

DIVERSITY OF BOLETES IN PAKISTAN – FOCUS ON *SUILLUS BREVIPES* AND *SUILLUS SIBIRICUS*

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ABSTRACT

During the exploration of diversity of non-gilled fungi and their ectomycorrhizal morphotypes from Pakistan, *Suillus brevipes* was found ectomycorrhizal with *Quercus incana* while *Suillus sibiricus* was found associated with roots of *Pinus wallichiana* and *Salix alba*. Basidiomes and ectomycorrhizae of the latter are characterized morpho-anatomically and by using rDNA-ITS and 5.8S gene while for the former, both the basidiome and the ectomycorrhizae have been identified and characterized morpho-anatomically. Previously, *S. sibiricus* was found associated only with *P. wallichiana*. In the present work *S. alba* has been found as new host for *S. sibiricus*.

Molecular analysis of *S. sibiricus* was used to describe the phylogenetic position of the fungus using Maximum Parsimony analysis. *S. brevipes* and morphotypes of both boletes are new records for Pakistan.

Keywords: Ectomycorrhizae; Non-gilled fungi; Pakistan; PCR-ITS; Sequence alignment

INTRODUCTION

Boletes (Basidiomycota; Agaricomycetes) are characterized by the presence of tubes instead of gills [1, 2]. There are 35 genera and more than 1,000 species of Boletes known worldwide [3] with only ten (10) genera and forty four (44) species reported from Pakistan [4-6]. They are distributed mostly in Europe, Asia, North America, in the temperate latitude in the northern hemisphere [7, 8] while mostly confined to moist temperate coniferous forests in Pakistan [4, 9]. More than 90% of members of this group form symbiotic associations with the root system of forest trees, which is the most important tool for germination and good development throughout the life of tree [10]. The plant families *Betulaceae*, *Caesalpiniaceae*, *Casuarinaceae*, *Dipterocarpaceae*, *Ericaceae*, *Fagaceae*, *Mimosaceae*, *Myrtaceae*, *Pinaceae* and *Salicaceae* form ectomycorrhizal symbioses with Boletes [11, 12]. This group is economically important because it contains edible, medicinally-important and poisonous mushrooms [13,14, 15].

Suillus P. Micheli ex Adans. is a genus of Boletes belonging to *Suillaceae* represented by 54 species worldwide [3] with only four species known from Pakistan [4, 5, 6]. Wu et al. [16] discussed the bio-geographic pattern and phylogenetic relationship of *Suillus* species from Eastern Asian (China and Nepal) and North American territories. During an investigation of the

diversity of boletes in Pakistan, *S. brevipes* and *S. sibiricus* were isolated and in the present work they are described morpho-anatomically along with their ectomycorrhizal morphotypes from Pakistan. *Suillus sibiricus* and its ectomycorrhizae have been described up to molecular level and identified by using nrRNA tandem repeats. Molecular phylogeny of *S. sibiricus* has also been discussed with Eastern Asian and Eastern North American isolates retrieved from Genbank.

MATERIALS AND METHODS

The study area is located in Himalayan moist temperate forests of Pakistan that are evergreen forests of conifers mixed with some oaks and other deciduous broad-leaved trees. Their undergrowth is rarely dense, and consists of both evergreen and deciduous species. These forests occur between 1,500 m and 3,000 m elevation with mean annual rainfall about 1,700 mm. Average aerial humidity is 57% and the mean annual temperature is 12°C [17]. Many Boletes form symbiotic association with the roots of these plants for their better growth [4].

Sampling was done during the rainy season from July to August 2008 - 2010. Sporocarps and soil samples containing their ectomycorrhizal morphotypes were collected. Small parts of both the fruiting body and ECM were stored in 2% CTAB buffer for DNA analysis. Soil blocks were wrapped in polythene bags to avoid evaporation and crumbling. Fresh characters of sporocarps were recorded in the field and photographed. Sporocarps were dried properly and the soil samples were washed to separate the ECM. Both sporocarps and ECM were characterized morpho-anatomically. The identification was done with the help of available literature [18, 19, 20]. The nomenclature was made according to the Index Fungorum. Special designation numbers were given to each of the samples. The specimens were deposited in Punjab University herbarium, Botany department (LAH).

Molecular Analysis. DNA was extracted by modified CTAB method following Gardes & Bruns [21]. The hymenial tissue was removed with sterile forceps and rinsed with sterile H₂O. Fresh and healthy ectomycorrhizal morphotypes from *Pinus wallichiana* A.B. Jack. and *Salix alba* L. were selected manually under a stereomicroscope (Olympus, SZ30, Japan). The extraction was modified for silica emulsion binding and purification (Gene-Clean; Q-Biogene, Irvine, CA, USA).

The ITS regions of nuclear rDNA were amplified by Polymerase Chain Reaction (PCR) using universal primers i.e. ITS1 and ITS4 primers [21, 22]. Purified PCR products were sequenced using a DNA sequencer (3730ABi). DNA sequence was submitted to BLAST and used to query the nucleotide collection using default settings. rDNA-ITS sequences of *S. sibiricus* were submitted to GenBank.

Phylogenetic position of *S. sibiricus* was deduced by Maximum Parsimony with bootstrapping using PAUP* Version 4.0b10. For that purpose, a tree was made by using seven (7) sequences obtained from *S. sibiricus* from Pakistan and 23 closely related rDNA ITS sequences of *Suillus* spp. were retrieved from the DNA database using the BLAST program from GenBank (Table 1). All sequences were aligned and corrected manually using MacClade 4.08 and Bioedit (version 7.0.9).

RESULTS

Suillus brevipes (Peck) Kuntze, *Revis. gen. pl.* (Leipzig) 3(2): 535 (1898) (Fig. 1. A-F). Pileus convex to hemispheric, 2.5-6 cm, chocolate brown, shiny, smooth, glabrous, sticky, flesh thick and off-white, margins slightly incurved, entire, smooth, of same color like pileus surface. Hymenium adnate and ascending, frequent pores, creamish to light yellowish, no color change upon bruising, pores rounded to irregular in shape. Stipe centric, clavate, 3.5-6.8 cm long, 1.3-2.2 cm wide, smooth, whitish with brown small patches at some points, semi-hollow, ring or volva absent. Basidia clavate, thin walled, contents brown in Meltzer's, 18-26 x 6-8 μ m, 2-4 sterigmate. Basidioles clavate, thin walled, 15-23 x 5.5-8 μ m. **Cystidia** cylindrical to clavate to subfusoid to ampullaceous, thick walled, dark brown contents, 35-49 x 5-9 μ m. Basidiospores ellipsoid to fusiform to subfusiform, smooth, thick walled, 6-10 x 3-6.5 μ m, honey brown to light yellowish.



Figure 1. *Suillus brevipes*, A-F. Views of the Sporocarp (A & B), Basidia (C), Basidioles (D), Cystidia (E), Basidiospores (F). **Scale Bars** for A & B = 1 cm, C = 6 μ m, D = 6 μ m, E = 8 μ m, F = 3 μ m.

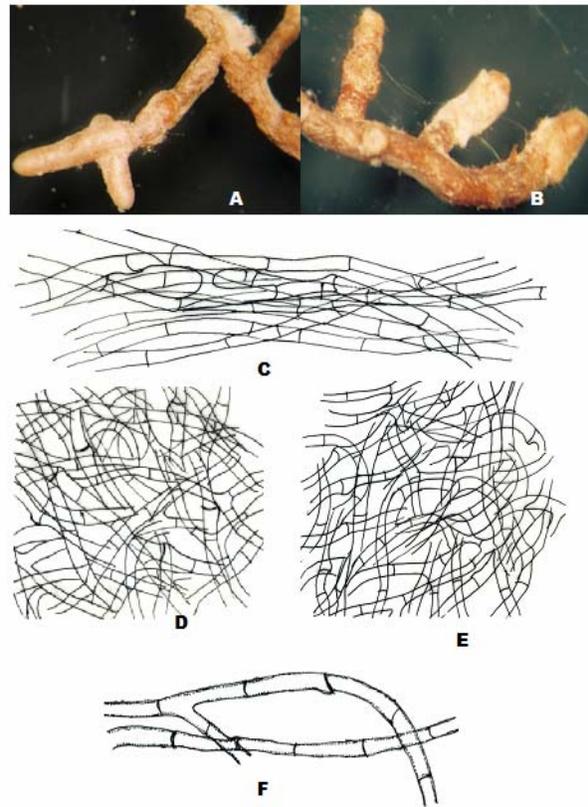


Figure 2. A-F. Ectomycorrhizae of *Suillus brevipes*. Views of ECM under stereomicroscope (A & B). Anatomical features of ECM under light microscope (C-F). Rhizomorph (C), Outer mantle (D), Inner mantle (E), Emanating hyphae (F). **Scale Bars** for A & B = 0.6 mm, C = 4.5 μ m, D = 7 μ m, E = 7.5 μ m, F = 5 μ m.

Habitat and Distribution of *Suillus brevipes*. On ground, near and solitary under *Quercus incana*, at 2250 m. a. s. l., Pakistan, KPK, Khanspur, 19th June, 2008. S. S. B. # 12.

Description of ectomycorrhizae of *Suillus brevipes*

Morphological characteristics: (Fig. 2. A & B). Mycorrhizal system: monopodial pinnate, system 3.5-4 mm long, main axis 0.5 mm wide, texture smooth, tips beaded to straight, tip length about 0.5 mm and <0.5 mm wide, young tips honey brown, older tips black and apices light honey brown, host tissue visible under mantle surface. Rare Rhizomorphs, at restricted points, whitish brown. Common emanating hyphae, straight, whitish.

Anatomical characteristics of mantle in plan views (Fig. 2. D & E). Plectenchymatous outer mantle layer (type E, Agerer) [23], matrix material granular, cells 3 µm wide and 11 µm long, honey brown, cell contents: granular, common septa and clamped septa, hyphal junction angle about 60°, rare anastomose. Plectenchymatous inner mantle layer (type E, Agerer) [23], granular cell contents, cells 5 µm wide and 12 µm long, yellowish brown, common septa and clamped septa, common hyphal junction, hyphal junction angle about 45°, rare anastomose and H-shaped. Anatomical characteristics of emanating elements (Fig. 2. C & F). Rhizomorphs highly differentiated (type B, Agerer) [23], cells 4 µm wide and 9 µm long, light brown in color, cell contents oil like bodies, common septa and clamped septa, common hyphal junction and Y-shaped, anastomose H-shaped. Rare Emanating hyphae, cells 5 µm wide and 8 µm long, reddish brown, septa and clamped septa present.

***Suillus sibiricus* (Singer) Singer, *Farlowia* 2: 260 (1945) (Fig. 3. A-F)**

MYCOPAK: EA15194, EA23904, EA24040, EA24104, EA17193, SS142010, SS022010.

GENBANK: JN119748-54

Pileus 4-8 cm, pulvinate to obtuse, sticky, slimy, shiny, glabrous, yellowish brown, smooth, margins smooth, entire, slightly darker color than pileus surface, deflexed. Hymenium: adnate and ascending, pores frequent, yellowish, angular pores. Stipe: 10 cm long, 1.8 cm wide, central, equal, ring present, yellowish to off-white from apex to ring, reddish brown from ring towards base, whitish near base, reticulated, reticulations white, rough, solid, curved. Basidia: long clavate, 2-4 sterigmate, thick walled, brownish contents visible in Meltzer's, 25-39 x 7-9 µm. Basidioles: clavate, thick walled, contents visible, (20-) 26-31 x 7.5-8 µm. Cystidia: cylindrical to subfusiform, thick walled, yellowish brown in Meltzer's, yellowish brown contents, 33-45 (-62) x 7-9 µm. Pileocystidia: a tangled layer of repent hyphae, hyaline to light honey in Meltzer's, thin walled, granular contents, septate, 60-74 x 8-10 µm. Caulocystidia: long, subclavate- clavate-cylindrical, some with pointed ends, septate, thick walled, hyaline in Meltzer's, 68-99 x 13-20 µm. Basidiospores: ellipsoid-fusiform, thin walled, amyloid, smooth, 10-12 x 3.5-5 µm.

Description of Ectomycorrhizae of *Suillus sibiricus* with *Pinus wallichiana*

Morphological Characters (Fig. 4. A & B). Ectomycorrhizal system frequently found under the fruiting bodies, dichotomously branched, 3-5 mm long, 0.5 mm in diam., length of tips 1.5 mm, dark honey brown when older. *Very tips* honey brown to reddish brown, tips straight. Mantle surface long-spiny to cottony; host tissue not visible under the sheath. Rhizomorphs:

common, connecting distinctly to the mantle surface, off-white to light brown, differentiated. Emanating hyphae: light brown, common, giving cottony appearance, straight.

Anatomical characteristics of mantle in plan views. (Fig. 4. C & D). Mantle: pseudoparenchymatous in all layers. Outer mantle layer: pseudoparenchymatous (type L, Agerer) [23], cells rounded to angular, cells 7-9 μm long, 7.3-10 μm in diam., no matrix material observed, cell contents clear, cell color hyaline to light yellowish. Inner mantle layer: pseudoparenchymatous (type H; Agerer) [23], without any ring like structures; cells colorless to yellowish, cells 6.5-7.4 μm in diam., cell walls 5.4-7.2 μm thick; cell contents not observed.

Anatomical characteristics of emanating elements (Fig. 4. E & F)

Rhizomorphs: highly differentiated, cells about 5.4 μm in diameter and about 87 μm length, light brown color, septa rare, clamps absent, cell contents clear. Emanating hyphae: common, 5 μm in diameter, 67 μm long hyphal cells, only clamped septa present.

Habitat and Distribution of *Suillus sibiricus*. On ground, in groups, under *Pinus wallichiana*, at 2347 m. a. s. l., Pakistan, KPK, Khera gali, 7th August, 2010. S. S. B. # 53.

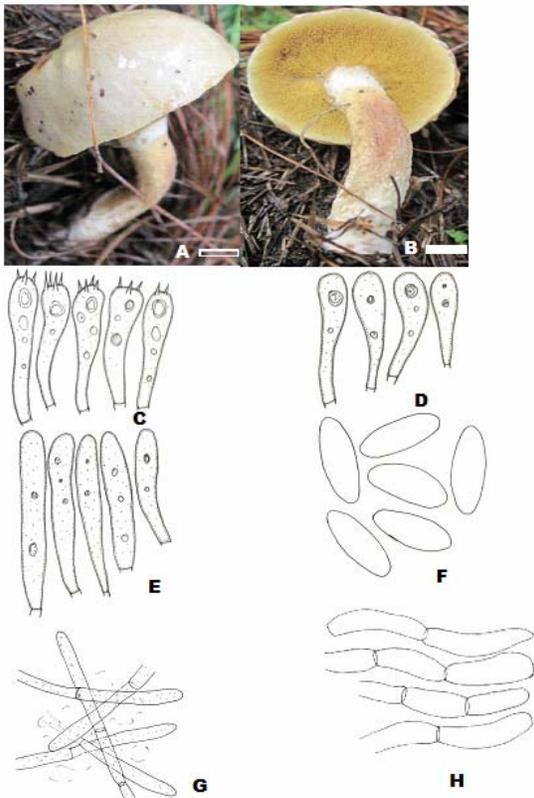


Figure 3. *Suillus sibiricus*, A-F. Views of the Sporocarp (A & B), Basidia (C), Basidioles (D), Cystidia (E), Basidiospores (F), Pileocystidia (G), Caulocystidia (H). Scale Bars for A & B = 1.4 cm, C = 7 μm , D = 7 μm , E = 8.5 μm , F = 4.5 μm , G = 12 μm , H = 24 μm .

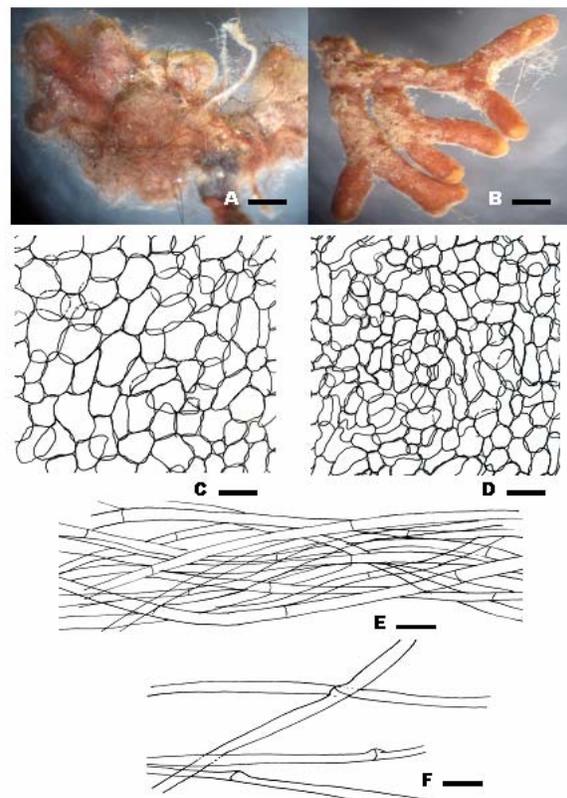


Figure 4. A-F. Ectomycorrhizae of *Suillus sibiricus* with *Pinus wallichiana*. Views of ECM under stereomicroscope (A & B). Anatomical features of ECM under light microscope (C-F). Outer mantle (C), Inner mantle (D), Rhizomorph (E), Emanating hyphae (F). Scale Bars for A & B = 1 mm, C = 7.5 μm , D = 10 μm , E = 16 μm , F = 9.5 μm .

Description of ectomycorrhizae of *Suillus sibiricus* with *Salix alba*.

Morphological characteristics (Fig. 5. A). Mycorrhizal system found under the fruiting bodies: monopodial pinnate, system up to 5.5 mm long, with 0.6 mm thick main axis, unramified tips 2.2 mm long and about 0.5 mm thick, color of system dark brown, younger tips honey brown, rounded and slightly curved, surface of mycorrhizal system smooth, host tissue visible under mantle surface. Rhizomorphs: rare, attached at restricted points, dark brown to black.

Anatomical characteristics of mantle in plan views (Fig. 5. C & D)

Mantle parenchymatous in all layers. Outer mantle layer: parenchymatous, (type L, Agerer) [23]; cells irregular in shape, 10 μm in diameter and 12.5 μm in length, light brown color of cells, no cell contents, no septa and clamps. Inner mantle layer: parenchymatous, (type M, Agerer) [23]; cells angular, smaller than outer mantle, 9 μm in diameter and 11 μm in length, honey brown color of cells, no matrix material, no septa and clamps.

Anatomical characteristics of emanating elements (Fig. 5. B). Rhizomorphs: differentiated (type B, Agerer) [23]; septa present and H-type anastomose type, cells 5 μm in diameter, 26 μm in length, cells thin walled, septa common, clamps and clamp septa absent.

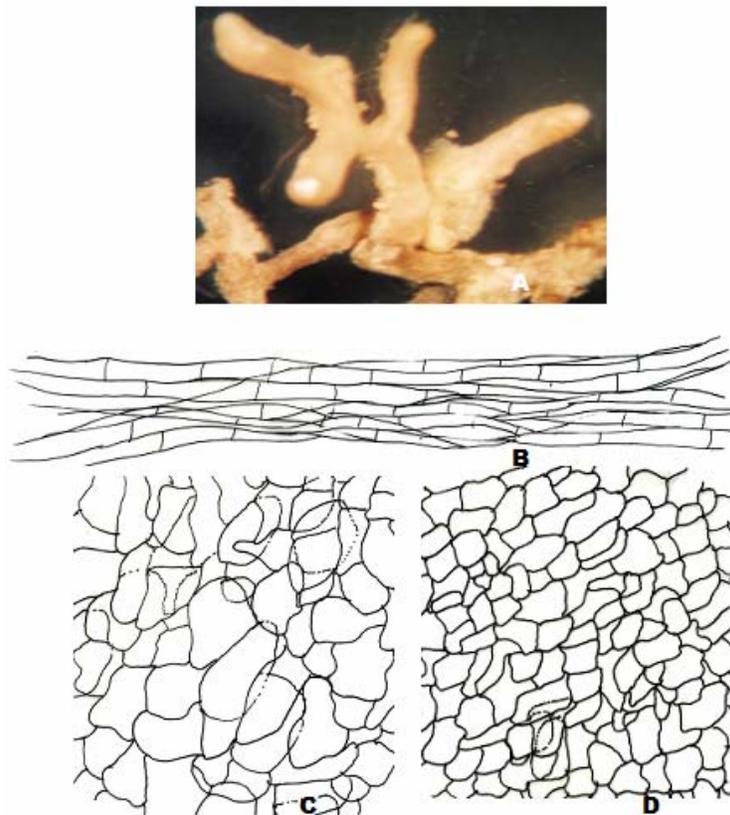


Figure 5. A-F. Ectomycorrhizae of *Suillus sibiricus* with *Salix alba*.

View of ECM under stereomicroscope (A). Anatomical features of ECM under light microscope (B-D). Rhizomorph (B), Outer mantle (C), Inner mantle (D). **Scale Bars** for A = 1 mm. B = 17 μm . C = 8.5 μm . D = 16 μm .

Molecular identification and characterization of *S. sibiricus* and its ectomycorrhizae. PCR-ITS products of rDNA obtained from basidiomes and their ectomycorrhizae with *Pinus wallichiana* and *Salix alba* were sequenced. The sequences showed 99% similarity with isolates of *S. sibiricus* from China and America, confirming the morphological identification.

The phylogenetic analysis for genus *Suillus* was carried out using parsimony as optimality criterion. The sequences included in this analysis had around 659 characters, from which 494 characters were used for further analysis after alignment and trimming from both 3' and 5' sites of rDNA-ITS. After that, none of characters were excluded from final analysis.

All characters were of type 'unord'. There were 53 parsimony-informative sites, 415 constant sites, 26 variable characters were parsimony-uninformative. All the gaps were treated as "missing" data. Multistate taxa were interpreted as uncertainty. The starting tree(s) was obtained via stepwise addition with random addition of sequence and 1000 number of replicates. There were 49145891 starting seeds for the tree generated. Only 01 tree held at each step during stepwise addition of the sequences. Tree-bisection-reconnection (TBR) was used as branch-swapping algorithm. A total of 6,457,051 rearrangements were tried for the best tree. Only 27 trees were retained for analysis. The genetic distance matrix was derived from Maximum Parsimony (MP) analysis generated a consensus tree from the best 144 trees showing the following scores: Tree length (TL) = 146, consistency index (CI) = 0.6438, homoplasy index (HI) = 0.3562, CI excluding uninformative characters = 0.5517, HI excluding uninformative characters = 0.4483, retention index (RI) = 0.7977, rescaled consistency index (RC) = 0.5136. Phylogenetic analysis showed the various species of *Suillus*. Maximum Parsimony consensus tree indicated three major clades and one independent clade. Maximum Parsimony consensus tree was constructed exclusively for *Suillus* species from geographically different localities, mostly from Eastern Asia (China and Nepal), Eastern North America and from Pakistan to resolve exact identification. The cladogram represents a major polytomous clade formed by *S. americanus* and *S. sibiricus* species (Table 01). All of the species of this clade shared 98-99% of characters studied so far for this analysis and thus identified as *S. sibiricus*. Both *S. sibiricus* and *S. americanus* occupied topologically different positions in the same polytomous clade (Fig.6).

The Maximum Parsimony analysis resulted in major polytomous clade comprising sixteen isolates of *S. americanus* and *S. sibiricus*. All these species are monophyletic along with *S. flavidus* (Accession No. FJ845439.1), *S. megaporinus* (Accession No. GQ249400.1) and *S. umbonatus* (Accession No. L541115.1).

DISCUSSION

Boletes are an important component of Himalayan Moist Temperate Forests of Pakistan and make ectomycorrhizal association with gymnosperm and angiosperm trees of this area. Out of ten (10) genera of 44 species of Boletes, *Suillus* is represented by 4 species viz. *S. granulatus* (L.) Roussel, *S. placidus* (Bonord.) Singer, *S. sibiricus* (Singer) Singer and *S. tomentosus* (Kauffman) Singer [4, 5, 6].

Suillus is very important genus of Boletes. Its phylogenetic and bio-geographic relationship comprehensively described by Wu et al. [16]. *Suillus sibiricus* was originally described by Rolf Singer in 1938 [24]. While *S. brevipes* was first described by Charles Frost in 1874 [25].

In the present studies *S. brevipes* and *S. sibiricus* have been found forming ectotrophic mycorrhizae with different host trees of Himalayan Moist Temperate Forests of Pakistan. The

basidiomata and ECM morphotypes of both mushrooms have been described and illustrated using morpho-anatomical methods. The *S. sibiricus* and its ECM mycorrhizae have been identified using rDNA-ITS and 5.8S gene. *S. sibiricus* was described as mycorrhizal from two different hosts viz; *Pinus wallichiana* and *Salix alba* while *S. brevipes* was found associated with *Quercus incana*.

Table 1. List of *Suillus* species sequences, length in base pairs, their Geographic Origin, Collection and Accession Numbers.

Scientific names	Genbank Accession No.	Origin country	Length (bp)
<i>Suillus americanus</i>	AF166503.2	USA	623
<i>Suillus americanus</i>	L54103.1	USA	644
<i>Suillus americanus</i>	AF166500.2	USA	619
<i>Suillus americanus</i>	AF166501.2	USA	648
<i>Suillus bresadolae</i>	GU187544.1	USA	711
<i>Suillus brevipes</i>	FJ845440.1	CANADA	947
<i>Suillus caerulescens</i>	EU486453.1	CANADA	1260
<i>Suillus cavipes</i>	AF166505.2	CHINA	721
<i>Suillus flavidus</i>	FJ845439.1	CANADA	943
<i>Suillus granulatus</i>	AY898617.1	SPAIN	799
<i>Suillus intermedius</i>	L54074.1	USA	643
<i>Suillus lakei</i>	DQ367917.1	CANADA	899
<i>Suillus luteus</i>	DQ440568.1	SPAIN	802
<i>Suillus megaporinus</i>	GQ249400.1	JAPAN	698
<i>Suillus paluster</i>	AB284451.1	JAPAN	792
<i>Suillus quiescens</i>	GQ249402.1	USA	792
<i>Suillus sibiricus</i>	AF166512.1	USA	709
<i>Suillus sibiricus</i>	AF166515.2	CHINA	677
<i>Suillus sibiricus</i>	AF166514.2	CHINA	617
<i>Suillus sibiricus</i>	L54117.1	NEPAL	644
<i>Suillus sibiricus</i>	AF166516.1	USA	694
	MYCOPAK EA15194	PAK	644
<i>Suillus sibiricus</i>	(<i>Salix alba</i> Root) JN119748.1		
	MYCOPAK EA17193	PAK	635
<i>Suillus sibiricus</i>	(<i>Salix alba</i> Root) JN119752.1		
	MYCOPAK EA23904	PAK	370
<i>Suillus sibiricus</i>	(<i>Pinus wallichiana</i> Root) JN119749.1		
	MYCOPAK EA24040	PAK	580
<i>Suillus sibiricus</i>	(<i>Pinus wallichiana</i> Root) JN119750.1		
	MYCOPAK EA24104	PAK	588
<i>Suillus sibiricus</i>	(<i>Pinus wallichiana</i> Root) JN119751.1		
	MYCOPAK S2 (<i>Pinus wallichiana</i>)	PAK	507
<i>Suillus sibiricus</i>	JN119754.1		
	MYCOPAK S14 (<i>Pinus wallichiana</i>)	PAK	642
<i>Suillus sibiricus</i>	JN119753.1		
<i>Suillus subalutaceus</i>	L54075.1	USA	644
<i>Suillus umbonatus</i>	L54115.1	USA	644

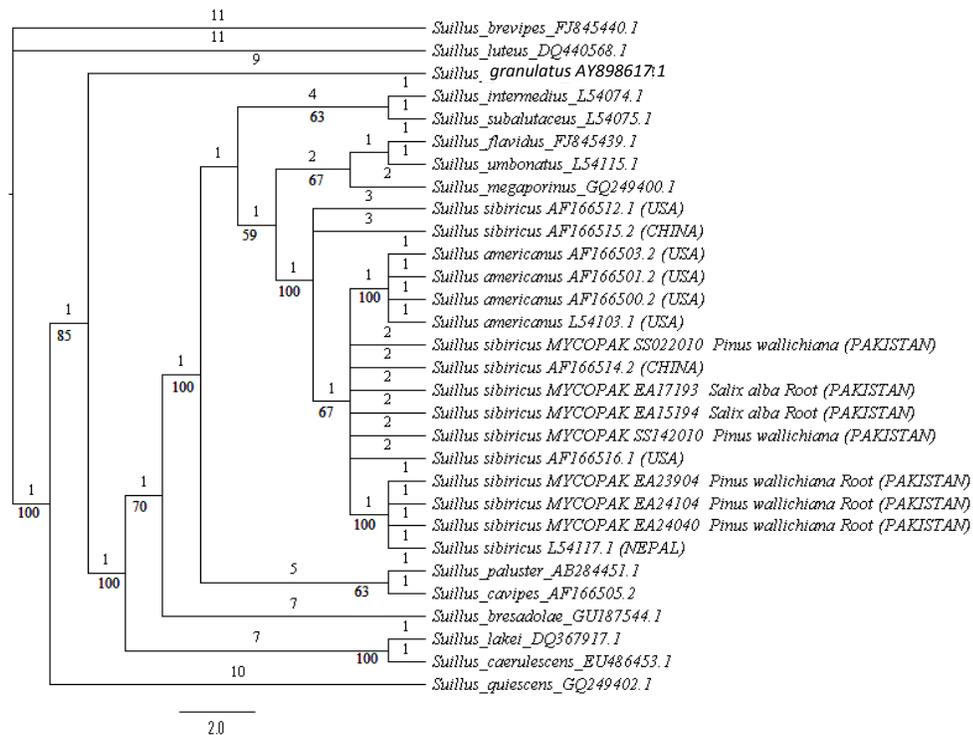


Figure 6. Bootstrap 50% majority-rule consensus tree. Cladogram based on parsimony analysis of rDNA-ITS region of different species of *Suillus*. MP tree generated by parsimony analysis of rDNA-ITS with 5.8s gene. The numbers above brackets refer to number of changes, those below to Bootstrap values. The accession numbers of analyzed sequences are shown after each taxon name.

S. brevipipes has similarity with *S. granulatus*, *S. albidipes* and *S. pallidiceps*. But the major differences from these species are *S. granulatus* has shorter stem, and distinct raised granules on the stem while *S. brevipipes* has smooth white stipe. Similarly, *S. brevipipes* is differentiated from *S. albidipes* by not having a cottony roll of velar tissue at the margin when young. A major difference of *S. brevipipes* from *S. pallidiceps* is a pale yellow cap color of the latter while the former has a chocolate brown cap [26]. Previously, it was found associated with *Pinus contorta* and *P. ponderosa* [27] and there is no report regarding their distribution in South Asia. The mycorrhizae of *S. brevipipes* were described with *P. contorta* which form tuberculate system and tortuous tips. While in present study, with *Q. incana* it forms monopodial pinnate and possess plectenchymatous type of mantle which is in contrast with sychymatous type of mantle in *P. contorta*.

S. sibiricus often confused with North American species, *S. americanus*. The latter has larger sporocarps as compared with *S. sibiricus*. Despite this minor difference, other macro and micro features resemble each other. The only thing separating them is their geographical distribution [16]. *Suillus flavidus* and *S. umbonatus* are also close relatives of *S. sibiricus* that form sister clade with *S. sibiricus* in constructed Cladogram (Fig. 6). The ectomycorrhizae of *S. sibiricus* were previously described with *Pinus cembra*, *P. banksiana*, *P. monticola*, *P. peuce*, *P. pumila* and *P. sibirica* [24, 28-32]. According to Agerer [23], *S. sibiricus* forms coralloid mycorrhizal with *P. cembra*. Mantle is typically of plectenchymatous type with club-shaped cells. Hyphae of mantle embedded in the pigment matrix and possess large hyphae in

rhizomorphs. In contrast to *P. cembra*, in present investigation *S. sibiricus* forms dichotomously branched mycorrhiza with *P. wallichiana*. The mycorrhizal system bears honey brown coloration with long spiny to cottony appearance. Mantel resembles with *P. cembra* mycorrhizal in its shape but cells varied from round to angular. The symbiotic range of *S. sibiricus* is revised in this investigation. A deciduous host, *Salix alba* L. is found symbiotically with *S. sibiricus*. It possesses monopodial pinnate type of mycorrhizal systems. These are different from *P. wallichiana* and *P. cembra* mycorrhiza. It posses parenchymatous mantle with irregular cells. Rare H-type rhizomorphs are present with dark brown- to black-colored septate hyphae.

During present investigation, it is concluded that *S. brevipes* and its ectomycorrhizae are new reports for Pakistan. In addition, molecular analysis of *S. sibiricus* and its ectomycorrhizae are being done for the first time in Pakistan and association of this fungus with *Salix alba* is new to science world and it seems that *S. sibiricus* extends its host range from conifers to deciduous trees.

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