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A preliminary study of the ectomycorrhizal fungi associated with banj oak and chir pine in the Garhwal Himalaya, India

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Abstract

The assemblages of ectomycorrhizal fungi associated with chir pine (*Pinus roxburghii*) and banj oak (*Quercus leucotrichophora*) in the Garhwal Himalaya were characterized using molecular techniques. The present study is one of the first efforts in India to identify ectomycorrhizal fungi from DNA extracted from root-tips. A total of 23 taxa were recorded, and 15 of these were taxa known to be ectomycorrhizal. Eleven ectomycorrhizal taxa were recorded from banj oak and six from chir pine. Only two taxa (*Russula cerolens* and an unidentified member of the Russulaceae) were recorded from both types of trees. In addition to fungi identified from root-tips, five other taxa of ectomycorrhizal fungi were identified from fruiting bodies collected in forests dominated by either banj oak or chir pine.

Key words – DNA sequences – Garhwal – India – macrofungi – root tips – Russulaceae

Introduction

Hawksworth & Rossman (1997) provided a conservative estimate of 1.5 million species of fungi worldwide and indicated that approximately 1.43 million of these were not yet described. Later, Blackwell (2011) suggested that the total number of fungi may exceed 5 million. In either case, it is clear that the fungi represent a highly diverse group of organisms. The Himalayan region is one of the understudied regions of the world in terms of the fungi present, and it seems likely that a majority of the species of macrofungi, including those that are ectomycorrhizal, have yet to be documented. One of the major problems for studies of fungi in this region is that the fruiting season for a majority of the macrofungi coincides with the occurrence of the monsoon. Since the Himalayan Mountains are geologically young and fragile, they are prone to landslides and rock falls, thereby severely affecting all forms of transportation during summer monsoon, which limits the scope of field work and collection of the fruiting bodies of macrofungi.

As mentioned by Tedersoo et al. (2010), the revolution in molecular technology which took place in 1990s resulted in a significant improvement with respect to *in situ* identification of ectomycorrhizal fungi (Gardes et al. 1991, Egger 1995, Horton and Bruns 2001). With the help of molecular techniques, those fungi associated with roots could be identified without the need of ever

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observing their fruiting bodies. Moreover, comparisons of fungal DNA sequences obtained from fruiting bodies with DNA extracted from root-tips could be used to substantiate the fact that particular taxa were indeed ectomycorrhizal.

Although the use of molecular techniques to characterize ectomycorrhizal fungal assemblages from root-tips has been the focus of a number of studies (e.g., Gardes et al 1991, Taylor and Bruns 1999, Landeweert et al 2005), there are apparently no such studies in India. The objective of the study reported herein was to examine the assemblages of ectomycorrhizal fungi associated with chir pine (*Pinus roxburghii* Sargent) and banj oak (*Quercus leucotrichophora* A. Camus) in the Garhwal Himalaya in the state of Uttarakhand (India). According to Singh and Singh (1992), these are the two dominant trees in the forests of this region. Of the two species, the former typically occurs at lower elevations (1000–1800 m) and the latter at somewhat higher elevations (1500–2100 m).

Materials & Methods

Sample Collection

A first set of 117 root-tip samples was collected in the summer of 2012. These were obtained from 100 different trees, 50 chir pine and 50 banj oak. These 100 trees occurred at four different localities, two dominated by chir pine and two dominated by banj oak. An effort was made to collect at least 25 samples from each locality. The samples (Fig. 1) were extracted by digging the soil around the roots with the help of a trowel and hoe. Fine roots were cut off with scissors and placed in zip-lock bags. Later, the roots were gently rinsed with water and placed in 1.5 ml tubes containing a 2% CTAB (Cetyl Trimethyl Ammonium Bromide) solution. Each root tip was treated as an individual sample. In December 2014, 100 additional samples were collected. These samples were obtained from a total 25 trees, 15 banj oak and 10 chir pine.

In addition to the root-tips, 14 fruiting bodies of ectomycorrhizal fungi were collected from the same localities. For preservation of fresh fungal tissue, 1.5 ml tubes containing CTAB were used. An effort was made to obtain a sample of uncontaminated tissue (the tissue was taken from the inner portion of the stipe with the help of sterilized forceps and a razor) from each fruiting body, and this tissue was placed in tubes containing the CTAB. In addition, the fruiting bodies were dried for preservation. A number of other fruiting bodies observed during the field work were documented with digital images for morphology-based identification.

DNA Extraction

The tubes containing the samples were stored at -20° C until they were used for DNA extraction. To prepare the samples for DNA extraction, they were thawed and rinsed with distilled water to remove soil particles and other impurities. With the help of a dissecting microscope, individual colonized root tips were selected and placed in new 1.5 ml tubes. An Invisorb spin plant mini kit was used for DNA extraction, following the manufacturer's protocol. A similar procedure was followed for the specimens of fruiting bodies.

PCR and Sequencing

ITS1F and ITS4B (Gardes & Bruns, 1993), ITS1 and ITS4 (Bonello et al., 1998) and ITS1F and ITS4 primer combinations were used to amplify the internal transcribed spacer (ITS) sequence of the fungal rDNA region. The PCR reaction was set up using a volume of 25 µl as follow: 12.5 µl GoTaq Green 2X master mix (Promega Madison, WI), 1.25 µl Forward Primer, 1.25 µl Reverse Primer, 9.0 µl Molecular grade water and 1.0 µl extracted DNA. PCR amplifications were performed using a Bio Rad T100 Thermal Cycler (Bio Rad Inc., Hercules, CA). The PCR program was carried out with an initial activation at 94° C for 3 minutes, followed by 38 cycles of denaturation at 94° C for 1 minute, annealing at 55° C for 45 seconds, and extension at 72° C for 45 seconds. The final elongation was done at 72° C for 10 minutes.

Fig. 1 – Ectomycorrhizal root-tips of banj oak.

After PCR, gel electrophoresis was carried out using a 1% agarose gel (1 gm Genepure LA Agarose/100 ml of 1×TA buffer) in order to verify the quality of the PCR product. SYBR safe dye (1: 10000) was added in order to make the bands visible. After this, the gel was examined under UV light to determine the progress of the bands. If the progress was satisfactory, then the gel was photographed using the Bio-Rad Imaging System. The samples that showed bands were sent to Sanger Sequencing (Beckman Coulter Genomics, Danver, MA). The results were received in the form of chromatogram files with forward and reverse reads for each sample.

The reads were then edited manually and contigs were assembled using SeqMan (Version 7.1.0) software program. The edited contiguous sequences were then identified using the BLAST (Basic Local Alignment Search Tool) search tool on the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and the results were verified with the help of the UNITE database (http://www2.dpes.gu.se/project/unite/UNITE_intro.htm).

Results

A total of 23 taxa were recorded, and 15 of these are taxa known to be ectomycorrhizal (Tables 1 & 2). Eleven ectomycorrhizal taxa were recorded from banj oak and six from chir pine. Only two taxa (*Russula cerolens* and an unidentified member of the Russulaceae) were recorded from both types of trees. Eight of the taxa identified from DNA sequences extracted from root-tips are fungi that are not ectomycorrhizal. Three of these (*Cladosporium cladosporioides*, *Cladosporium tenuissimum*, and *Clavispora lusitaniae*) were recorded from both types of trees. *Delicatula integrella* was recorded only from banj oak, whereas *Mycena leptocephala*, *Oidiodendron maius*, *Penicillium restrictum* and *Trichoderma hamatum* were recorded only from chir pine. Most of these are common soil fungi that undoubtedly occur in the rhizosphere of many different kinds of trees. Therefore, there is no reason to attach any particular significance to their occurrence in the assemblage of fungi associated with either type of tree.

Table 1 Fungal taxa identified from root-tip samples collected from banj oak.

Taxon	Group of Fungi	Ecology
Cladosporium cladosporioides (Fresen.) G.A. de Vries	Ascomycota	Saprotrophic
Cladosporium tenuissimum Cooke	Ascomycota	Saprotrophic
Clavispora lusitaniae Rodr. Mir.	Ascomycota	Yeast
Delicatula integrella (Pers.) Fayod	Basidiomycota	Saprotrophic
Russula cerolens Shaffer	Basidiomycota	Ectomycorrhizal
Russula cf. amoenolens Romagn.	Basidiomycota	Ectomycorrhizal
Russula fulvograminea Ruots., Sarnari & Vauras	Basidiomycota	Ectomycorrhizal
Russula illota Romagn.	Basidiomycota	Ectomycorrhizal
Russula senecis S. Imai	Basidiomycota	Ectomycorrhizal
Russula sp. 1	Basidiomycota	Ectomycorrhizal
Russula sp. 2	Basidiomycota	Ectomycorrhizal
Scleroderma areolatum Ehrenb.	Basidiomycota	Ectomycorrhizal
Tomentella parmastoana Suvi & Kõljalg	Basidiomycota	Ectomycorrhizal
Tomentella sp.	Basidiomycota	Ectomycorrhizal
Unidentified member of the Russulaceae	Basidiomycota	Ectomycorrhizal

Thirteen of the 15 taxa of ectomycorrhizal fungi are members of the Basidiomycota, and only two taxa (*Delastria* sp. and *Tuber* sp.) are members of the Ascomycota. Both of the latter are hypogeous fungi, and their subterranean fruiting bodies are not easily observed in the field. Members of the Russulaceae were especially prominent as ectomycorrhizal associates of banj oak. Eight of 11 taxa recorded from the latter were members of this family, including one taxon that could be identified only to the level of family. Other ectomycorrhizal fungi recorded from banj oak were *Scleroderma areolatum, Tomentella parmastoana* and a second species of *Tomentella*. Six of the 13 taxa recorded for chir pine were ectomycorrhizal. These were *Delastria* sp., two different species of *Russula*, the same unidentified member of the Russulaceae recorded for banj oak, *Suillus triacicularis* and *Tuber* sp.

DNA sequences obtained from fruiting bodies collected in the field allowed five different taxa to be identified. These were *Lactarius chichuensis* W.F. Chiu, *Russula livescens* (Batsch) Bataille, *Suillus triacicularis* B. Verma & M.S. Reddy, *Xerocomus* sp. 1, and *Xerocomus* sp. 2. Identification of a particular fruiting body to species is often impossible if only an image is available, but many genera are distinct enough to be recognized. The images obtained in the present study included fungi from such ectomycorrhizal genera as *Amanita, Cantharellus, Cortinarius, Geastrum, Laccaria, Lactarius, Russula, Scleroderma, Suillus* and *Xerocomus*.

In a few instances, a tentative morphology-based identification to species was possible (Fig. 2). *Lactarius* cf. *sanjappae* K. Das, J. R. Sharma & Montoya, *Russula aeruginea* Lindbland ex Fr., *Russula* cf. *amoenolens* Romagn., *Russula* cf. *mariae* Peck, *Scleroderma areolatum* Ehrenb. And *Xerocomus* cf. *subtomentosus* (L.) Quél. were associated with banj oak, whereas *Cantharellus cibarius* Fr. and *Russula* cf. *amoenolens* were associated with chir pine.

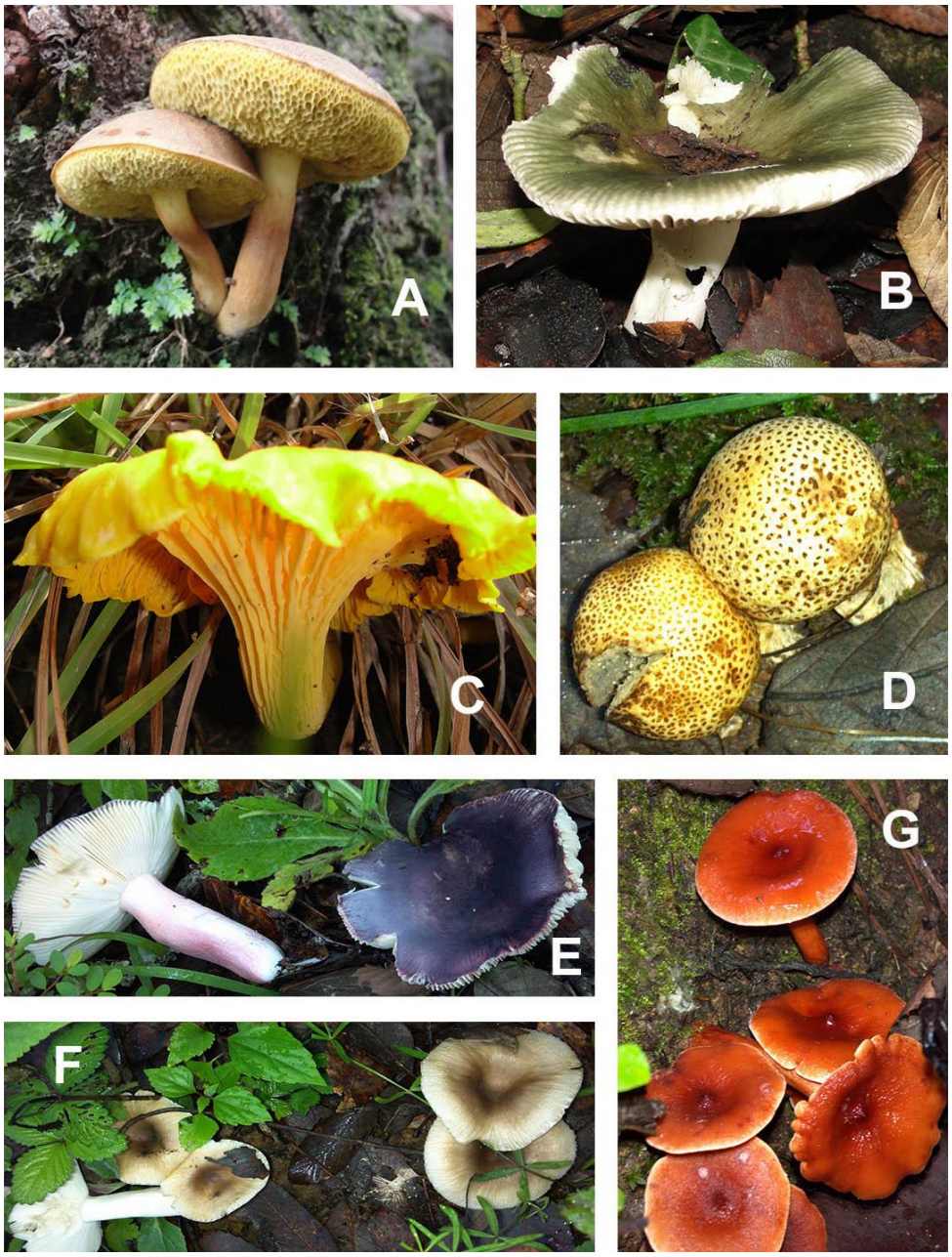

Fig. 2 – Morphology-identified fungal taxa associated with banj oak and chir pine in the Garhwal Himalaya: (A) *Xerocomus* cf. *subtomentosus*, (B) *Russula aeruginea*, (C) *Cantharellus cibarius*, (D) *Scleroderma areolatum*, (E) *Russula* cf. *mariae*, (F) *Russula* cf. *amoenolens*, and (G) *Lactarius* cf. *sanjappae*.

Discussion

The overall results obtained in the present study indicate that members of the Russulaceae (an agaric family that contains the genera *Russula* and *Lactarius*) are among the more ecologically important ectomycorrhizal associates of banj oak and chir pine in the forests of the Garhwal Himalaya. This is especially true for banj oak. The DNA sequences obtained in the present study were compared with those available on public databases (e.g., GenBank). However, the number of sequences of ectomycorrhizal fungi from northern India is relatively low, and an exact match for some of the sequences was not possible. This at least suggests that some of the sequences we obtained either represent described taxa for which sequences do not yet exist or taxa that are new to science. The latter seems likely be the case for at least a couple of the fungi represented by collections of fruiting bodies and is almost undoubtedly true for fungi identified from sequences extracted from root-tips.

Table 2 Fungal taxa identified from root-tip samples collected from chir pine.

Taxon	Group of Fungi	Ecology
Cladosporium cladosporioides (Fresen.) G.A. de Vries	Ascomycota	Saprotrophic
Cladosporium tenuissimum Cooke	Ascomycota	Saprotrophic
Clavispora lusitaniae Rodr. Mir.	Ascomycota	Yeast
Delastria sp.	Ascomycota	Ectomycorrhizal
Mycena leptocephala (Pers.) Gillet	Basidiomycota	Saprotrophic
Oidiodendron maius G.L. Barron	Ascomycota	Saprotrophic
Penicillium restrictum J.C. Gilman & E.V. Abbott	Ascomycota	Soil fungus
Russula cerolens Shaffer	Basidiomycota	Ectomycorrhizal
Russula sp. 3	Basidiomycota	Ectomycorrhizal
Suillus triacicularis B. Verma & M.S. Reddy	Basidiomycota	Ectomycorrhizal
Trichoderma hamatum (Bonord.) Bainier	Ascomycota	Soil fungus
Tuber sp.	Ascomycota	Ectomycorrhizal
Unidentified member of the Russulaceae	Basidiomycota	Ectomycorrhizal

As noted earlier in this paper, the monsoon season is the main fruiting period for the majority of macrofungi in the Himalayan region, and this is certainly the case for ectomycorrhizal fungi. Carrying out field studies of fungi during the monsoon season is particularly difficult because of the often exceedingly rugged terrain. However, the use of molecular techniques that allow the fungi present to be identified from DNA sequences extracted from root-tips represents an approach that does not depend upon collecting fruiting bodies in the field.

Although there have been a number of previous studies of ectomycorrhizal fungi carried out in India (e.g., Kumar et al. 1990, Bhatt et al. 2003, Pande et al. 2004), the present study apparently represents the first effort in which molecular techniques have been used to characterize the assemblages of such fungi associated with particular tree species. Although the results presented herein are preliminary, they clearly indicate that it is possible to characterize the ectomycorrhizal fungi present in the Himalayan region by using the DNA extracted from root-tips. As such, the present study represents a potential starting point for other more comprehensive studies that are needed to develop a more complete understanding of the assemblages of ectomycorrhizal fungi associated with the forests of the Himalayan region of India.

A number of studies (e.g., Saxena & Singh 1984, Singh et al. 1984, Singh & Singh 1986, Ralhan & Singh 1987, Singh & Singh 1992) have suggested that chir pine is expanding at the expense of banj oak in the Central Himalaya. It would be interesting to know just what impact this is having on the assemblages of ectomycorrhizal fungi associated with the two trees. Our data suggest that the assemblage associated with each of the two trees is compositionally distinct, with only a few taxa shared in common (Jaccard similarity coefficient = 0.22). Moreover, the assemblage associated with banj oak appears to be more biodiverse than the assemblage associated with chir pine. Just how these apparent patterns relate to the successional dynamics of forest communities dominated by banj oak and chir pine is not known. This aspect of the ecology of banj oak and chir pine certainly warrants additional study. Such studies could determine if there are any host-specific ectomycorrhizal fungi for these two tree species. A more complete knowledge of the fungi present could provide some additional insight into the ongoing ecological problem involving the apparent displacement of banj

oak by chir pine. For example, if the usual ectomycorrhizal associates of chir pine are already present in a banj oak forest, would this increase the chances of the former species becoming established? More importantly, the type of study reported herein has the potential of revealing many species of fungi (including non-ectomycorrhizal forms) not yet recorded as fruiting bodies from the forests of India. This would lead to a much more complete understanding of these organisms in this still understudied region of the world.

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