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SUILLUS HIMALAYENSIS (BOLETALES: BASIDIOMYCOTA: FUNGI) AND ITS SYMBIOTIC ASSOCIATION WITH ROOTS OF *PINUS WALLICHIANA*, FIRST REPORT FROM CONIFEROUS FORESTS OF PAKISTAN.

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ABSTRACT

Suillus himalayensis (*Boletales, Suillaceae*) was found associated with *Pinus wallichiana* during a survey of macrofungi from moist coniferous forests of Pakistan. Both, the fruiting body and ectomycorrhizae were characterized morphoanatomically as well as by molecular analysis. The identification of the fungal symbiont as *Suillus himalayensis* was confirmed by Internal Transcribed Spacer of ribosomal DNA (ITS rDNA) sequence comparison between mycorrhizas and sporocarps. Sporocarps were matched with published data available from India, where it was first time reported. Phylogenetic analyses and morphological descriptions are provided. This represents the first description of *S. himalayensis* as an ectomycorrhizal fungus; and, the first report of this species in Pakistan.

Key words: BLAST; moist temperate; phylogeny; rhizosphere.

INTRODUCTION

The forests of Pakistan is a main source of timber, paper, fuel wood, medicine, shelter as well as food and provide ecotourism and wildlife conservation purposes. Less than 4% of land in Pakistan is covered with forests (Introduction to landscapes of Pakistan, 2010). Himalayan moist temperate forests are a major part of this percentage. These forests are located at an elevation of 1373 to 3050 m a.s.l. and the main vegetation of these forests are conifers such as *Abies pindrow* Royle (1836), *Cedrus deodara* (Roxb. ex D. Don) G. Don (1830), *Picea smithiana* Boiss. (1884), *Pinus roxburghii* Sarg. (1897), *P. wallichiana* A.B. Jack. (1938), and *Taxus wallichiana* Zucc. (1843). Among these conifers, some deciduous trees and shrubs of different species also occur (Hussain, 1995). High rainfall occurs here during summer (July–September). High rainfall and moderate temperature make an environment suitable for the growth of mushrooms. Most of these fungi form mutualistic symbiotic associations with forest trees in the form of ectomycorrhizae that facilitate tree growth through enhanced nutrient absorption and protection of roots from root pathogens (Marx, 1991). These forests are considered one of the biodiversity rich hotspots. The Himalayan temperate forests are under severe pressure from logging for timber and firewood, clearing for agriculture, and the increasing population pressure; hence deforestation is occurring at an increasing rate. There is need to increase forested area in this region. In this regard, it is important to know the ectomycorrhizal diversity, which can guide more intelligent sylvicultural practices such the selection of robust mycorrhizal fungi for inoculating tree seedlings in the nursery. .

Among ectomycorrhizal mushrooms, *Suillus* Gray (1821: 646) is a very important genus forming symbiotic associations with many conifers and also with some deciduous trees (Wu *et al*., 2000). The development of mycorrhizae result in better growth, survival, enhanced mineral nutrition, and tolerance against pathogens and heavy metals (Krznaric *et al*., 2009). Due to their such importance in forest trees, scientists have been researching this genus for their role as ectomycorrhizae, their diversity and phylogeny (Kretzer and Bruns, 1997; Wu *et al*., 2000; Manian *et al.,* 2001; Beatriz *et al*., 2006; Feng *et al*., 2008; Sarwar *et al*., 2011, 2012a, b; Sarwar, 2013; Sarwar and Khalid, 2014; Sarwar *et al.,* 2015). About 53 different species of *Suillus* has been documented throughout the world so far (Kirk *et al.,* 2008; Bruns *et al.,* 2010; Verma and Reddy, 2014a, b, c) but from Pakistan only a little work has been published, mostly by the corresponding author (Ahmad *et al*., 1997; Razaq, 2007; Niazi, 2008; Sarwar *et al*., 2011, 2012; Sarwar, 2013; Sarwar and Khalid, 2014; Sarwar *et al*., 2015).

During the present study, DNA sequences of the internal transcribed spacer (ITS) region of the collected specimens and ectomycorrhizae were analyzed because the ITS region has been used to identify a large number of species in the genus *Suillus*. The aim of the present work is to describe, for the first time, the symbiotic association of conifers with *Suillus himalayensis* Verma and Reddy (2014), a new report from Pakistan, as well as to confirm its ectomycorrhizal association with local coniferous trees.

MATERIALS AND METHODS

The sampling was carried out in coniferous forests of Pakistan dominated by *Pinus wallichiana, Abies pindrow, Pinus roxburghii* and some deciduous trees (Fig. 1) during the rainy season (July–September, 2010-2012). Three taxa of *S. himalayensis* were collected after during visits to all selected sites. The sporocarps and blocks of soil directly beneath the fruiting bodies were taken from the rhizosphere of *Pinus wallichiana*. The sporocarps were air dried while the soil blocks containing ectomycorrhizae (ECM) were wrapped in polythene bags and brought to the lab for further analysis. Field data on basidiocarps (site, habitat, association, etc.) was noted. Specimens were studied macroscopically and microscopically in the laboratory following the methods of Bessette *et al*. (2000). For the spore dimensions, the first values present the range of lengths and widths, and the values in parentheses present mean spore lengths and widths ± standard deviations followed *Qm* ± standard deviation, where *Qm* is the mean of *Q* (= length/width ratio of an individual spore). Other measurements are given as a range with exceptional values in parentheses. ECM were carefully placed in water to clean off soil particles and characterized morphologically under the stereomicroscope. The microscopic description of the ECM follows the terminology of Agerer (1991, 1999). Mantle views, emanating elements, and illustrations were documented with the help of a camera lucida. Voucher specimens were deposited in the Herbarium, Department of Botany, University of the Punjab, Lahore, Pakistan (LAH).

Molecular Analysis: DNA was extracted from dried basidiocarps as well as from ectomycorrhizae using Extract N. Amp.TM Plant kit (SIGMA), and the nrDNA was amplified using fungal specific primers pair ITS1 (5′–TCCGTAGGTGAACCTTGCGG–3′) and ITS4 (5′–TCCTCCGCTTATTGATATGC–3′). Amplification parameters were denaturation at 94°C for 4 min., then 35 cycles of 45 s at 94°C, 45 s at 54°C, and 1 min 30 s at 72°C, and a final extension at 72°C for 2 min. The purified polymerase chain reaction (PCR) products were sequenced bidirectionally by Macrogen (South Korea). The sequence was basic local alignment search tool (BLAST) searched in GenBank for comparison with available sequences.

To calculate percent identity, similarity, and divergence, selected sequences were aligned using Clustal W and corrected manually. All ambiguous insertions and deletions were removed prior to further analyses. Percent Identities (PID) and DNA divergence were calculated by DNAStar. Maximum Likelihood criteria was used to describe the phylogenetic placement with other species included in the present study. Phylograms were made using Mega5.

RESULTS

Molecular Phylogeny: Blast match of ITS-rDNA sequences both from fruiting bodies and ectomycorrhizae showed maximum similarity with *Suillus himalayensis* (Verma and Reddy, 2014) reported from India. From GenBank, ITS sequences of closely related *Suillus* spp., and *Rhizopogon subcaerulescens* A.H. Sm. (1966) as an out-group were retrieved for phylogenetic analysis. For phylogenetic analysis, 670 genetic characters were used in aligned datasheet containing 443 conserved sites, 204 variable sites, and 132 parsimony sites. The phylogram based on maximum likelihood criterion is represented by five clades (Fig. 1). Sequences of ectomycorrhizae and fruiting body of *S. himalayensis* from Pakistan (KR056819, KR056820, KR056821, KR056822) clustered with same species from India (KF699850, KJ472765).

Taxonomy: ***Suillus himalayensis*** B. Verma & M. S. Reddy, Nova Hedwigia,

GenBank Accession no.: KR056819, KR056820, KR056821

Morpho-anatomical Description: Basidioma 3.5–10 cm in height. Pileus 3–9cm wide, convex to hemispherical to flattened; margins straight and flaring to slightly deflexed, sometimes irregular; pileal veil white, cottony, collapsing with age and finally evanescent; pileus surface moist to viscid, yellowish white to pale yellow to yellowish brown, scales brown colored appressed fibrillose throughout the surface, more prominent towards the margin. Context light yellow, changing to brown when bruising; odor and taste not distinctive. Stipe 3–9 cm long, 8–17 mm thick, central, rarely ecentric, cylindrical, equal in diameter, straight to curved to irregular, solid, covered with whitish glandular dots when immature which becomes brownish black with maturity, color yellow at top, pale yellow to reddish yellow in middle and white at base; annulus lacking, sometimes a whitish ring present above stipe centre, veil remnants attached to stipe in rare specimens; whitish mycelium at base; Stipe context yellow. Pore surface yellow when young, becomes slightly brown upon bruising; tubes 3-9 mm deep, decurrent to adnate and horizontal, frequent pores, pores angular, large, about 2–3 per mm; Basidiospores 7.5–11.3 × 4–5 µm, ellipsoid or oblong, thin walled, guttulate, smooth, hyaline or pale brown in Potassium hydroxide (KOH), inamyloid. Basidia clavate, 4 spored, brown in KOH, 31.6–35.1 × 6.8–10 µm. Cheliocystidia cylindrical or clavate, thin walled, brown in KOH, 33–92 × 6.5–9.4 µm. Pleurocystidia cylindrical or clavate, scattered, brown in KOH, 33–70 × 6–10 µm. Pileipellis a tangled layer of hyphae, gelatinized, thin walled, cylindrical, 3–7 µm.

Habitat and Host plant: Solitary to scattered on soil under *Pinus wallichiana* and *Abies pindrow* trees.

Distribution: Himalayan Moist Temperate Forests

Material examined: *Suillus himalayensis* Pakistan, Khyber Pakhtunkhwa (KPK), Mansehra, Chattar plain, 2250 m a.s.l., under *Pinus wallichiana* A.B. Jack., solitary, on ground, 15 Sept, 2012, Malka M.S.M.# 18 (LAH0612; GenBank: KR056821); Sharan (Kaghan valley), 2011 m a.s.l., under *Pinus wallichiana,* solitary, on ground, 19th July, 2011, Sarwar S.B.# 81 (LAH19611; GenBank: KR056820); Helipad, 2250 m a.s.l., under *Abies pindrow* Royle, scattered, on ground, 5th July, 2010, Sarwar S.B. #103 (LAH5710; GenBank: KR056819).

Description of ectomycorrhizae of *Suillus himalayensis*

Morphological characteristics: Mycorrhizal system dichotomously branched, system ≤ 8 mm long, with ≤ 0.8 mm thick main axis, unramified ends straight, ≤ 2 mm long, ≤ 0.5 mm diam., color of system reddish brown to dull yellow, apices light brown to honey brown, host tissue not visible under the sheath. Emanating elements present; Rhizomorphs prominent, attached at restricted points, whitish, Emanating hyphae whitish to light brown.

Anatomical characteristics of mantle: Mantle plectenchymatous in all layers**.** Outer mantle layer loosely plectenchymatous (type E, Agerer, 1987–2008); hyphae light honey brown with no matrix material, rarely septate and clamps absent. Inner mantle layer loosely plectenchymatous, (type E, Agerer, 1987–2008); light brown colour, no matrix material, septa rare, clamped septa absent.

Anatomical characteristics of emanating elements: Rhizomorphs differentiated (type B, Agerer, 1987–2008); septa present, cells 4 µm in diameter, 73 µm in length, cells thin walled, septa common, clamps and clamp septa absent. Emanating hyphae septate, hyaline, thin, hyphal width about 1.7–2.1µm, cell length up to 81 µm, clamp connections and anastomosis not observed among hyphae.

Material examined: Ectomycorrhizae of *Suillus himalayensis* Pakistan, Khyber Pakhtunkhwa (KPK), Sharan (Kaghan valley), 2011 m a.s.l., near rhizosphere of *Pinus wallichiana*, 19th July, 2012, Malka M.S.M.# 21 (GenBank: KR056822).

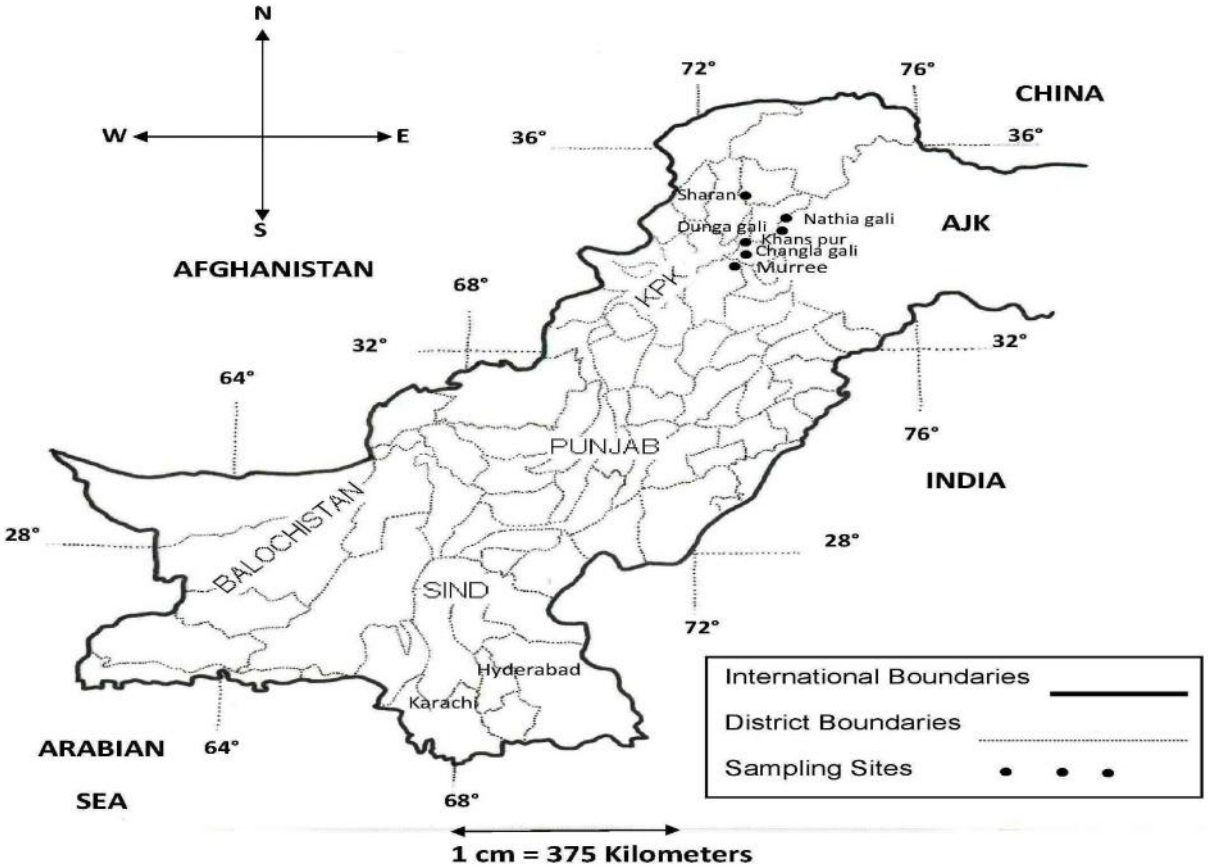


FIG. 1: Map of Pakistan showing sampling sites

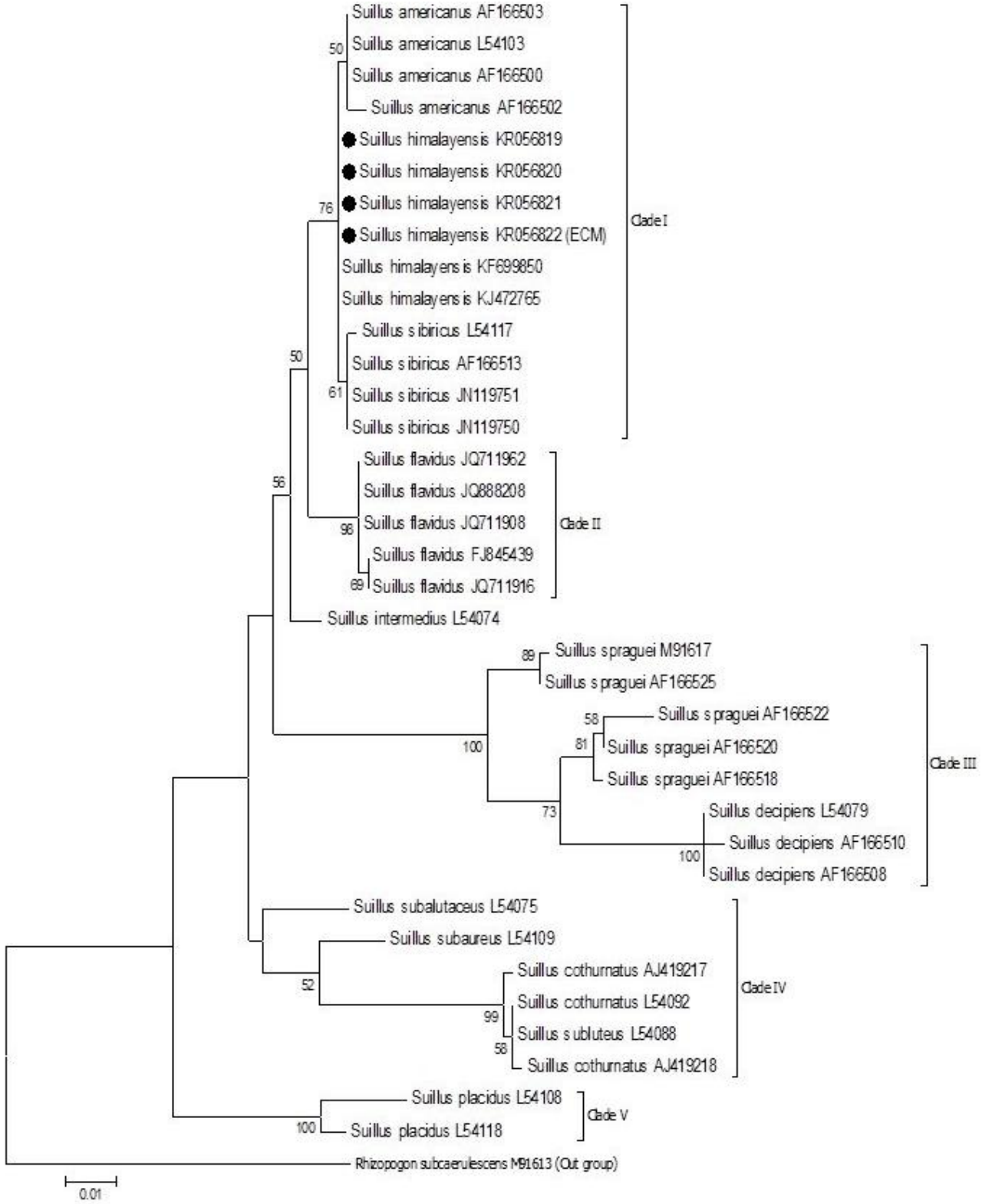


Plate 1. Phylogenetic position of *Suillus himalayensis* and its ectomycorrhizae from Pakistan with respect to other *Suillus* spp. Tree inferred by maximum likelihood analysis based on rDNA sequences, including *ITS* region. *Rhizopogon subcaerulescens* was used as outgroup. The numbers against branches indicate the percentage (>50%) at which a given branch was supported in 1000 bootstrap replications. GenBank accession numbers are given at the end of species names. ● indicate species reported from Pakistan.



Plate 2. Figs. 1-6. *Suillus himalayensis*. Fresh basidiomata showing different Morphological features (1-6). Scale bars= 2 cm in 1-6.

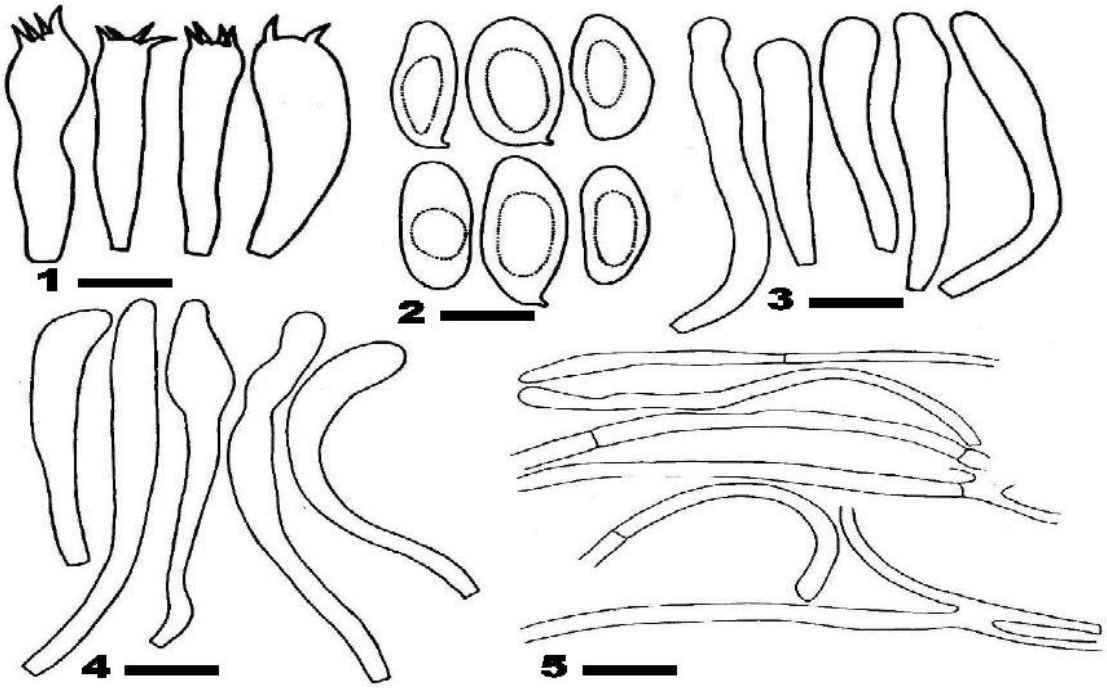


Plate 3. Figs. 1-5. Anatomical features of *Suillus himalayensis:* 1. Basidia (bar = 6 µm). 2. Basidiospores (bar = 6 µm). 3. Pleurocystidia (bar = 7 µm). 4. Cheilocystidia (bar = 9 µm). 5. Pilepellis (bar = 11 µm).

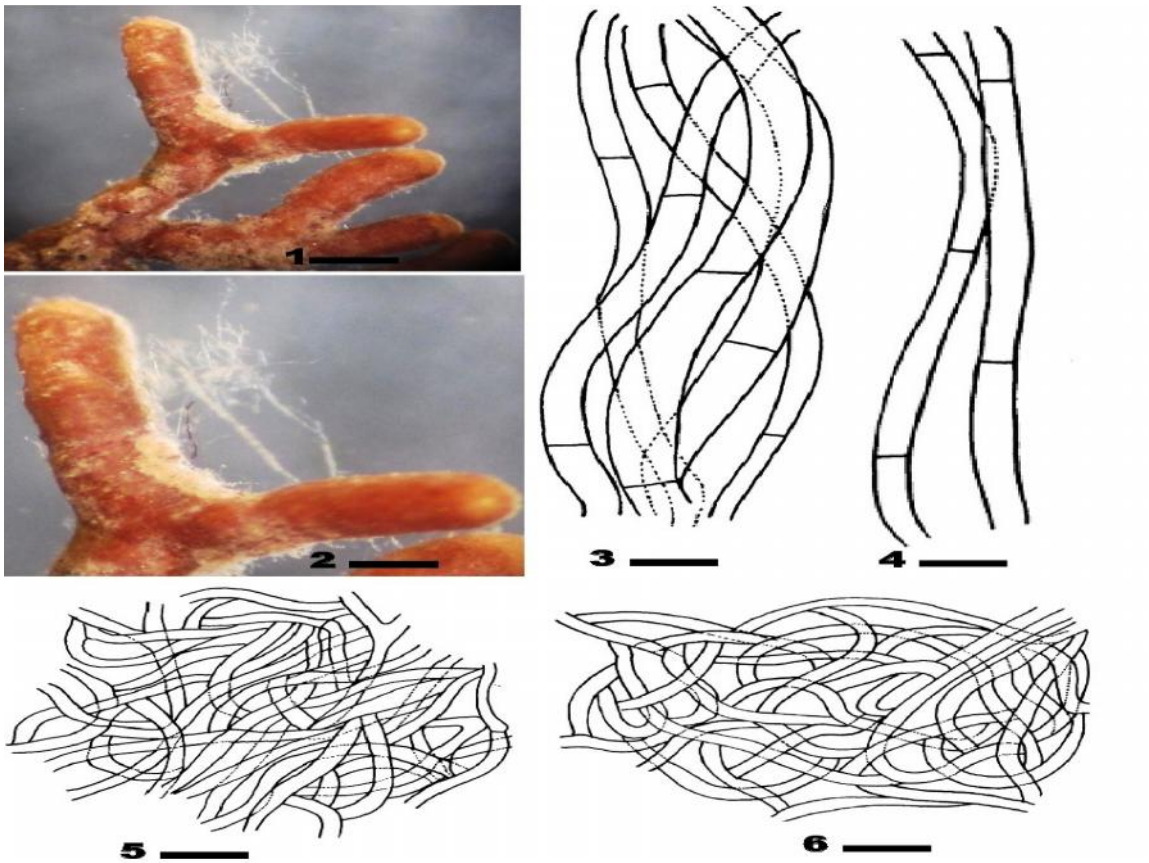


Plate 4. Figs. 1-6. ECM of *Suillus himalayensis* with *Pinus wallichiana* and its anatomical features. 1-2. Morphological features (Habit) (bar = 0.2 cm). 3. Rhizomorphs (bar = 12 µm). 4. Emanating hyphae (bar = 11.5 µm). 5. Outer mantle (bar = 5.3 µm). 6. Inner mantle (bar = 5.5 µm).

DISCUSSION

During the present investigation ectomycorrhizae of *S. himalayensis* associated with *P. wallichiana* were reported for the first time and described both by morphological as well as molecular methods. These ectomycorrhizae are characterized morphologically by dichotomously branched ramifications with plectenchymatous inner and outer mantle surfaces, differentiated rhizomorphs, and emanating hyphae. This is an addition to the literature of molecular descriptions of ectomycorrhizae of boletes from Pakistan.

Morphological characteristics of collected fruiting bodies were compared with descriptions of *S. himalayensis* and closely related speceis i.e. *S. americanus* (Peck) Snell (1959) and *S. sibiricus* (Singer) Singer (1945) and literature was analyzed (Nguyen *et al*., 2016) but species reported during this study form a separate clade phylogenetically from *S. americanus.* Morphologically, the species closely resembled *S. himalayensis* due to its yellow white to pale yellow pileus with light greenish tinge, brownish squamules and white pileal veil (Verma and Reddy, 2014c). Phylogenetic results showed that *S. himalayensis* forms a clade with sequences of the same species in GenBank. We report *S. himalayensis* as a new record for Pakistan and its ectomycorrhizae for the first time in the world. We hope that this research work will contribute to sylvicultural practices such the selection of robust mycorrhizal fungi for inoculating tree seedlings in the nursery for the express interest of reestablishing forest areas in the Himalayan range.

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