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ECTOMYCORRHIZAL STATUS OF *PINUS WALLICHIANA* (BLUE PINE) GROWING IN HIMALAYAN MOIST TEMPERATE FORESTS OF PAKISTAN

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Abstract

The root system and morphotypes of *Pinus wallichiana* were investigated for symbiotic association of fungal communities by analyzing them at molecular level for the first time in Pakistan. From twenty one (21) soil cores, twenty three (23) molecular operational taxonomic units (MOTUs) belonging to 11 genera were identified. These included *Cortinarius*, *Helvella*, *Hymenoscyphus*, *Hypocrea*, *Inocybe*, *Nectria*, *Ophiocordyceps*, *Russula*, *Sebacinaceae*, *Suillus* and *Tomentella*. Biodiversity, species richness and community structure of these fungi have been described with reference to this novel host. *Hymenoschyphus* sp. Trtsf14 was found to be most abundant taxon (30.61%), followed by *Inocybe* sp. MHNIT-01 (35.41%) and *Suillus sibiricus* (18.75%), respectively. Neither accumulation curve nor any species richness estimators approached an asymptote and thus more extensive sampling is suggested for completion of below ground fungal communities.

Key words: Biodiversity, Fungal communities, MOUs, Species richness.

Introduction

Pinaceae is among the most commercially and economically important plant family, valued for its timber and wood pulp, throughout the world. It is native to Northern Hemisphere and is present in Himalayan, Karakoram and Hindu Kush mountain ranges of Pakistan (Champion *et al*., 1965). Members of this family are obligatory ectomycorrhizal. Pinaceae in Pakistan is represented by 4 genera and 9 species (Nasir *et al*., 1969). The Pinaceae being the most invasive family of and shrubs for ectomycorrhizal associations, with *Pinus* being the most invasive genus of this family (Richardson & Rejmánek, 2004). *P. wallichiana* constitutes 10% of total vegetation of Himalayan Moist Temperate Forest (Champion *et al*., 1965). Forests in Pakistan are declining with annual reduction of 2.5% (Economic Survey of Pakistan, 2016-17) due to local fuel wood collection, commercial harvesting and mismanagement. So, it is a dire need of time to reforest these rapidly declining forests. Growth of forestry increased to 6.47 percent becasue of increased timber production in Khyber Pakhtunkhwa in the range of 26.7 to 36.1 thousand cubic meters (Economic Survey of Pakistan, 2018-19). Ectomycorrhizal fungi are an important constituent of many forest ecosystems where they are playing vital roles in maintenance of soil properties, plant community dynamicsrecycling of nutrients, plant community dynamics and recycling of nutrients maintenance of soil properties (Smith & Read, 2008). The communities formed by these fungi can have direct or indirect impact on the ecosystem (Staddon *et al*., 2002). There are only a few reports concerning ectomycorrhizal fungi in association with *P. wallichiana* from Pakistan. These studies are based on morpho-anatomical and direct tracing methods for identification of ectomycorrhizal morphotypes (Ilyas, 2013). However, molecular data of ectomycorrhizal fungi associated with this host is absolutely lacking. Present investigation is first such attempt from Pakistan in which diversity and species richness of mycoflora associated with blue pine roots have been assessed. During this study, 23 fungal morphotypes have been identified using molecular tools based on ITS-nrDNA sequence data and their phylogenetic position using Maximum Likelihood method has been described. The belowground community structure and composition of mycoflora associated with same host have also been investigated for the first time.

Materials and Methods

Description of the sampling site: Selected sampling areas of Himalayan Moist Temperate forest included Ayubia National Park, Helipad-Khanspur, Nathia gali and adjoining areas. Sampling was made frequently during June to August (2008-2010). These areas receive 1520 mm to 127 mm annual rain fall with mean annual temperature ranging from -4 to 25°C.

Collection of the morphotypes and extraction of DNA: Soil cores, 6 × 12 cm cm^2 containing ectomycorrhizal roots of *P. wallichiana* were sampled randomly at various depths (6-12 cm) beneath sporocarps growing near vicinity (1-2 meters) of *P. wallichiana* with the help of digger. Soil cores were vouchered and brought in the laboratory for further analysis. Root samples were washed under running shower of water using 10-50 mm^2 sieves. Isolated EcM morphotypes were counted and classified according to morphology using a dissecting microscope. Characters such as colour, texture, presence or absence of emanating hyphae and rhizomorphs were noted. Randomly selected four morphotypes per each soil core were analyzed further. DNA was extracted using modified CTAB method following Gardes and Bruns (1993) blended by Q biogen Clean Kit protocol and RED Extract-N-Amp ™ Plant PCR kit (Sigma, St. Louis, Missouri, USA).

rDNA-ITS amplification and sequencing: ITS-nrDNA region was amplified through Polymerase Chain Reaction (PCR) following Gardes and Bruns (1993) and Extract–N–AmpTM Plant PCR Kit (SIGMA) using ITS1F/ITS1 & ITS4 primers. The amplified PCR bands were analysed and visualized on agarose gel with the help of Gel Documentation System (Model: UVtec, Avebury House, Cambridge CB4 1QB UK) using default manufacturer's settings. PCR products were sequenced using cycle sequencing reaction based on Sanger method (Sanger *et al*., 1977). All the obtained DNA sequences were BLAST searched in genbank. The DNA sequences were matched with ≥ 97% similarity cut-off value for ECM fungal species identification (Ryberg *et al*., 2008; Walker *et al*., 2008).

Phylogenetic analyses: Retreived ITS-nrDNA sequences were aligned for phylogenetic analyses using MEGA 6 with 100 replicates (Tamura *et al*., 2011). Parsimony criterion with 1000 bootstrap replicates was used as a support for individual branches for phylogenetic analysis.

Biodiversity calculation: Species richness, species accumulation and other diversity parameters of ectomycorrhizal morphotypes associated with *P. wallichiana* were calculated by using the statistical software programme "Estimate S". Shannon–Wiener (H′) Diversity Index was used to assess species diversity. Species accumulation curve was plotted for measuring species richness to represent the cumulative number of observed myco-flora sequences as a function of sampling effort (Colwell, 2006). Different species richness estimators have been calculated: Observed Species, Chao1, Chao2, Jack1, Jack2, ACE and ICE were used for the present study.

Results

Belowground community structure and composition (Figs 1-6, Table 1)

Pooled roots of *P. wallichiana* were found to be colonized by 23 species, from which 20 were identified as ectomycorrhizal and three as non-mycorrhizal. Among them, eight taxa were identified at species level, fifteen taxa at genus level and two at family level (Table 1). For the present investigation, all species belonging to Basidiomycota were identified as ectomycorrhizal following Tedersoo (2017), Tedersoo *et al.*, (2009) and Rinaldi *et al*., (2008) and their association with *P. wallichiana* is being documented for the first time from Pakistan. Among species belonging to Ascomycota, *Helvella albella* and *Hymenoschyphus* sp. were identified as ectomycorrhizal. *Hymenoschyphus* sp. dominated the Ascomycetous group over all fungal community associated with living roots and were treated as ectomycorrhizal.

From 23 MOTUs/ species, *Hymenoschyphus* sp. (Trtsf14), *Inocybe* sp. (MHNIT-01) and *Suillus sibiricus* were the most abundant (Fig. 3). Percentage abundance of *Hymenoscyphus* sp., *Inocybe*, *Russula* and *Tomentella* in the community was 32.65%, 14.28%, 16.2% and 10.2%, respectively (Table 1).

Table 1. Distribution of belowground mycoflora of *P. wallichiana* with their observed and percent abundance.

	Species name	Observed abundance	% Abundance
	Ascomycetes		
1.	*Helvella albella*	1	2.04
2.	*Hymenoscyphus* sp. Trtsf14	15	30.61
3.	*Hymenoscyphus* sp.	1	2.04
4.	*Hypocrea* sp. MHVIR-01	1	2.04
5.	*Nectria haematococca*	1	2.04
6.	*Ophiocordyceps* sp. MHSIN-01	1	2.04
	Basidiomycetes		
7.	*Cortinarius leucopus*	2	4.08
8.	*Inocybe cryptocystis*	2	4.08
9.	*Inocybe* sp. MHCRY-01	2	4.08
10.	*Inocybe* sp. MHNIT-01	3	6.12
11.	*Inocybe* sp. MHTAR-01	1	2.04
12.	*Russula cessans*	2	4.08
13.	*Russula lutea*	2	4.08
14.	*Russula nigricans*	1	2.04
15.	*Russula* sp. MHCER-01	1	2.04
16.	*Russula* sp. MHNAU-01	1	2.04
17.	*Sebacinaceae* sp. 2ENA24	1	2.04
18.	*Sebacinaceae* sp. F42	2	4.08
19.	*Suillus sibiricus*	3	6.12
20.	*Thelephoraceae* sp.	1	2.04
21.	*Tomentella* sp. MH13	1	2.04
22.	*Tomentella* sp. MH291689	1	2.04
23.	*Tomentella* sp. MHFUS-01	2	4.08
24.	*Tomentella* sp. MHPAK-01	1	2.04

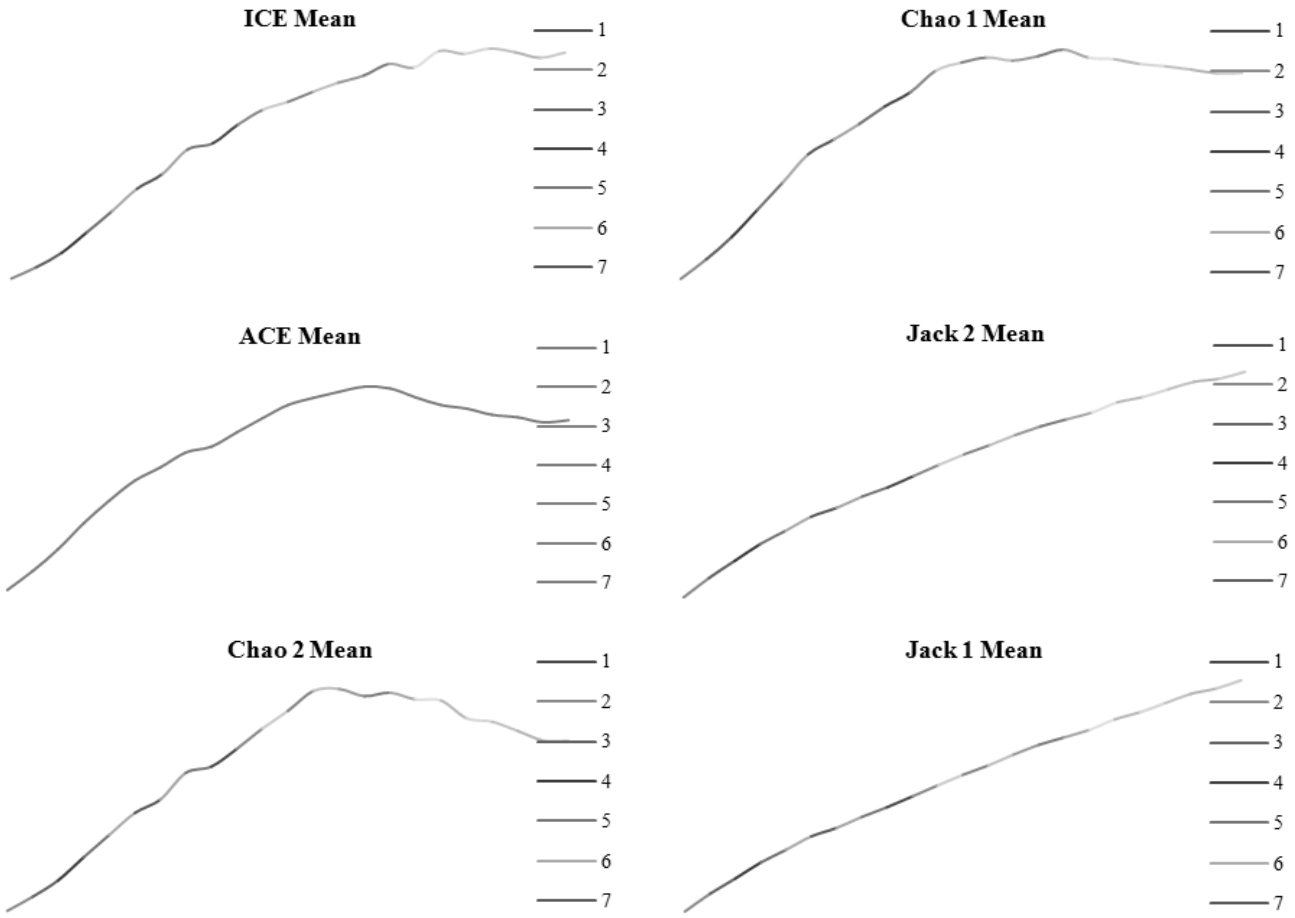

Fig. 1. Species Richness Indices (ACE, ICE, Chao 1, Chao 2, Jack 1and Jack 2) indicating estimated mycoflora associated with *Pinus wallichiana.*

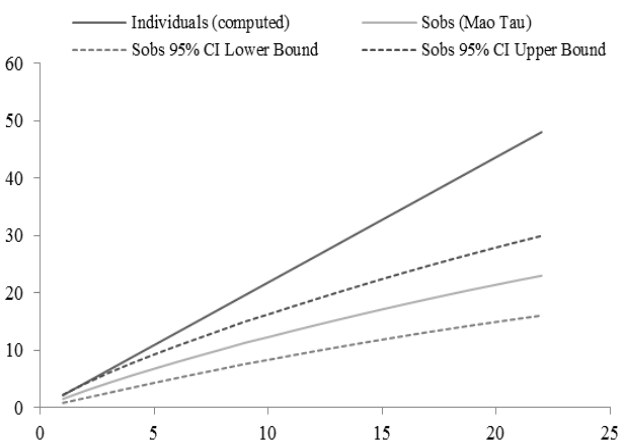

Fig. 2. Species accumulation Curve for mycoflora associated with *Pinus wallichiana*.

The species accumulation curve (Fig. 2) indicated the incompleteness of fungal community and did not reach the platue. Similar behaviour was observed with Chao 1, Chao 2, Jack 1, Jack 2, ICE and ACE curve (Fig. 1) indicating insufficient sampling. The species richness indices Jack 1, Jack 2, Chao 1, Chao 2, ICE and ACE predicted that 39.33, 51.9, 39.26, 50.42, 85.7 and 40.11 fungi could be associated with *P. wallichiana*, respectively. In present exploration, neither accumulation curve nor any species richness estimator approached an asymptote with increasing sampling size (Figs. 1 & 2).

For the present study, the sampling size maintained at three root tips per soil revealing 2.08 species per core. Ten taxa occurred as singletons in some soil cores. We measured the species diversity by Shannon Index for the mycoflora associated with root system of *P. wallichiana*. The value of this index (2.67) showed low to intermediate biodiversity.

Molecular identification and phylogenetic analysis of fungal taxa: From the living roots system of *P. wallichiana*, 47 ITS-nrDNA sequences were obtained. These were grouped into 23 fungal taxa and BLAST searched for closest match in GenBank (Table 1). The Phylogenetic relatedness was drawn using the Maximum Likelihood criterion (Tamura & Nei, 1993). Total 77 sequences were subjected for the analysis. All ambiguous nucleotide positions were excluded for each sequence pair. A total of 858 nucleotide positions in the final analysis were included. Phylogenetic analyses were conducted in MEGA6 (Tamura *et al*., 2011).

The mycoflora associated with the *P. wallichiana* roots were identified using rDNA-ITS sequences. In the present investigation, 22 phylotypes were listed on the basis of cladistic distribution of fungal taxa associated with *P. wallichiana* excluding *Ophiocoryceps* sp. MHSIN-01 because of its very short rDNA-ITS sequence. The cladogram included 22 ectomycorrhizal and non-mycorrhizal phylotypes distributed in 3 major clades/ 8 sub-clades.

Agaricales clade included 05 ectomycorrhizal fungal species from genus *Cortinarius* and *Inocybe* from *P. wallichiana*. Among these, *C. leucopus* (Bull.) Fr. shared 97.24% of analyzed genetic characters with *C. leucopus* (HQ604721.1, Canada). To the best of our knowledge, this species is not the part of belowground community associated with any member of Pinaceae in Pakistan. The other four species in Agaricales clade belonged to genus *Inocybe* shared by 04 rDNA sequences viz; *Inocybe* sp. MHCRY-01, *I. cryptocystis*, *Inocybe* sp. MHNIT-01, *Inocybe* sp. MHTAR-01 and these phylotypes of *Inocybe* are also being described first time in association with *P. wallichiana* in the present study. All members of Agaricales formed a monophyletic clade with strong support of bootstrapping (93%) and are addition to the mycoflora of Pakistan. With the addition of four new phylotypes, the number of *Inocybe* species increased to nineteen (27). Boletales clade represented exclusively by genus *Suillus* with strong bootstrap support (98%). From Pakistan, only *Suillus sibiricus* (Singer) Singer was found to occur in the belowground community associated with *P. wallichiana* and it shared 99.13% of analyzed genetic characters with *S. sibiricus* retrieved from GenBank (L54117.1, USA). In present study, Thelephorales clade was not highly resolved by bootstrapping (45%), this polytomous clade was dominated by *Tomentella* spp., observed in ectomycorrhizal communities of *P. wallichiana*. We documented four (04) phylotypes belonging to Thelephorales viz; Thelephoraceae sp. MHR291689, *Tomentella* sp. MH13, *Tomentella* sp. MHFUS-01 and *Tomentella* sp. MHPAK-01. Ectomycorrhizal status of family Sebacinaceae was not known previously from Himalayan region. Both Sebacinaceae spp. were found ectomycorrhizal with blue pine in Pakistan's part of Himalayan Moist Temperate forests. Members of Russulales are well represented in forests of Pakistan. This clade formed by species clustered together with strong bootstrap support (99%) and included five phylotypes isolated from pine roots. In the phylogram, species of Russulales formed short branches except *Russula* sp. MHNUA01. *R. cessans*, *R. lutea*, *R. nigricans*, *Russula* sp. MHCER-01 and *Russula* sp. MHNAU-01 are reported as ectomycorrhizal with *Pinus wallichiana*. Clade Pezizales formed by *Helvella* spp. evolved separately from Hypocreales and Helotiales (Fig. 5) supported by high percentage of bootstrapping (98%). *H. albella* (ENA22938) from Pakistan shared 97.98% genetic characters with *H. albella* (FJ859343) and this is first report of its ectomycorrhizal status from Pakistan. Clade Hypocreales is composed of seven species of order Pezizales (Fig. 5) including 2 species (*Hypocrea* sp. MHVIR-01, *Nectria haematococca*) of Hypocreales from the present investigation. Both MOTUs/ species were reported as root endophytes for first time with respect to Pinaceae. *N. haematococca* is new plant endophyte from Pakistan. Clade Helotiales includes only two phylotypes (*Hymenoschyphus* sp. Trtsf14 and *Hymenoschyphus* sp. 1) from Pakistan.

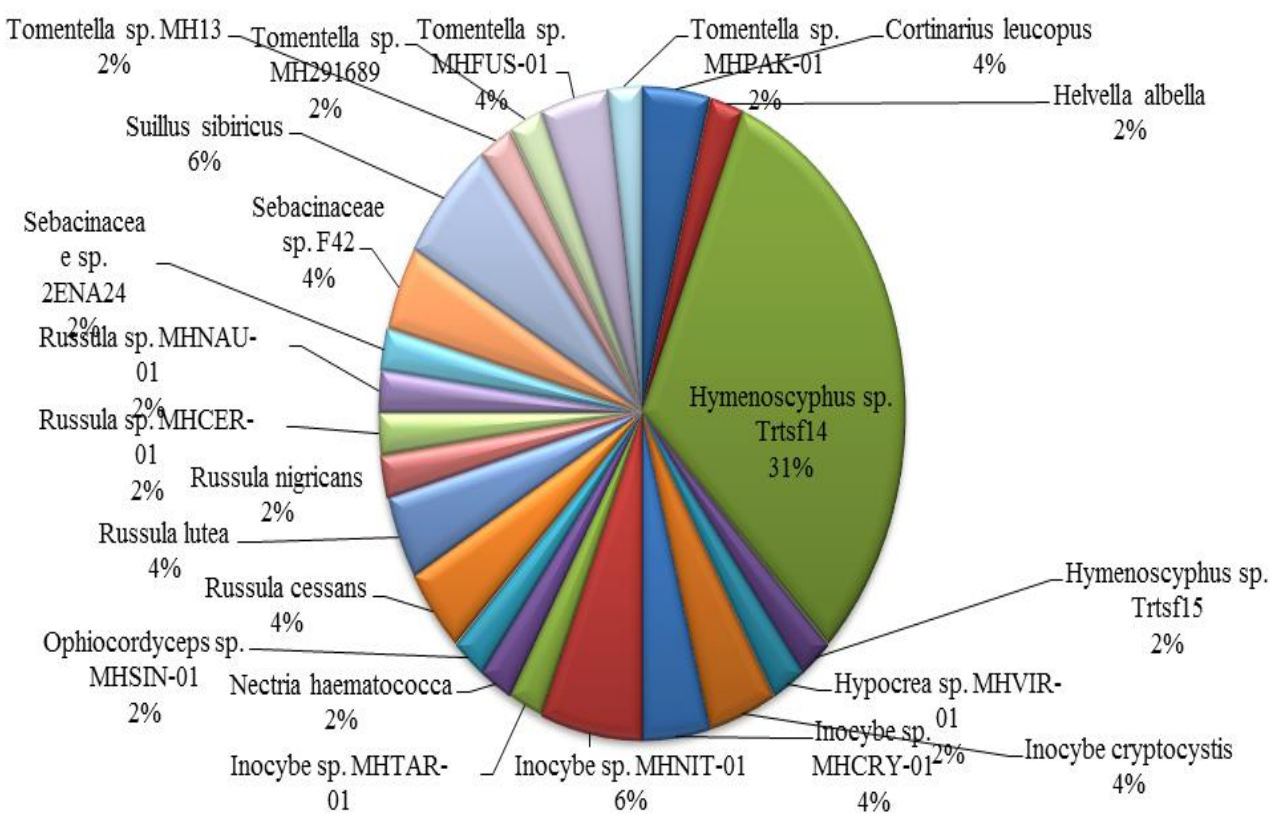

Fig. 3. Community composition of mycoflora associated with *Pinus wallichiana*.

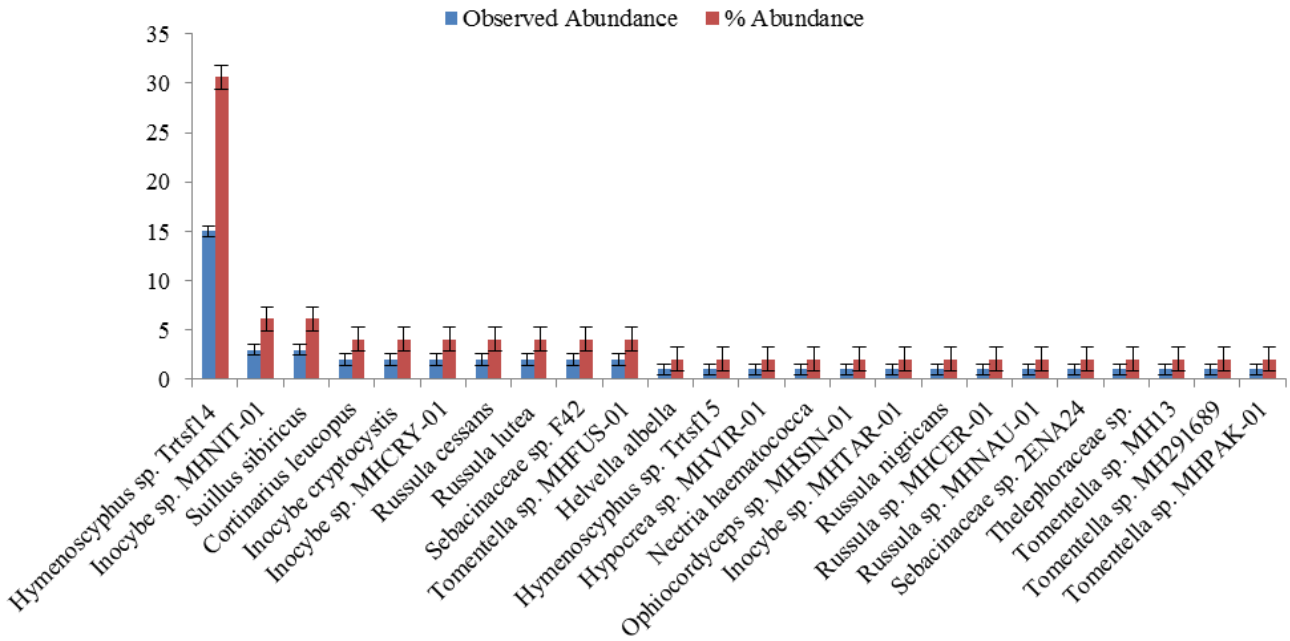

Fig. 4. Observed abundance and percent abundance of mycoflora associated with *Pinus wallichiana.*

Discussion

Structure and composition of belowground community: There were no previous reports about the community structure and composition of ectomycorrhizae associated with *P. wallichiana* from Himalayan Moist Temperate forests of Pakistan employing barcoding of rDNA-ITS region despite the fact that this tree dominates in this region. There are a few records (from Pakistan) of ectomycorrhizae (Niazi *et al*., 2006; 2010; Saba, 2016; Sarwar *et al*., 2016; Jabeen *et al*., 2017). In the present investigation high species diversity and richness was observed as indicated by diversity and species richness indices (Fig. 1). rDNA-ITS sequences also indicated some unreported and undescribed fungal species in association with *P. wallichiana*. There are several reports worldwide about the belowground ECM community structure and diversity associated with pines. Among them, *P. sylvestris* has been studied extensively and many fungal taxa were recorded (Luis & Cecilia, 2017; Menkis & Vasaitis, 2011) with 89.9% Ascomycetous fungi. The mentioned reports also showed low abundance of ectomycorrhizal fungi form the same host. Present study is also in line with their findings concerning the relatively higher percentage abundance of Ascomycetes associated with *P. wallichiana* (Fig. 6). Similarly, Obase *et al*., (2009) compared the ectomycorrhizal fungal communities of mature *P. thunbergii* Parl. of varying age trees of coastal forest in Samcheok, South Korea. Belowground community exclusively dominated by *Cenococcum geophilum* Fr. in contrast to our study. Moreover, Meagan *et al*., (2010) isolated 41 ectomycorrhizal fungi from root system of *P. sabiniana*.

According to available data, *Ophiocordyceps* sp. MHSIN-01 and *O. sinensis* have been first time reported from Pakistan associated with *P. wallichiana* roots. The later is endemic to Himalayan forests and has been previously reported from Bhutan, China, India and Nepal (Winkler, 2005; Lo *et al*., 2013; Xia *et al*., 2017). Importantly, all fungal species associated with *P. wallichiana* are not recorded before and are not in common to communities associated with other pines. But with exception of *Cenococcum geophilum*, *Suillus luteus* (L.) Roussel and *Tomentella* spp., which are evenly expressed in all fungal communities. Present investigation is in line with many other reports and suggests intra specific mycobiont specificity within different species of pines.

Molecular and phylogenetic identification of fungal taxa: Molecular tools are being used worldwide for rapid identification of environmental samples specially isolated from soil (Head *et al*., 1998) and have revolutionized the microbial diversity associated with roots (Horton & Bruns, 2001). As other methods may be laborious and time taking especially when samples obtained from pure cultures. In present study, % cut off value for species identification was considered. In phylogenetic inference, only 21 sequences were used out of 23 rDNA-ITS sequences obtained. Furthermore, the taxonomic placements of these phylotypes were confirmed based on phylogenetic relatedness using 'Neighbour Joining' as optimal criterion.

Cortinarius leucopus and its ectomycorrhizal phylotype was not described previously from Pakistan, in the present investigation, *Pinus wallichiana* is being reported as new host for this species and mycobiont as new record for Pakistan. There are about 2000 species of *Cortinarius* worldwide (Kirk *et al*., 2008) and Ahmad *et al*., (1997) reported only four species of *Cortinarius* from Pakistan, while three species of this genus have been reported as ectomycorrhizal from Kashmir Himalaya, India (Itoo *et al*., 2015).

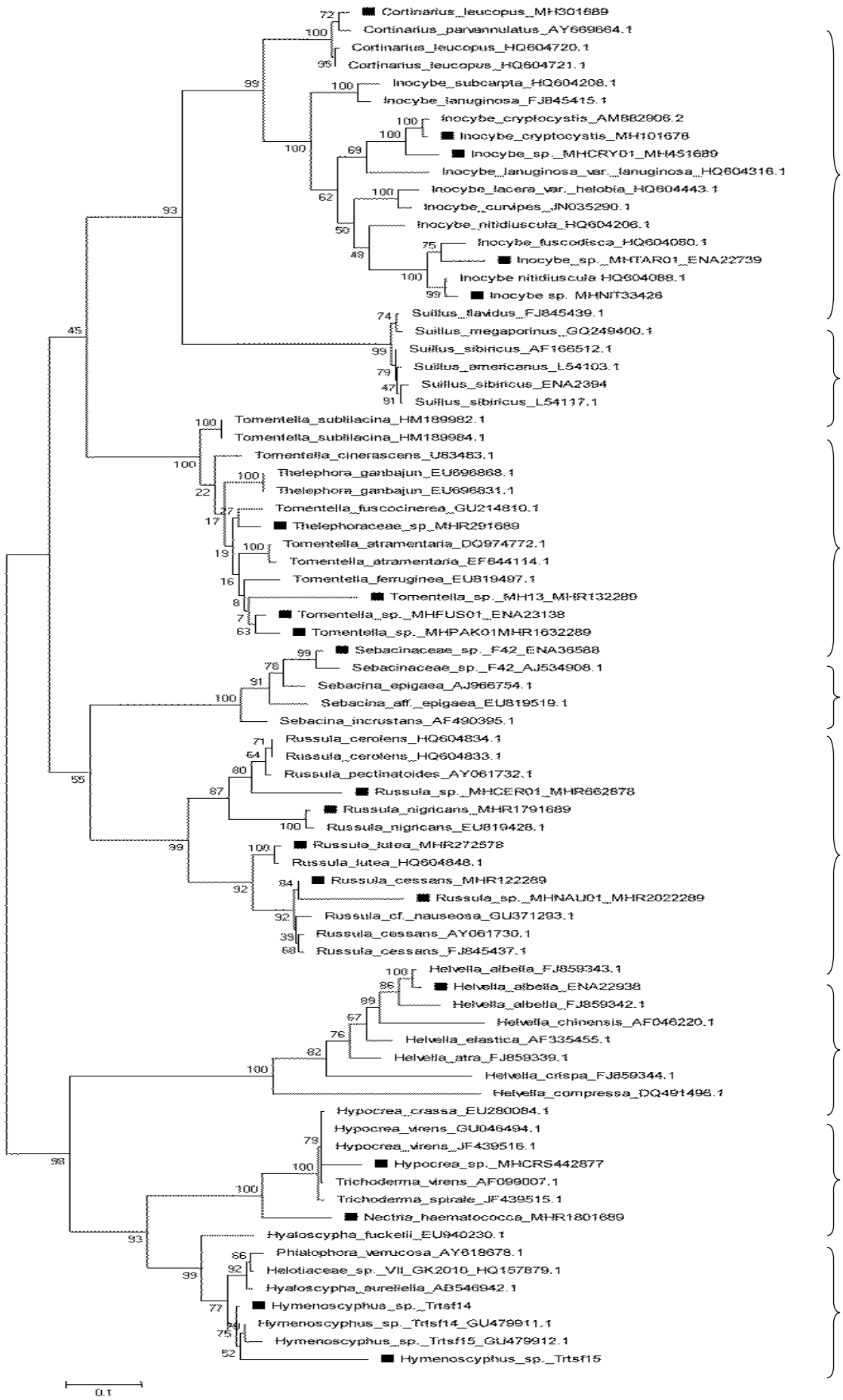

Fig. 5. Molecular Phylogenetic analyses by Maximum Likelihood method.

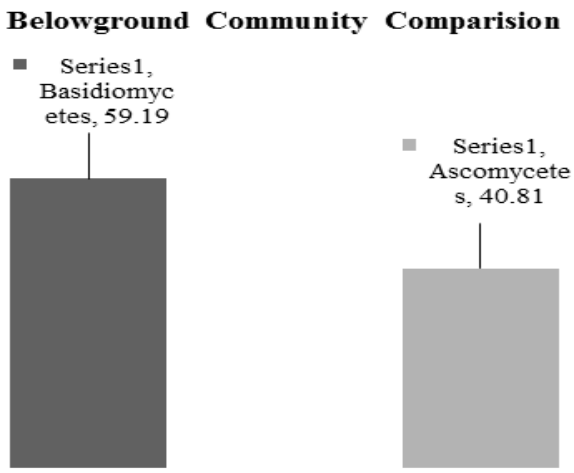

Fig. 6. Below ground fungal community comparison associated with *P. wallichiana* roots.

Inocybe cryptocystis has not previously been reported from Pakistan with any host. Seress *et al*., (2016) described 7 ectomycorrhizal morphotypes of genus *Inocybe* with different hosts; Ekaterina *et al*., (2016) reported 10 species of *Inocybe* with *Pinus koraiensis*. There are 850 species *Inocybe* worldwide (Kirk *et al*., 2008; Matheny *et al.,* 2020) and only thirty (30) species of *Inocybe* were reported from Pakistan (Ahmad *et al*., 1997; Naseer *et al.,* 2019). While its ectomycorrhizas have never been documented from Pakistan with *P. wallichiana*. Similarly *Suillus sibiricus* was found symbiotically associated with *Pinus cembra*, *P. siberical*, *P. wallichiana* and *Salix alba* Kern. (Sarwar *et al*., 2011) and with *P. koraiensis* along with other four species of genus *Suillus* (Ekaterina *et al*., 2016).

Thelephoraceae has a cosmopolitan distribution, however, it has been frequently reported with abundant species richness from coniferous forests of temperate Asia, Europe and North America with (Larsen, 1974; Kõljalg *et al*., 2000). Ekaterina *et al*., (2016) reported 24 ectomycorrhizal species of this family symbiotically associated with *Pinus koraiensis* and very little is known about this from Pakistan (Khalid & Hanif, 2017). Hanif *et al*., (2012) reported three MOTUs/ species of *Tomentella* from Pakistan forming ectomycorrhizal association with Himalayan Cedar.

Family Sebacinaceae is represented by 8 genera and 29 species, including 9 species of *Sebacina* (Kirk *et al*., 2008). This clade is poorly documented from Pakistan. Only one species, *Sebacina incrustans* (Pers.) Tul. & C. Tul. is previously reported (Ahmad, 1969) but lacking the report about its ectomycorrhizal status. This genus was found ectomycorrhizal with *Pinus cembra* (Rainer *et al*., 2015), *P. tabuliformis* Carr.) (Wei & Agerer, 2011) and *P. koraiensis* (Ekaterina *et al*., 2016). The present study added 2 more ectomycorrhizal MOTUs of *Sebacina* to mycoflora of Pakistan. In the present report, we documented *P. wallichiana* as a new host for ectomycorrhizal Sebacinoid fungi.

Three species from genus *Russula* were identified viz., *Russula cessans*, *R. lutea* and *R. nigricans*. *R. cessans* and *R. lutea* are being reported ectomycorrhizal with *P. wallichiana* for very first time from Pakistan. *R. nigricans* is also found in association with blue pine. This was previously reported from Sharan and Malakundi, Pakistan (Shibata, 1992) growing under pine forests. Ahmad *et al*., (1997) reported 23 species of *Russula* from Pakistan. rDNA based identified phylotypes of *Russula* were not previously documented from Pakistan with *P. wallichiana*. There were only a few reports of ectomycorrhizal morphotypes of *Russula* spp. which were identified morpho-anatomically (Niazi, 2008). With the addition of these four phylotypes, the number of *Russula* species from Pakistan has increased to 33.

Clade Pezizales is represented by only one ectomycorrhizal phylotype, *Helvella albella*. To our knowledge, there is not a single report about ectomycorrhizal status of *H. albella* with *P. wallichiana* except few records (Landeros *et al*., 2015). These were reported from the mixed conifers and hardwood vegetations. Hence *H. albella* is being reported first time forming association with root system of *P. wallichiana* and is an addition to the mycoflora of Pakistan. Recently, Hanif *et al*., (2012) described another member of Pezizales, *Peziza* sp. MHSUC-01 (JN836754) from Pakistan associated symbiotically with *Cedrus deodara* (Roxb.) G.Don (Pinaceae). In our present findings regarding Hypocreales, a few of root endophytes were also observed associated with pine roots. These were not among the dominant (8%) taxa (Fig. 3, 4). Similar co-relation was found by Chaverii and Vilchez (2006). There is no previous report of *Hymenoschyphus* with any host in Pakistan. In the current work, this fungus was found associated with *P. wallichiana*. There are many reports which focused on the doubtful trophic status of *Hymenoschyphus* (Rinaldi *et al*., 2008). Tedersoo *et al*., (2010) treated this species as pseudomycorrhizal to mycorrhizal. There are several scattered reports about the mycoflora associated with different pines but not a single comprehensive report on blue pine.

Conclusion

Present work is an insight about the ectomycorrhizal species richness and diversity associated with *P. wallichiana* in Himalayan Moist Temperate forests in Pakistan. This study also highlights the necessity for long term, frequent surveys and monitoring to explore the complete ECM fungal diversity in association with *P. wallichiana*. High species richness with low number of soil cores suggests more sampling efforts for the completion of belowground mycoflora. Despite the fact that sampling effort was not extensive, yet a high diversity of mycoflora was found to be associated with *P. wallichiana*. This is possibly due to warm and humid climate of Himalayan region. Our present sampling approach was to sample ectomycorrhizae just below sporocarp and so sampling was incidence based and random. A thorough and systematic sampling would be needed for the complete description of fungal community associated with this host. It is also important to evaluate that which type of ECM fungal species are most essential and useful for its host tree, more comprehensive study would be required that would increase our knowledge about the abundance and

community structure of ECM species associated the root tips for nutrient exchange and is of the greater benefit to the host. It seems from the present investigation that there could be most dominant fungal lineages (at family or order level) which could be very old and have global distributions. Some ectomycorrhizal fungal genera like *Russula*, *Tomentella* and *Inocybe* are dominant ectomycorrhizal representatives in moist temperate forests.

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