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Additions to the Ectomycorrhizae Associated with Himalayan Cedar (*Cedrus deodara*) using rDNA-ITS

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ABSTRACT

Himalayan cedar (*Cedrus deoadara* Roxb. Loud.), a member of Pinaceae, is an ever green tree of Himalayan origin and distributed from western Himalayan in Eastern Afghanistan, Pakistan, India and Western Nepal. Despite its usage in manufacturing of building material and furniture, it is an ectomycorrhizal host. In the present study, its four ectomycorrhizal fungi are characterized and identified using morpho-anatomic and molecular methods targeting its rDNA. The morphotypes of *Peziza* sp. MHSUC-01, *Russula livescens* and three species of *Tomentella* (*Tomentella* sp. 2ENA19_11, *Tomentella* sp. ENA35_13 & *Tomentella* sp. 2ENA35_13) are described first time from Pakistan. © 2012 Friends Science Publishers

Key Words: Himalayan moist temperate forests; rDNA; Mantle; PCR; Convergent

INTRODUCTION

Cedrus deodara (Roxb.) Loud. (Deodar cedar or Himalayan cedar), a member of Pinaceae, is native to Western Himalayas in Eastern Afghanistan, North Pakistan, North Central India, south western-most Tibat and Wesetrn Nipal. The trees are found to flourish at 1500-3200 m.a.s.l (Farjon, 1990). It is third most frequent (10%) after Abies pindrow Royle (60%) and *Picea simthiana* (15%) in Pakistan's part of Himalayan forests (Champion *et al.,* 1968). Ahmed *et al.* (2011) recorded *C. deodara* from 23 localities from Hindukush and Himalayan Moist Temperate forests of Pakistan. This extensive survey emphasizes its importance in ecosystem. This association of fungi, especially macrofungi, is of vital importance for tree growth and survival. This association is present in 85% of trees of world (Kirk *et al*., 2008).

Himalayan cedar has long been used in Eastern medicines and its wood extract is carminative, diaphoretic and useful in fever, flatulence, pulmonary and urinary disorders (Baquar, 1989). Several alkaloids have been reported (Zhang *et al.,* 1990; Digrak *et al.,* 1999; Shinde *et al*., 1999a, b; Rawat *et al*., 2000; Wolff *et al.,* 2001; Dimri & Sharma, 2004b) and extracted from different parts of *C. deodara* tree, which are of very useful medicinal importance.

The ectomycorrhizal symbioses have a different host range allowing the formation of ectomycorrhiza on a limited set of trees and shrubs. In temperate and boreal forests, up to 95% of the short roots form ectomycorrhizae (Smith & Read, 2008). Ectomycorrhizae have a beneficial impact on plant growth in natural and agroforestry ecosystems. Fundamental to the success of these symbioses is the switch of nutrients between the symbionts. In addition, the establishment of the symbiosis is required for the completion of the fungal life cycle. Himalayan cedar is also an excellent photobiont that makes ectomycorrhizal association with several groups of fungi. A few species have been reported forming ectomycorrhizae with *C. deodara* only from India (Singh & Lakhanpal, 2000; Deepika *et al.,* 2011; Saini & Singh, 2011) and from Pakistan (Niazi *et al.,* 2006, 2008). All these reports were based on cultural studies and morpho-anatomic characterization.

To understand the functioning of forest ecosystem, it is very important to characterize and identify the ectomycorrhizal fungi (Brand, 1992). Ectomycorrhizae are being identified using color, shape, macroscopic (ramification & presence of rhizomorphs) and microscopic (mantle organization & features of cystidia) characters (Agerer, 2002). But these features are not supportive to identify all the fungal species associated with roots, especially the mycorrhizae of related species. These often have similar morphological characters and are confused. The ectomycorrhizae formed by *Tuber* spp. (*T. maculatum* & *T. melanosporum*) morphologically appears to be similar to *T. indicum* (Comandini & Pacioni, 1997; Zambonelli *et al*., 1997, 1999). To resolve the problem encountered, DNA based methods have been introduced for correct identification of ectomycorrhizal fungi (Lanfranco *et al*., 1998; Horton & Bruns, 2001; Landweert *et al.,* 2003). These methods are based on targeting ribosomal DNA regions and preferred, because of their specificity and sensitivity. For this purpose, species specific primers have been designed for precise and accurate identification. Buncci *et al*. (2011) designed species specific primers to identify the *Tuber macrosporum*.

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In this study, focused on exploration of ectomycorrhizal morphotypes in Pakistan's Himalayan Moist Temperate forests, *C. deodara* morphotypes were sampled to measure the biodiversity of associated ectomycorrhizal fungi and indentify them using morpho-anatomic features and its rDNA genes. We used fungal specific primer (ITS1F) and universal primer (ITS4) to amplify its rDNA to confirm our morpho-anatomic identification.

MATERIALS AND METHODS

Himalayn cedar (*Cedrus deodara*) roots were sampled from Ayubia National Park, KPK. Sampled roots were voucher and wrapped in polythene bags. Morphotypes of *C. deodara* were manually selected after removing the soil particles from the morphotypes and kept in 2% CTAB buffer for DNA extraction and in distilled autoclaved H_2O for morpho-anatomic characterization. The selected morphotypes were characterized morpho-anatomically following Agerer (2002) and were deposited in Herbarium of Botany Department (LAH), University of the Punjab, Lahore. Morphological and anatomical characterization of *ectomycorrhizal system* was carried out under stereo and compound microscopes, photographed and illustrations were made with the help of Camera Lucida.

For molecular characterization, DNA was extracted from the selected morphotypes using modified CTAB method given by Bruns (1995). Amplification of the extracted DNA was performed using fungal specific and universal primers (ITS1F, ITS1 & ITS4). The hot-start enzyme JumpStart (Sigma, St Louis, MO, USA) was used to catalyse the PCR with 2 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 53°C, 40 s + 5 s per cycle at 72°C, and finishing with 5 min at 72°C. The PCR products were purified with QIA quick (Qiagen Inc., Valencia, CA, USA), sequenced bi-directionally using the reverse and forward primers and Big Dye Terminator Cycle Sequenceing V3.1 on an ABI 3730 DNA sequencer (Applied Biosystems, Foster City, CA, USA) and edited in sequencher 4.5 (Gene Codes, Ann Arbor, MI, USA) in Jodrell Laboratory, Royal Botanical Gardens, Kew, UK. DNA sequences were submitted to BLAST and used to query the nucleotide collection using default settings. The divergence in rDNA-ITS was measured by comparing sequence pairs reconstructed by using MegAlign (DNASTAR). DNA sequences obtained from *Cedrus deodara* morphotypes were submitted in GenBank. These sequences were manually edited using MacClade 4.08 and Bioedit (version 7.0.9).

RESULTS

***Peziza* sp. ENASUC_4.11 Gen Bank Accession No. JN836754**

Morphological Description: *Ectomycorrhizal system* this is highly dichotomous to coralloid. 5–7 mm long, axis 0.7–1.2 mm in diam. *Unramified ends* rounded to bent, bifurcate, 2–4 mm long and 1–1.5 mm in diam., younger tips creamy white, older tips brown to black. Texture of system was finely grainy, host tissue not visible under the sheath; *Rhizomorphs* absent, *Emanating hyphae* absent (Fig. 1a).

Anatomical characteristics of *mantle* in plan views: Mantle parenchymatous in all layers. *Outer mantle layers* parenchymatous with irregular shape of cells, light yellowish, no cell contents visible, cells 14–15 µm in length, 10–14 µm in width (Fig. 1b). *Inner mantle layer* parenchymatous, cells colorless to light yellowish, cells irregular in shape, no matrix material observed, cells 13–15 µm in length, 11–14 µm in width (Fig. 1c).

Molecular Description

Sequence data evidence: rDNA sequence of *Peziza* sp. ENASUC_4.11 was BLAST searched. This species shared 91% identity with *P. succosella* (DQ200841.1) and 89% with *P. cf. succosa* (EU819417.1) and identified as *Peziza* sp. ENASUC_4.11 (Table I). The genus *Peziza* (*Pezizales*) has first time been reported as ectomycorrhizal with *C. deodara*.

***Tomentella* spp. Gen Bank Accession Nos. JN836750, JN836751, JN836752**

Morphological Description: *Mycorrhizal system* the system is dichotomous, 2.5 mm long, main axis less than 1 mm wide, unramified tips straight, length of tips more than 1 mm and about 0.5 mm wide, colour of young and older tips dark brown to black, apices yellowish brown, texture rough, no visibility of host tissue through mantle. *Rhizomorphs* absent. *Emanating hyphae* frequent, restricted at tips, straight, blackish brown (Fig. 2a).

Anatomical characteristics of mantle in plan views: *Outer mantle layer* parenchymatous, cells 11–12 µm in length and 11.5 µm wide, no matrix material, no ornamation of cells, cell contents clear, brownish cells, cells rounded to irregular shape (Fig. 2b). *Inner mantle layer* also parenchymatous, no matrix material visible, cells 6.5–7 µm long and about 6 µm wide, cells smaller than outer mantle, cell contents clear (Fig. 2c).

Anatomical characteristics of emanating elements: *Rhizomorphs* absent. *Emanating hyphae* frequent have no clamps, septate, branched, cell contents clear, cells width about 4 µm and 25 µm long, cells thick walled (Fig. 2d).

Molecular Description: In the present investigation, genus *Tomentella* (*Thelephoraceae*) has also been reported as new mycobiont associated with roots of *C. deodara*. Three new isolates of *Tomentella* were described as ectomycorrhizal with *C. deodara.* rDNA from these three isolates when BLAST searched, they matched very close (92% & 93%) to *Thelephoraceae* sp. 'Taylor #2' (U83467.1) (Table I) and *Thelephoraceae* sp. P184 (FN669273.1) and second closest matched with *Tomentella* spp., (*Tomentella fuscocinerea* GU214810.1 & *Tomentella* sp., AJ534914.1). rDNA of these isolates were also found different from each other and showed divergence in ITS-rDNA ranging from 0.9-2.5 (Fig. 4a).

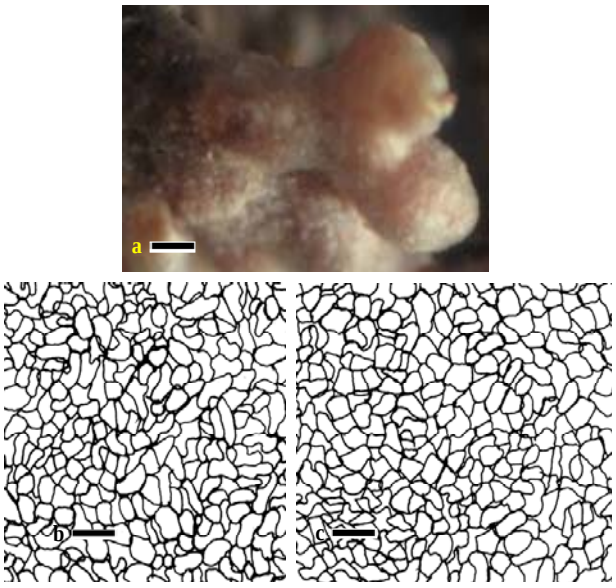
Table I: BLAST results of rDNA sequences from Pakistan along with closest matches and other parameters. Matching results of each morphotype based on rDNA ITS sequences

Fungal species	Voucher No.	GenBank Accession No.	Sequence length	Closest match in GenBank	Origin Country	GenBank Accession No.	Max Score	Query coverage	E Value	Max. Identity (%)
Tomentella sp.	ENA35_13	JN836750	605bp	*Thelephoraceae* sp. P184	Estonia	FN669273.1	911	100%	0.0	93%
Tomentella sp.	2ENA35_13	JN836751	816bp	*Thelephoraceae* sp. P184	Estonia	FN669273.1	911	99%	0.0	93%
Tomentella sp.	2ENA19_11	JN836752	618bp	*Thelephoraceae* sp. 'Taylor #2'	USA	U83467.1	887	99%	0.0	92%
Russula livescens	ENA27.12	JN836753	585bp	*Russula livescens*	China	GU371297.1	1027	100%	0.0	98%
Peziza sp. MHSUC 01	ENASUC_4.11	JN836754	420bp	*Peziza succosella*	USA	DQ200841.1	612	99%	0.0	91%

Table II: *Tomentella* spp. indicating the variations in molecular weight and G+C and A+T percentages

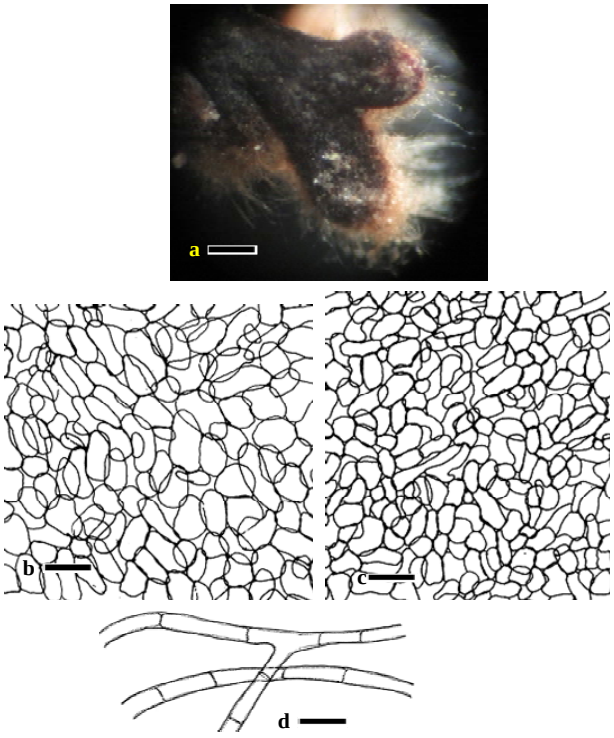
Fungal isolates	GenBank Accession No.	Sequence Length (bp)	Molecular Weight (Single stranded) in Daltons	G+C Contents	A+T Contents
Tomentella sp. ENA35_13	JN836750	605	183367	47.60%	52.40%
Tomentella sp. 2ENA35	JN836751	618	187225	47.90%	52.1%
Tomentella sp. 2ENA19_11	JN836752	618	187172	47.57%	52.43

Fig. 1a-c: *ECM of Peziza* sp. ENASUC_4.11 (a) Habit, (b) Outer *Mantle* and (c) Inner Mantle
Scale Bar for Fig. 1=0.53 mm, 2=2.25 µm, 3=2.25 µm



The G+C/A+T contents of these isolates showed slight variations (Fig. 3). When genetic characters of these isolates compared with each other, these were found to be different. *Tomentella* sp. 2ENA19_11, *Tomentella* sp. ENA35_13 and *Tomentella* sp. 2ENA35_13 shared 96.80-98.82% of genetic characters analyzed and showed 89.8% identity. Intra-specific variations in rDNA-ITS of these isolates observed in 671 base pairs rDNA sequence alignment length. There were total of 20 sites in the alignment of *Tomentella* spp. Sequences, which represented rDNA intra-specific variations in ITS and 5.8S gene of rDNA (Fig. 5) and hence treated as different isolates. But morphotypes of all these isolates possess similar morpho-anatomic features.

Fig. 2a-d: *ECM of Tomentella* spp., (2ENA19_11, ENA35_13, 2ENA35_13) (a) Habit, (b) Outer Mantle, (c) Inner *Mantle* and (d) Emanating hyphae
Scale Bar for 4= 0.39 mm, 5= 0.96 µm, 6= 0.96 µm, 7= 3.57 µm

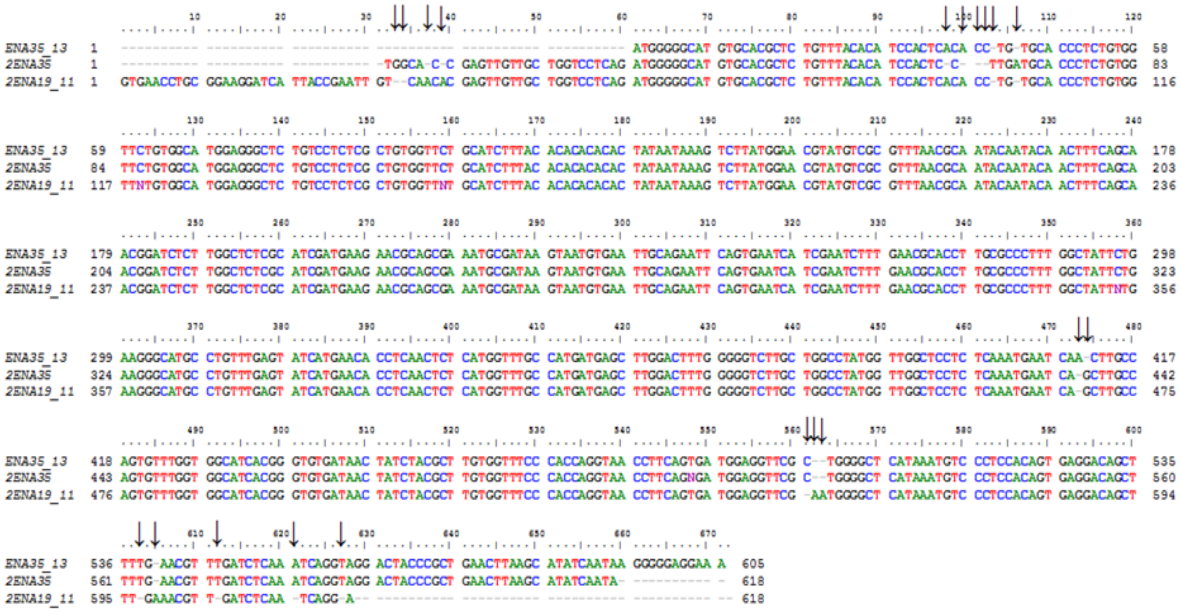


Thus, these seemed to be convergent morpho-anatomically and divergent phylogenetically.

***Russula livescens* (Batsch) Quélet Gen Bank Accession No. JN836753**

Morphological Description: *Ectomycorrhizal system* dichotomously branched, system up to 7 mm long with 2 mm thick main axis, unramified tips 2-3 mm long and

Fig. 3: Intra-specific variations among three isolates of *Tomentella* spp. from Pakistan. Arrow heads indicating the insertions and deletions of nucleotides in rDNA segments of three isolates of *Tomentella* spp



0.3-0.5 mm thick, colour of system dark yellowish brown, younger tips dark honey brown, straight to bent slightly, surface of mycorrhizal system velvety, host tissue not visible under mantle surface. *Rhizomorphs* absent. *Emanating hyphae* rare, branched, brownish (Fig. 4a).

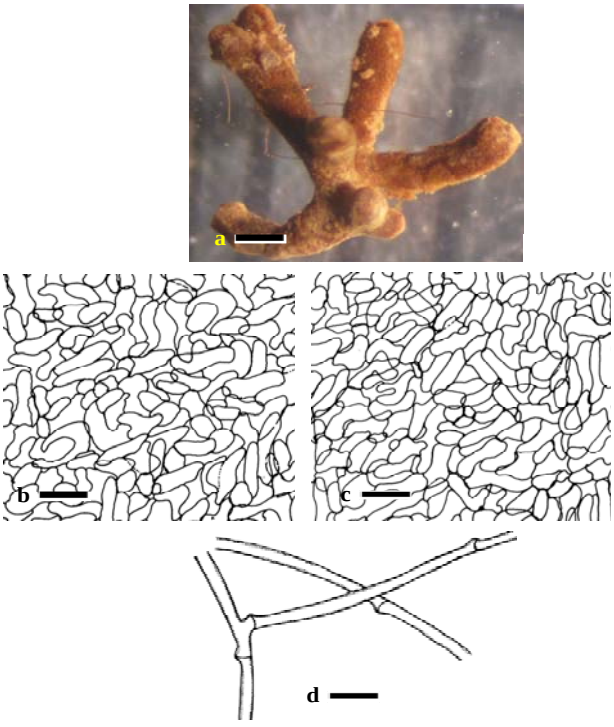
Anatomical characteristics of mantle in plan views: Mantle parenchymatous in all layers. *Outer mantle layer* is parenchymatous, cells elongated to irregular in shape, 17-18 µm in length and 6–7 µm in width, light yellowish, no cell contents, no septa and clamps (Fig. 4b). *Inner mantle layer* also parenchymatous, cells elongated to irregular in shape, 18.4 µm long and 5.6 µm in width, honey to light yellowish in color, no matrix material, no septa and clamps (Fig. 4c).

Anatomical characteristics of emanating elements: *Rhizomorphs* absent. *Emanating hyphae* branched, septa only at clamps, light yellowish, cells longer, thick walled, septa also thick, smooth, cells 3.5–4.3 µm in diam., 66.5 µm in length (Fig. 4d).

Molecular Description: *Russula livescens* is also reported as new mycobiont for *Cedrus deodara*. rDNA sequence of *Russual* ssp. from Pakistan when BLAST searched, it matched 98% with *R. livescens* (GU371297.1) (Table I) and a new record for Pakistan.

Fig. 4a-d: *ECM of Russula livescens*. (a) Habit, (b) Outer *Mantle*, (c) Inner *Mantle* and (d) Emanating hyphae

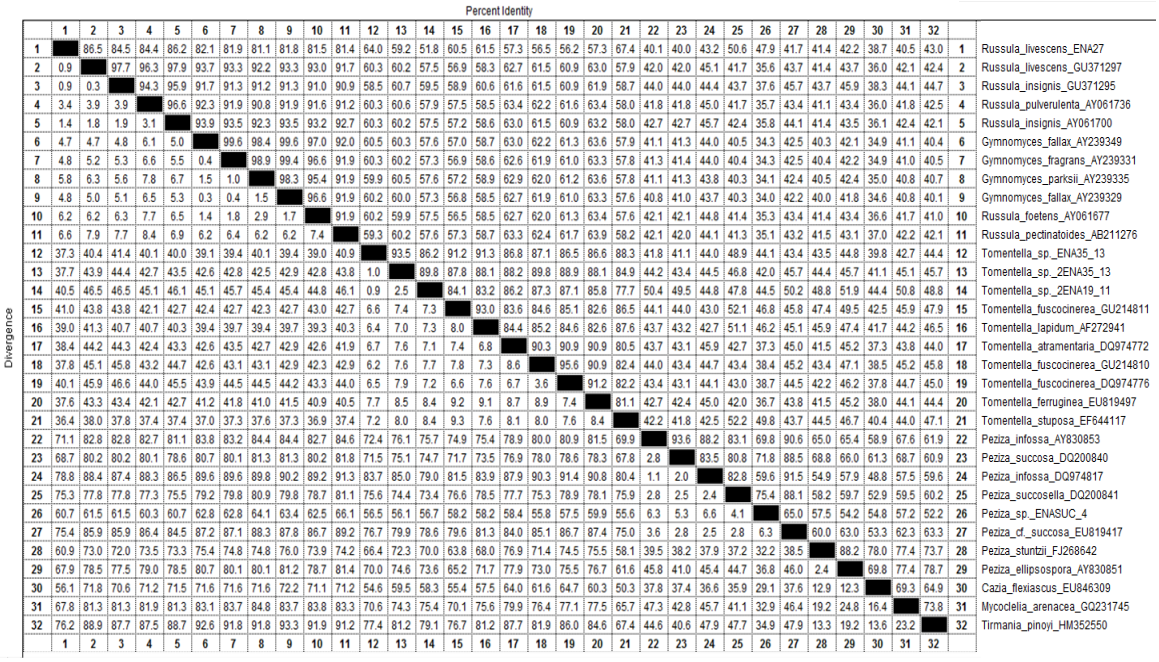
Scale Bar for 9= 0.48 mm, 10= 3.22 µm, 11= 3.22 µm, 12= 10 µm



DISCUSSION

In the present study, we documented the ectomycorrhizal forming fungi from the roots of Himalayan cedar first time from Pakistan based on molecular methods. These fungi on the roots were described morpho-anatomically and identified using rDNA sequences from these molecular operational taxonomic units (MOTUs)/species. We found this technique quite effective in the identification of fungi found symbiotically on roots of *C. deodara*. Our findings are in agreement with the study conducted by Iotti and Zambonelli (2006). They used the similar technique to identify *Tuber* ectomycorrhiza.

Fig. 5: Percent divergence and percent identity calculated by comparing sequence pairs reconstructed by MegAlign (DNASTAR)



We report five ectomycorrhizal MOTUs/species associated with Himalayan Cedar first time from Pakistan viz; *Peziza* sp. MHSUC-01, *Russula livescens*, *Tomentella* sp. 2ENA19_11, *Tomentella* sp. ENA35_13 and *Tomentella* sp. 2ENA35_13. Genus *Peziza* has 104 species worldwide and is represented by eight species in Pakistan (Ahmad *at al.,* 1997). *Peziza* sp. MHSUC-01 characterized and identified morpho-anatomically and using rDNA sequence data is found very close to *P. succosella* (DQ200841.1). To our knowledge, *P. succosella* is not reported as ectomycorrhizal. Another ally of *Peziza* sp. MHSUC-01 is *P. succosa,* which is reported as ectomycorrhizal by Tedersoo (2006). It has whitish cottony morphology, with pseudoparenchymatous mantle having spherical to rectangular, blunt-angled and thick walled cells in outer mantle and rectangular to polygonal thin walled cells in inner mantle (Tedersoo, 2006) as compared to *Peziza* sp. MHSUC-01. So, *Peziza* sp. MHSUC-01 is being treated as new MOTU/species. With this addition, the number of *Peziza* spp., is raised to nine from Pakistan.

We reported three MOTUs/ species of *Tomentella* viz; *Tomentella* sp. 2ENA19_11, *Tomentella* sp. ENA35_13 and *Tomentella* sp. 2ENA35_13 as ectomycorrhizal. Genus *Tomentella* is also not reported as ectomycorrhizal from Pakistan. Previously, only seven species were reported by Ahmad *et al.* (1997). With the present report, this number has increased to ten. The morphotypes of these three ectomycorrhizal isolates has striking similarities in morpho-anatomic features (Fig. 2a-d) but differ from other related species of *Tomentella*. Raidl and Müller (1996) described *T. ferrugenea,* a close ally of *Tomentella* spp. from Pakistan, as ectomycorrhizal with *Fagus sylvatica*. It has monopodial pinnate or monopodial pyramidal ramification with plectenchymatous mantle organization (Raidl & Müller, 1996) in contrast with dichotomous branched ramification and parenchymatous mantle organization in all *Tomentella* isolates from Pakistan.

Species of *Tomentella* included in present study has intraspecific variations in their rDNA region. There are several insertions and deletions indicated in the sequenced and aligned data (Fig. 3). Intraspecific variations in their repeat regions were also observed by the relative measuring the G+C contents. Maoxian *et al.* (2005) studied the intraspecific variations in Pearl Oyster by measuring G+C contents. Another study conducted by Muthumeenakshi *et al.* (2001) showed the similar rDNA-ITS variations in *Coniothyrium minitans* (*Ascomycota; Pleosporales*) and its related species viz; *C. sporulosum* and *C. cerealis*. They differentiated *C. minitans* and *C. cerealis* on the basis of their G+C contents.

Himalayan Cedar has another mycobiont, *Russula livescens,* found symbiotically with its roots. *R. livescens* reported as ectomycorrhizal with *Pinus yunnanensis* (Xue-Dan, 2010) and *Cistus* sp. (Contu, 1984). Occurrence of *R. livescens* with *C. deodara* is also a new record. Previously, 23 species of *Russula* have been reported by Ahmad *et al.* (1997). There are some other reports (Khalid, 1998; Niazi, 2008) regarding the *Russula* from Pakistan, but not a single report about their ectomycorrhiza especially with *C.*

deodara. R. livescens is reported as new record from Pakistan, raising their number to 24.

The present investigation emphasizes the use of rDNA based technology for the precise and accurate identification of ectomycorrhizal fungi. Ectomycorrhiza of *C. deodara* have been first time reported from Pakistan using molecular methods. This host tree is unexplored from Pakistan and comprehensive study could add the mycoflora associated with it and of Pakistan.

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