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MOLECULAR DIVERSITY OF ECTOMYCORRHIZAL FUNGI OF CENTRAL HIMALAYA OF INDIA: AN IMPORTANT COMPONENT OF FOREST ECOSYSTEM

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ABSTRACT

Ectomycorrhizae are symbiotic organ formed between a fungus and the roots of tree. These fungi are very important for the growth and development of higher tree species. Ectomycorrhizal diversity through effective plant growth would affect tree species composition and ecosystem functioning. In a preliminary study the comparative symbiotic efficiencies of two ectomycorrhizal fungi viz., *Amanita hemibapha* and *Russula vesca* was assessed in association with oak (*Q. leucotrichophora*) and pine (*P. roxburghii*) seedlings. Our studies indicated that the fungal species had a marked influence on competitive outcome of the seedlings, their growth and health status. Uttarakhand state (India) is very rich in tree biodiversity however; the ECM fungal diversity of Himalayan region and how they may be impacting the tree diversity is not well studied. It is essential to document this important wealth and to find out the ways to conserve it. This will essentially require a precise and authentic characterization of the fungal species and molecular approaches can meet the challenge .We have used a molecular approach using RAPD techniques on genomic DNA from different ectomycorrhizal (EM) fungal species belonging to the genus *Cortinarius, Boletus, Inocybe* and *Xerocomus* of Kumaun region. Divergence analysis using RAPD markers of different ectomycorrhizal species was done using UPGMA method (Unweighted Pair Group Method with Arithmetic Mean). Divergence analysis of six genotypes revealed that species 1(*Boletus appendiculatus)* and species 5 (*Boletus variegates*) were found to be located on a single cluster reflecting their homology whereas species 1 (*Boletus appendiculatus)* and species 2 (*Inocybe species*) were found to be distantly related. Species 4 *(Xerocomus chrysenteron)* share near homology with species 1 (*Boletus appendiculatus)* and 5 (*Boletus variegates)* whereas species 3 (*Cortinarious species* 1) and species 6 (*Cortinarious species 2)* also share near homology. The study indicates that if assay conditions are carefully controlled, the RAPD methodology may provide a cheap, rapid and effective means to evaluate the genetic diversity among a large number of fungal species. When coupled to using primers specific to fungal ITS region more accurate identification can be made all the way species and strain level.

Key Words: Ectomycorrhizae, *Q. leucotrichophora*, *P. roxburghii,* Molecular diversity.

INTRODUCTION

Mycorrhizae are symbiotic (occasionally weakly pathogenic) association between a fungus and the roots of a plant (Kirk *et al*. 2001). These fungi are very important for the growth and development of higher tree species. Ectomycorrhizal fungi can account for 25% or more of the root mass of forest, thus representing a major belowground structural component of the forest ecosystem. Ectomycorrhizal diversity through effective plant growth would affect tree species composition and ecosystem functioning. Mycorrhizae, due to their key position at the plant-soil interface, are important to be considered in

the study of ecosystems. Mycorrhizae are known to influence plant performance through the benefits they confer on their hosts. Their benefits, as an example, lead to improve growth of host plants as well as increase tolerance to drought and disease (Molina *et al.* 1992). More than 90% plant species have association with mycorrhizal fungi (Trappe 1987), but not much is known about the effects of mycorrhizal symbiosis on plant species composition and competition (Ozinga *et al.* 1997). Until recently the importance of mycorrhizal fungi in determining plant diversity relative to other mechanisms such as species competition and species coexistence remained largely unattended. In a preliminary study the comparative symbiotic efficiencies of two ectomycorrhizal fungi viz., *Amanita hemibapha* and *Russula vesca* assessed in association with oak (Q. *leucotrichophora*) and pine(*P. roxburghii*) seedlings indicated that the fungal species seem to have a marked influence on competitive outcome of the seedlings, their growth and health status(Pande *et al* .2004) . In view of the great potential of suitable mycorrhizal fungi, the pure culture technique as developed and commercialized in other countries need urgent attention for harnessing this natural source of biofertilizer for growth, improvement and greening wasteland in our country also. Another aspect of these fungi is they could be an alternative of costly protein rich food because of their higher protein content and presence of essential amino acids (Pande & Pande, 2004). They are excellent source of Vitamins also (Hayes & Hand, 1981). By providing this knowledge to villagers economic upliftment of the society could be done by economy generation through selection of sporocarp of edible ectomycorrhizal fungi.

Uttarakhand state is very rich in biodiversity but a little work has been done in this state in the area of ectomycorrhizal fungi. The fungal diversity of India and particularly of Himalayan region is poorly understood. It is essential to document this important wealth and to find out the ways to conserve it. This will essentially require a precise and authentic characterization of the fungal species and a molecular approach can meet the challenge. An attempt was made to analyze inter generic and intra generic molecular diversity of different ectomycorrhizal (EM) fungal species belonging to the genus *Cortinarius, Boletus, Inocybe* and *Xerocomus* of Kumaun region of India using RAPD markers.

MATERIAL AND METHODS

For the culture of ectomycorrhizae, fruiting bodies were collected from the different places near Nainital during the month of June -July. All the samples were taken into lab and identified on the basis of various macroscopic, microscopic and chemical characteristics following Pande, V. (2004). Total six species of genus *Cortinarius, Boletus, Inocybe* and *Xerocomus* were selected for genetic diversity analysis by RAPD markers. Different species selected for study were:

1. *Boletus appendiculatus*	Species 1
2. *Inocybe sp*.	Species 2
3. *Cortinarius sp. 1*	Species 3
4. *Xerocomus chrysenteron*	Species 4
5. *Boletus variegates*	Species 5
6. *Cortinarius sp. 2*	Species 6

DNA was isolated from dried fruiting bodies of different EM fungal species using four different protocols viz. Modified CTAB (Doyle &Doyle,1990), Qiagen DNeasy Miniplant Kit, Wizard®Genomic DNA Purification Kit and Fermentas genomic DNA Purification Kit. The qualitative analysis of DNA was carried out by submerged gel electrophoresis using. 8% agarose gels (Sambrook, 1987).The quantity of purified genomic DNA was estimated by measuring optical density at 260nm. The O.D. at 280 nm was also recorded for determining protein contamination. The ratio of O.D. 260/O.D. 280 gives the amount of RNA or protein in the preparation. A value of 1.8 is optimum for best DNA preparation.

For RAPD analysis ten Random tenmer primers were used (Table 1). PCR amplification of DNA extracted from seven species was done following the same standard protocol for all the primers. PCR products were run in agarose gel and bands were scored manually. The amplification profile generated by each primer was compared and the relatively molecular size of each band was examined by comparing the position of bands with markers. Divergence analysis using RAPD markers of different ectomycorrhizal species was done using UPGMA (Unweighted Pair Group Method with Arithmetic Mean) method.

RESULTS

The quality and quantity of extracted DNA from various protocols was checked by UV spectrophotometery and agarose gel electrophoresis. The ratio of optical density at 260 nm and 280 nm for ideal solution should be around 1.7 to 1.8.By comparing the data on successful completion of DNA extraction by various protocols, it was observed that among the four different protocols used, DNA was extracted successfully in all the samples ranging from good to very good by using all the methods but protocol D(Modified Doyle &Doyle method) was found best among four in terms of quality of DNA (Table 2) .

Among the 10 different 10 mer primers used, only four primers produced scorable products of which only two primers bound efficiently to all the six species (Table 3). The four primers generated a total of 39 PCR products ranging in size from 0.08-4.25 kb out of which 38 were polymorphic (Table 4).

The results were further analyzed by plotting dendrogram using UPGMA method. The results were further analysed by plotting dendrogram using UPGMA method. Hierarchical cluster analysis showed total five clusters.

Dissimilarity matrix

Dissimilarity coefficient ranged from 0.00 – 3.162 in different fungal species selected in present experiment (Table 5). This matrix was used to generate a tree for cluster analysis using UPGMA method (Fig 1). Species 2 and 3 have been found to have maximum value of dissimilarity coefficient, hence they have maximum dissimilarity.

DISCUSSION

Of the 24 samples analysed, only 13samples showed clear bands on agarose gel under UV light. The samples obtained by means of various protocols ,were evaluated according to DNA quality, colour, spectral absorbance ratio and final concentration .Out of four methods used, Quizen DNeasy Miniplant Kit method (protocol A) which uses silica gel-membrane technology and simple spin procedures to isolate high quality DNA ,was found not much better on quality basis for these species which is clear from spectroscopic analysis(A260/A280ratio).By comparing the results with reference to yield of the DNA,

Table 1. Sequence of Primers Used

Primer No.	Primer sequence (5'-3')
A.	CTCACGTTGG
B.	GGTACTCCCC
C.	TCGTGCGGGT
D.	CAGCGACTGT
E.	GTCCGTACTG
F.	GGTGCTCCGT
G.	CCTCTGACTG
H.	CACCCCCTTG
I.	GATACCTCGG
J.	TCCGATGCTG

Fermentas genomic DNA Purification Kit method(protocol B) was found best with good yield with more samples whereas with reference to quality of DNA Modified Doyle &Doyle method (protocol)was found best.

It is evident from the morphological traits that the species selected in this study showed considerable variations. The molecular analysis showed significant DNA polymorphism among these species. The dendrogram constructed with electrophoresis generated banding pattern from PCR amplification (RAPD) of six genomic DNA of different ectomycorrhizal fungal species, reveals the following:

Among total set of analyzed genotypes 5 genotypes can be clustered into one major group whereas one genotype (Species 2) is entirely different

The group is composed of genotypes.1, 3, 4, 5 and 6. In this species 3 was found to be segregated from rest of the other four members of analyzing collection. In this group a sub cluster was present which comprised species 1, 5, 4 & 6 In this sub cluster species 1,5 & 4 were further placed in another sub cluster whereas species 6 was found to be segregated from rest of the other three members of analyzing collection.

In the sub cluster comprising species 1,5 & 4, the genotype 1 and 5 found to be located on a single cluster, reflecting their homology of genomes. The genotype 1 is found to be distantly related to genotype 2 in evolutionary terms PCR based DNA markers such as RAPD is known to be very useful for investigating genetic relatedness

Table 2. Evaluation of DNA samples extracted using different protocols:

Extraction methods	Species	A260/A280	Concentration (μg/ml)
Protocol A			
(Quizen DNeasy Miniplant Kit)	*Boletus appendiculatus*	0.842	0.80
	Inocybe sp.	1.242	-
	Cortinarius sp.1	1.750	-
	Xerocomus chrysenteron	1.752	7.45
	Boletus variegates	1.369	-
	Cortinarius sp.2	0.615	-
Protocol B			
(Fermentas genomic DNA	*Boletus appendiculatus*	1.356	-
Purification Kit)	*Inocybe sp.*	1.425	6.20
	Cortinarius sp.1	1.769	2.30
	Xerocomus chrysenteron	1.791	10.30
	Boletus variegates	1.450	4.35
	Cortinarius sp.2	1.250	-
Protocol C			
(Wizard®Genomic DNA	*Boletus appendiculatus*	1.480	7.55
Purification Kit)	*Inocybe sp.*	1.680	2.10
	Cortinarius sp.1	1.937	3.10
	Xerocomus chrysenteron	1.913	2.2
	Boletus variegates	1.064	-
	Cortinarius sp.2	1.232	-
Protocol D			
(Modified Doyle &Doyle method)	*Boletus appendiculatus*	1.887	6.70
	Inocybe sp.	1.137	-
	Cortinarius sp.1	1.189	-
	Xerocomus chrysenteron	1.448	2.10
	Boletus variegates	1.750	3.15
	Cortinarius sp.2	1.791	4.30

Table 3. Sequence of Primer with gave positive result during RAPD analysis of DNA extracted from different ectomycorrhizal species

S.No.	Primers	Species 1	Species 2	Species 3	Species 4	Species 5	Species 6
1	5'-CAGCGACTGT-3'(D)	+	-	+	+	-	-
2	5'- GGTGCTCCGT -3'(F)	+	+	+	+	+	+
3	5'-CACCCCCTTG-3' (H)	+	+	+	+	+	+
4	5'-TCCGATGCTG-3'(J)	-	-	+	+	-	-

Table 4. Total Number of Amplified Products and Number of Polymorphic Products Generated By PCR Using RAPD Markers

Sl. no.	Sequence of Primers	Total number of amplified products	Total number of polymorphic products	Size range (kb)
1.	5'-CAGCGACTGT-3'(D)	5	5	0.7-2.0
2.	5'-GGTGCTCCGT -3'(F)	12	12	0.08-4.25
3.	5'-CACCCCCTTG-3' (H)	15	15	0.08-4.5
4.	5'-TCCGATGCTG-3'(J)	7	6	0.08-2.5

Table 5. Dissimilarity Matrix For Nei And Li's Coefficient Of Seven Species Of Ectomycorrhizal Fungi Based On RAPD Analysis:

	Species 1	Species 2	Species 3	Species 4	Species 5	Species 6
Species 1		2.828	2.449	2.236	1.000	2.236
Species 2	2.828		3.162	2.646	2.646	2.646
Species 3	2.449	3.162		2.646	2.646	2.646
Species 4	2.236	2.646	2.646		2.000	2.828
Species 5	1.000	2.646	2.646	2.000		2.449
Species 6	2.236	2.646	2.646	2.828	2.449	

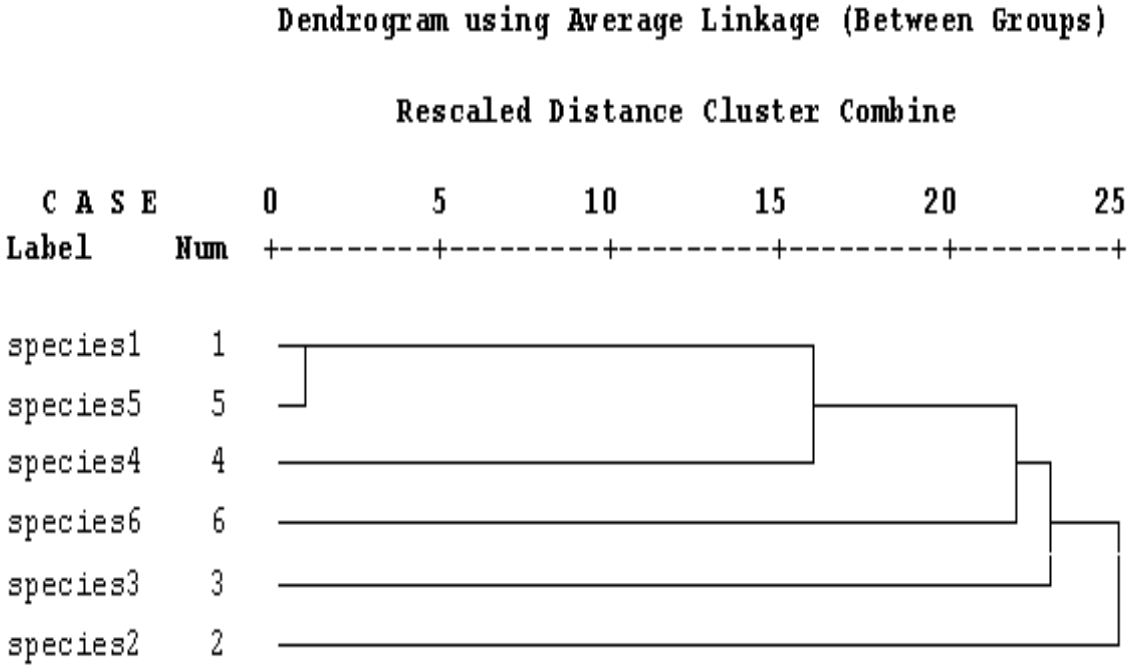

Fig. 1. Hierarchical Cluster Analysis

and diversity in fungal species. This study revealed that considerable differences could be observed between results from molecular markers and those from morphological traits for selected fungal species. This shows that RAPD method is capable of revealing appreciable levels of polymorphism in case of different ectomycorrhizal fungal species.

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