presence of multiple bands which have never been reported till date proved to be more useful for verifying fungal species associated with the conifer. Hence, this method speeds up the process of identification and characterization of ectomycorrhizal fungi. The present method resolves the problems associated with the DNA

isolation phase, such as the simultaneous extraction of DNA of multiple fungal species. Usually this occurs because adjacent root tips are frequently colonized by different ECM fungi, and more than one fungal species can be present on the same root tip (Brand, 1992 and Kaldorf et al., 2004). Moreover, mycelia of competing ECM species can often be seen growing along roots and on the mantle surface of other ectomycorrhizas. In addition to speeding up of the identification process of ectomycorrhizal fungi this method also reduces the chances of contamination coming from other fungi as endophytes (Jonsson et al., 1999) and fungi which can contaminate the cultures of these ectomycorrhizae. Due to reproducibility, rapidity and low cost this method can be very useful for the characterization and identification of the various ectomycorrhizal species associated with a range of hosts.

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