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Full Length Article

Diversity and Phylogeny of *Suillus* (Suillaceae; Boletales; Basidiomycota) from Coniferous Forests of Pakistan

Samina Sarwar* **and Abdul Nasir Khalid**
Department of Botany, University of the Punjab, Quaid-e-Azam Campus, Lahore, 54950, Pakistan
*For correspondence: samina_boletus@yahoo.com

Abstract

Suillus (Boletales; Basidiomycota) is an ectomycorrhizal genus, generally associated with Pinaceae. Coniferous forests of Pakistan are rich in mycodiversity and *Suillus* species are found as early appearing fungi in the vicinity of conifers. This study reports the diversity of *Suillus* collected during a period of three (3) years (2008-2011). From 32 basidiomata of *Suillus* collected, 12 species of this genus were identified. These basidiomata were characterized morphologically, and phylogenetically by amplifying and sequencing the ITS region of rDNA. © 2014 Friends Science Publishers

Keywords: Moist temperate forests; PCR; rDNA; Ectomycorrhizae

Introduction

Suillus (*Suillaceae, Basidiomycota, Boletales*) forms ectomycorrhizal associations mostly with members of the *Pinaceae* and is characterized by having slimy caps, glandular dots on the stipe, large pore openings that are often arranged radially and a partial veil that leaves a ring or tissue hanging from the cap margin (Kuo, 2004). This genus is mostly distributed in northern temperate locations, although some species have been reported in the southern hemisphere as well (Kirk *et al*., 2008). Wu *et al*. (2000) discussed the bio-geographic pattern and phylogenetic relationship of *Suillus* species from Eastern Asian (China and Nepal) and North American territories. Knowledge of *Suillus* species diversity is important because of their major roles in natural and managed ecosystems as ectomycorrhizal fungi. This fungal group becomes an important factor for reforestation program worldwide. Furthermore, they are important as a food source for human being and animals (Brundrett *et al*., 1996). They are also used as a bio-indicator of environmental quality. Studies on the diversity and taxonomy (base on morphological characters and molecular analyses) of *Suillus* is lacking and needs more investigation.

The purpose of this research was to study the diversity and phylogeny of *Suillus* collected from coniferous forests of Pakistan, which are located at 1373 to 3050 m. altitude. The most important component of these forests are coniferous trees i.e., *Pinus wallichiana* A.B. Jackson, *P. roxburghii* Sargent, *Abies pindrow* Royb. (Royle), *Cedrus deodara* (Roxb.) Loud., *Picea smithiana* (Wall.) Boiss., *Taxus wallichiana* Zucc mixed with deciduous trees (Hussain, 1995). Heavy rainfall and adequate temperature make the environment suitable for the growth of mushrooms in these forests.

This paper described the diversity of *Suillus* (Boletes, Fungi) with the help of the anatomical, morphological and genetic analyses as little knowledge is available from forests in Pakistan.

Materials and Methods

Sporocarp Collection

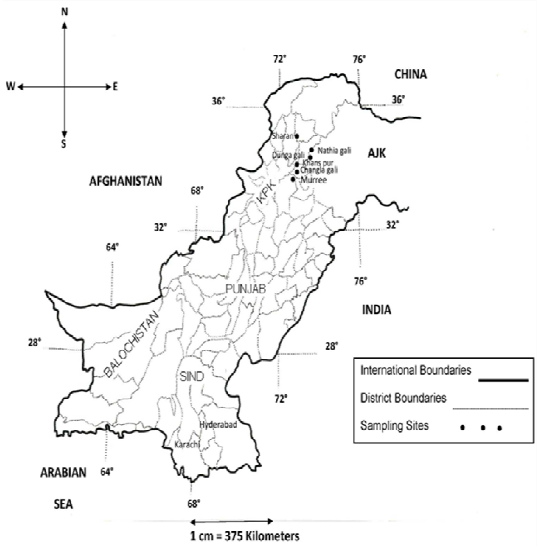

Fig. 1: Map of Pakistan showing sampling sites

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Table 1: Distribution of *Suillus* taxa collected from different sites of coniferous forests of Pakistan

Scientific names	Host tree/Substrate	Locality	Date of collection	Collector name	Identification method	Comments
Suillus bovinus	Coniferous trees	Mushkin forests, District Astore	2007	Razaq	Morphological	Previously reported
Suillus grevillei	Coniferous trees	Khanspur	1996	Iqbal & Khalid	Morphological	Previously reported
Suillus granulatus	cf *Abies pindrow, Pinus wallichiana*	Khanspur, Helipad	2008	Sarwar	Morphological & Molecular	New record and Molecular analysis first time from Pakistan
Suillus granulatus	coniferous trees	Murree, Malakundi, Pirchinasi	1969, 1992, 1993 & 2010	Ahmad, Shibata, Murakami &Sarwar	Morphological	Previously reported
Suillus luteus	On soil, along sides of canals	Dashkin, District Astore	2007	Razaq	Morphological	Previously reported
Suillus placidus	*Juglans regia*, *Pinus wallichiana* and *Abies pindrow*	Dhirkot (AJK), Sharan, Nathiagali, Dungagali	1993 & 1996	Murakami, Iqbal & Khalid	Morphological	Previously reported
Suillus sibiricus	*Abies pindrow, Pinus wallichiana, Populus sp& Salix alba*	KPK, Ayubia, Khera gali, Kuzagali, Banjoosa (AJK), Batakundi, Pirchinasi	1962, 1993, 2008 & 2010	Ahmad, Murakami, Niazi, Sarwar	Morphological & Molecular	Molecular analysis first time from Pakistan
Suillus tomentosus	Coniferous trees, under *Abies pindrow* and herbaceous vegetation	Nathiagali, Malakundi, Dungagali	1992, 1996 & 2008	Shibata, Iqbal & Khalid, Niazi	Morphological	Previously reported
Suillus viscidus	Various trees especially pine trees	Mushkin forests, District Astore	2007	Razaq	Morphological	Previously reported
Suillus collinitus	*Pinus wallichiana*	Helipad & Khanspur (KPK)	2008, 2010	Sarwar	Morphological & Molecular	Molecular analysis first time from Pakistan
Suillus brevipes	*Quercus incana*	KPK, Khanspur	2008	Sarwar	Morphological	New record
Suillus flavidus	*Pinus wallichiana*	KPK Khanspur, Ayubia	2008, 2010	Sarwar	Morphological	New record

Specimens were collected from the selected areas (Fig. 1; Table 1) beginning early summer (2008-2011) when sporocarp production was first observed in July until production ceased at the end of September. Sporocarps were taken with the help of sharp digger. A special designation (collection number) was given to each sample. Field notes were made of fresh fruiting bodies, including color, measurements, shape and bruising reactions. Photographs of fresh sporocarps were also taken to view various parts such as pileus surface, stipe and pore surface. After photographing, the sporocarps were dried by keeping them near a fan heater. After drying, each specimen was placed in a separate paper bag and labeled.

Morphoanatomic Characterization of Sporocarps

For morphological characterization the following characteristics of fresh sporocarps were taken: Color, shape, measurements (width, length, thickness) of pileus and stipe; context of pileus and stipe and color changing of context upon bruising; ornamentation of stipe and pileus surface; attachment of stipe; shape of pileus margin, color; presence of ring on stipe; color of pore surface, pore and tube size, and bruising reaction of pore surface.

For anatomical characterization of sporocarps, a compound microscope was used and the following characters were noted by preparing slides in KOH, Meltzer's, Trypan Blue and Lactic Acid: Shape, length, width, cytoplasmic contents of basidia, cystidia, basidiospores, pileipellis and terminal cells of pileipellis, and color reaction.

Molecular Characterization

DNA Extraction and Amplification

DNA was extracted from dried sporocarps and ectomycorrhizae (ECM) using the enzymatic digestion and glass–fibre filtration (EDGF) protocol in Dentinger *et al*. (2010). The nuclear ribosomal internal transcribed spacer (ITS) region was amplified following PCR conditions in Dentinger *et al*. (2010) using the fungal specific and universal primers (White *et al*., 1990; Dentinger *et al.,* 2010). PCR products were purified using ExoSAP–IT® (Affymetrix, High Wycombe, UK) and dye–terminated unidirectional sequencing was performed using a BigDye® Terminator V3.1 Cycle Sequencing Kit (Life Technologies/ABI, California, USA) in 10 µL reactions with respective primers following the protocol in Dentinger *et al.* (2010). Sequencing reactions were cleaned using ethanol precipitation following the manufacturer's instructions, re-suspended in 30 µL of distilled water, and run on an ABI 3730 DNA sequencer in the Jodrell Laboratory, Royal Botanic Gardens Kew.

Editing of Sequences and BLAST Analysis of ITS Sequences

ITS sequences were compared using Basic Local Alignment Search Tool (BLAST) network service using National Center for Biotechnology Information (NCBI) to compare or confirm identifications. These sequences were edited and

cleaned at BioEdit, where required and were aligned with other sequences present in GenBank, using the muscle alignment tool (www.ebi.ac.uk/Tools/msa/muscle). In aligned sequences, all characters were equally weighed and gap positions were treated as missing data. Percent Identity and divergence of species was calculated using the computer program MegAlign (DNASTAR Inc.) and percent genetic characters of different species were calculated with Jalview software. The preferable cutoff value for species delimitation was 97%, below which the sequences were considered to represent different species.

Phylogenetic Analysis

Phylogenetic trees were made separately for each species sequence(s) because either their ITS1, ITS2 parts or complete ITS region was amplified successfully so combine phylogenetic tree was not reliable. Maximum Likelihood (ML) analysis was done using Molecular Evolutionary Genetic Analysis (MEGA 5.0) with default settings of program i.e. Jukes–Cantor Model and for ML Heuristic Nearest–Neighbor–Interchange (NNI) method was used (Tamura *et al*., 2011). 1000 bootstrapping replicates were performed for analysis. Phylogenetic position of some species was confirmed by making Maximum Parsimony Tree with bootstrapping using PAUP* Version 4.0b10.

Results

Tweleve *Suillus* species are identified morpho-anatomically for this study; species which had previously been described are listed at the end of the results. Attempts were made to identify all species by sequencing but only 4 species were characterized successfully by molecular analysis. Results are given alphabetically.

Suillus brevipes (Peck) Kuntze, *Revis. Gen. Pl.* (Leipzig) 3(2): 535 (1898) Fig. 2.

Pileus 2.5–6 cm, convex to hemispheric to plane, chocolate brown, shiny, smooth, glabrous, sticky, flesh thick and off–white, margins slightly incurved, entire, smooth, of same color like pileus surface.

Context whitish to light yellowish, no color change upon bruising.

Stipe 3–6 cm long, 1–2 cm thick, centric, clavate, smooth, whitish with brown small patches at some points, semi–hollow, ring and volva absent, context whitish, no color change upon bruising.

Pore surface adnate and ascending, whitish to cream to light yellowish, pores rounded to irregular, about 2 per mm, tubes 4–9 mm deep, yellowish, no color change upon bruising.

Basidispores ellipsoid to fusiform to subfusiform, smooth, thick walled, 6–10 × 3–6 µm, (8.2 ± 1.27 × 4.8 ± 0.93; Q_m= 2.1 ± 0.76).

Basidia clavate, 2–4 sterigmate, thin walled, 18–26 × 6–8 µm.

Cystidia cylindrical to clavate to subfusoid to ampullaceous, thick walled, dark brown contents, 35–49 × 5–9 µm.

Pileipellis cylindrical with rounded ends, thin–walled, 40–55 × 5–8 µm, most terminal elements subclavate to cylindrical, 49–58 × 7–9 µm.

Smell and Taste not distinctive.

Edibility edible.

Chemical reactions pileipellis stains olive in $FeSO_4$, dark brown in KOH, Meltzer reagent and Lactic acid, spores brown in Meltzer reagent.

MATERIAL EXAMINED: Pakistan: *KHYBER PAKHTUNKHWA*, Khanspur, 2250 m.a.s.l., under *Quercus incana* Roxb., solitary, on ground, 19th June 2008, Sarwar S.B. # 12 (LAH0608).

Suillus c.f. granulatus (L.) Roussel, *Fl. Calvados*, Edn 2: 34 (1806) Fig. 3.

Pileus 6–13 cm wide, plane to convex, surface viscid, sticky, smooth, yellowish brown to camel brown, margins smooth, straight or flaring.

Context yellowish, no color change upon bruising.

Stipe: 4–7 cm long, 1–2 cm thick, equal, centric, solid, yellowish to yellowish brown, brownish glandular dots on upper half, ring absent.

Pore surface yellowish, adnate and horizontal, color change to brownish when bruised, pores angular to irregular and frequent, about 1–2 per mm, tubes 3–11 mm deep.

Basidispores subfusiform, smooth, 8–11 × 4–6 µm, (9.4 ± 0.97 × 5.1 ± 0.7; Q_m= 1.9 ± 0.5).

Basidia clavate, 3–4 sharp sterigmate, thick walled, 16–18 × 6–7.5 µm. Cystidia cylindrical to fusoid to ampullaceous, granular contents visible, thick walled, dark brown, 50–55 × 7.5–9 µm. Pileipellis clavate to irregular, 53–69×11–13 µm, terminal elements of Pileipellis clavate to irregular, hyphae septate at end, 49–62 × 10–12 µm.

Smell and Taste not distinctive.

Edibility edible.

Chemical reactions pileipellis stains bluish gray in $FeSO_4$, olive gray in KOH; spores brown in Meltzer reagent.

Material examined: Pakistan: Khyber Pakhtunkhwa, Khanspur, 2350 m a.s.l., under *P. wallichiana* solitary, on ground, 17th June 2008, Sarwar S.B. # 72(LAH0608), (Holotype); Helipad, 2350 m a.s.l., under *A. pindrow*, solitary, on ground, 18th June 2008, Sarwar S.B. # 72A(LAH0608).

Suillus collinitus (Fr.) Kuntze, *Revis Gen. Pl.* (Leipzig) 3(2): 536 (1898) Fig. 4.

Pileus 2 cm wide, hemispheric to convex, viscid when wet, brown to dark brown, margins smooth, deflexed to straight. Context yellowish white, no color change upon bruising.

Stipe 5.6–7.4 cm long, about 1 cm thick, nearly equal, cylindrical, yellowish to brownish yellow, whitish pink near base with pinkish mycelia at base, ring absent, dry, centric, ocassionally slightly curved with brown glandular dots.

Pore surface bright yellow to yellowish, brownish

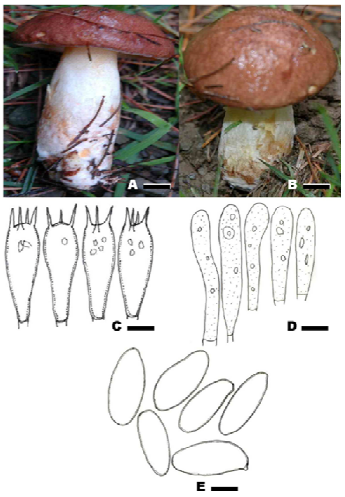

Fig. 2: *Suillus brevipes*. A & B, Sporocarps; C, Basidia; D, Cystidia; E, Basidiospores. Scale Bars: for A and B = 1 cm; C = 6 µm; D = 8 µm; E = 3 µm

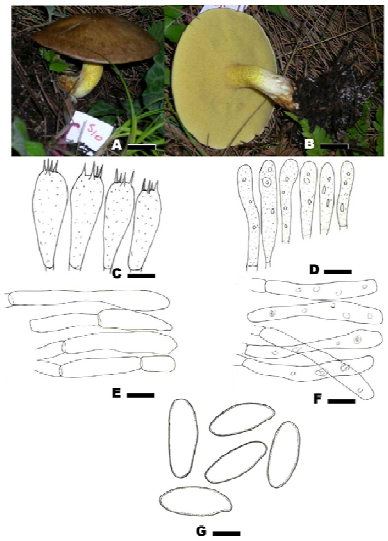

Fig. 3: *Suillus c.f. granulatus*. A & B, Sporocarps; C, Basidia; D, Cystidia; E, Terminal elements of pileipellis hyphae; F, Pileipellis hyphae; G, Basidiospores. Scale Bars: for A and B = 2 cm; C = 4 µm; D = 15 µm; E = 12 µm; F =15 µm; G = 3.5 µm

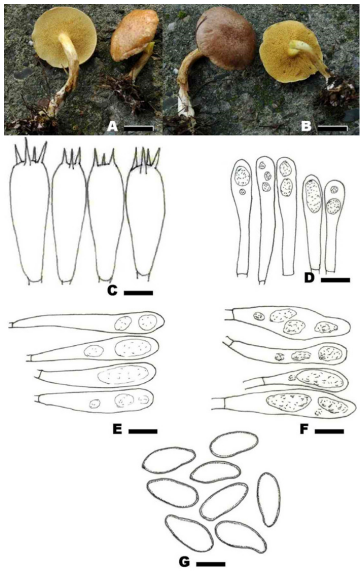

Fig. 4: *Suillus collinitus*. A & B, Sporocarps; C, Basidia; D, Cystidia; E, Pileipellis hyphae; F, Terminal elements of pileipellis hyphae; G, Basidiospores. Scale Bars: for A & B =2 cm; C =3.5 µm; D = 11µm; E = 19 µm; F =16 µm; G = 7 µm

upon bruising, adnate to decurrent, pores rounded to angular, 1–2 per mm, tubes shorter near margins of pileus.

Basidispores ellipsoid to fusiform, thick walled, smooth, light honey brown, (7–) 9–13 × 5–7 µm, (11.4 ± 1.25 × 6.12 ± 0.75; Q_m= 1.9 ± 0.4).

Basidia clavate, 2–4 sterigmate, 13–15 × 7–9 µm. Cystidia clavate to sub–globose, dark brown, thick walled with brownish contents, (26–) 35–41 × 7–10 µm. Pileipellis cylindrical to slightly clavate, thick walled, 70–79 × 13–17 µm, most terminal elements of pileipellis cylindrical to clavate, some are globose from above, dark brown, contents visible, thick walled, 60–67 × 9–12 µm.

Smell and Taste: not distinctive.

Edibility edible.

Chemical reactions pileipellis stains olive in $FeSO_4$, dark brown in KOH, Meltzer reagent and Lactic acid, spores brown to brown in Meltzer reagent.

Material examined: Pakistan: Khyber Pakhtunkhwa, Khanspur Helipad, 2250 m a.s.l., under *P. wallichiana,* solitary, on ground, 26th July 2008, Sarwar S.B. # 03(LAH0708), (Holotype); 18th June 2010, Sarwar S.B. # 03A(LAH0610).

Suillus flavidus (Fr.) J. Presl, *Wsobecny Rostl.* (Praha) 2: 1917 (1846) Fig. 5.

Pileus 3–9 cm wide, convex to hemispherical to nearly plane, occasionally slightly umbonate at maturity, occasionally margins straight and flaring to slightly deflexed with whitish remnants of veil, surface viscid to glutinous when wet, glabrous, yellow to yellowish brown. Context light yellow, changes brown when bruising, not bluing.

Stipe 3–10 cm long, 1.5–2 cm thick, nearly equal, cylindrical, centric and curved, solid, slightly dry, reddish when young, yellow to white with reddish tinge when mature, whitish glandular dots in some case, whitish thick band like ring present above centre of stipe, color above ring yellow.

Pore surface yellow becomes slightly brown upon bruising, adnate and horizontal, pores angular to irregular, infrequent, about 2 per mm, tubes 3–9 mm deep.

Basidispores ellipsoid to fusoid, smooth, 9–13 × 4–6 µm, (11.3 ± 1.2 × 5.2 ± 0.6; Q_m= 2.26 ± 0.17).

Basidia cylindric to long clavate, thick walled, yellowish brown contents visible in Meltzer reagent, 1–4 sterigmate, 22–26 × 8–10 µm. Cystidia cylindrical to fusoid–ventricose, brown contents visible, thick walled, dark brown, 32–34 × 9–10 µm. Pileipellis long, cylindrical to slightly clavate, thick walled, brown, 77–84 × 18–20 µm, most terminal elements of pileipellis cylindrical to clavate, in clusters and separate also, some are globose from above, dark brown, thick walled, 71–77 × 8–10 (–14) µm.

Smell and Taste not distinctive.

Edibility edible.

Chemical reactions pileipellis stains reddish in KOH,

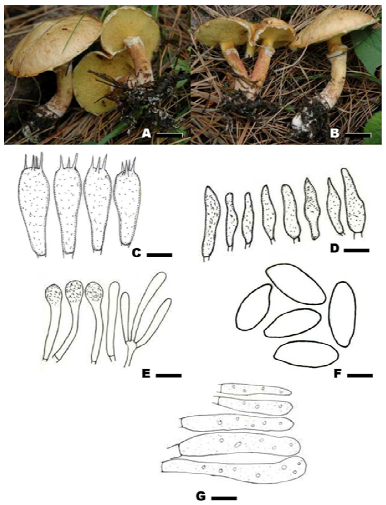

Fig. 5: *Suillus flavidus*. A & B, Sporocarps; C, Basidia; D, Cystidia; E, Terminal elements of pileipellis hyphae; F, Basidiospres; G, Pileipellis hyphae. Scale Bars: for A & B =2.5 cm; C =8 µm; D = 11 µm; E = 25 µm; F =6 µm; G = 16 µm

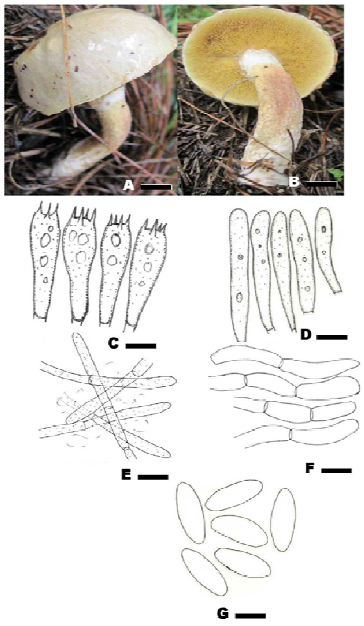

Fig. 6: *Suillus sibiricus*. A & B, Sporocarps; C, Basidia; D, Cystidia; E, pileipellis hyphae; F, Terminal elements of pileipellis hyphae; G, Basidiospores. Scale Bars: for A and B = 1.5 cm; C = 7 µm; D = 8.5 µm; E = 12 µm; F = 24 µm; G = 4.5 µm

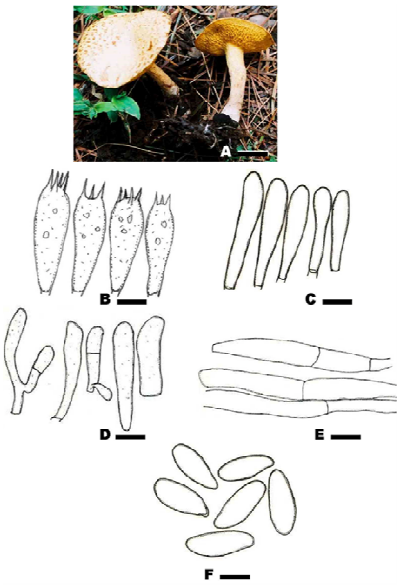

Fig. 7: *Suillus tomentosus*. A, Sporocarp; B, Basidia; C, Cystidia; D, Terminal elements of pileipellis hyphae; E, Pileipellis hyphae; F, Basidiospores. Scale Bars: for A =2 cm; B =11 µm; C= 11 µm; D= 24 µm; E =25 µm; F = 7 µm

spores brownish in Meltzer reagent, light yellow to honey yellow in Lactic acid.

Material examined: Pakistan: Khyber Pakhtunkhwa, Ayubia, 2350 m a.s.l., under *P. wallichiana*, gregarious, on ground, 19th June 2008, Sarwar S.B. # 06(LAH0608), (Holotype); Khanspur, 2250 m a.s.l., solitary, on ground, 17th July 2010, Sarwar S.B. # 06A(LAH0710).

Suillus sibiricus (Singer) Singer, *Farlowia,* 2: 260 (1945) Fig. 6

Genbank: JN119748–54

Pileus 4–8 cm, pulvinate to obtuse, sticky, slimy, shiny, glabrous, yellowish brown, smooth, sometimes with brownish scales on yellowish to dull yellowish color, margins entire, slightly darker color than pileus surface, deflexed. Context pale yellowish, not bluing on exposing.

Stipe about 10 cm long, 1.2–1.8 cm thick, central, equal, ring present, yellowish to off–white from apex to ring, reddish brown from ring towards base, whitish near base, whitish to brown glandular dots, rough, solid, curved.

Pore surface yellowish, adnate and ascending, color change to brownish when bruised, pores angular and frequent, about 2 per mm, tubes 7–17 mm deep.

Basidispores ellipsoid–fusiform, thin walled, smooth, 10–12 × 3.5–5 µm, (10.6 ± 2.5 × 4.1 ± 0.53; Q_m = 2.94 ± 0.34).

Basidia clavate, 2–4 sterigmate, thick walled, brownish contents visible in Meltzer reagent, 25–39 × 7–9 µm. Cystidia cylindrical to subfusiform, thick walled, yellowish brown in Meltzer reagent, yellowish brown contents, 33–45 (–62) × 7–9 µm. Pileipellis a tangled layer of repent hyphae, thin walled, granular contents, septate, 60–74 × 8–10 µm, most terminal elements subclavate–clavate–cylindrical with pointed ends, thick walled, 68–99 × 13–20 µm.

Smell and Taste not distinctive.

Edibility edible.

Chemical reactions pileipellis stains yellowish brown in $FeSO_4$, dark brown to black in KOH, hyaline to light honey in Meltzer reagent; spores light yellowish brown in Meltzer reagent, light yellow to honey yellow in Lactic acid.

Material examined: Pakistan: Khyber Pakhtunkhwa, Khaira Gali, 2347 m a.s.l., under *P. wallichiana*, solitary, on ground, 18th June 2010, Sarwar S.B. # 53(LAH0610), (Holotype); Nathiagali, 2520 m a.s.l., 19th July 2010, Sarwar S.B. # 53A(LAH0710); Khaira gali, 2347 m a.s.l., under *Salix alba* L., scattered or in groups, on ground, 7th August 2010, Sarwar S.B. # 53B(LAH0810).

Suillus tomentosus (Kauffman) Singer, *Mycologia* 51(4): 570 (1960) [1959] Fig. 7.

Pileus 4–6 cm wide, convex, becoming nearly plane with age, yellowish, surface viscid, irregular circular patches of gray–brown to dark brown tomentum or squamules all over the pileus surface, margins incurved when young,

gradually becoming deflexed to straight to uplifted with age, smooth. Context light yellowish, bluing when exposed. Odor and taste not distinctive.

Stipe 4–7 cm long, 1–2 cm thick, nearly equal, dry, solid, centric, cylindrical, yellowish, with brown patches sometimes, yellowish glandular dots near apex, basal mycelium salmon–buff, volva and annulus absent, context yellowish, bluing upon exposure.

Pore surface adnate and horizontal to arcuate, bright yellowish, pores infrequent, pores angular to irregular with wide openings, 1 per mm, tubes 6–11 mm deep, changing slightly blue then brown upon bruising.

Basidispores oblong–ellipsoid–inequilatteral, slightly apiculate, thin walled, smooth, 14–15 × 5–7 µm, (14.5 ± 0.40 × 5.9 ± 0.73; Q_m= 2.4 ± 0.39).

Basidia clavate to irregular, 2–3 sterigmate, hyaline, thin walled, contents visible, 33–36 × 14–15 µm. Cystidia elongated, cylindrical to sub clavate, 34–42 × 9–10 µm. Pileipellis cylindrical elongated, thin walled, septate, 72–103 × 16–21 µm, terminal elements of pileipellis cylindrical to subclavate to irregular shaped, septate in some cases, 59–95 × 15–21 µm.

$FeSO_4$ creamish to pinkish in KOH.

Material examined: Pakistan: Khyber Pakhtunkhwa, Ayubia, 2350 m a.s.l., under *P. wallichiana*, in groups, on ground, 15th August 2006, A.R. Niazi # 38(LAH0806).

Phylogenetic Analysis Figs. 8–11

Four *Suillus* species *S. c.f. granulatus*, *S. collinitus, S. flavidus* and *S. sibiricus* were characterized molecularly and phylogenetically. During molecular analysis of *S. c.f. granulatus* 379 bp long sequence belonging to 5.8S and ITS2 showed maximum 99% similarity and 99% query coverage with sequence of *S. c.f. granulatus* (L54121) during BLAST. For phylogenetic analysis 31 sequences containing 412 genetic characters were used after aligning and trimming at both ends. These contains 275 conserved, 113 variable and 67 parsimony informative sites. All characters were equally weighted and unordered. Phylogenetic tree was made by maximum likelihood criteria. *S. c.f. granulatus* from Pakistan form clade with *S. c.f. granulatus* (L54121) (Fig. 8) supported by 92% bootstrap value and shared maximum 100% genetic

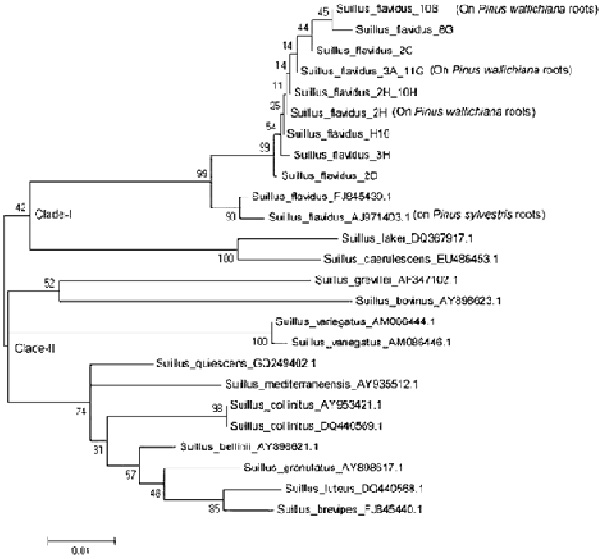

Fig. 10: Phylogenetic position of *Suillus flavidus* and its ectomycorrhizae from Pakistan with respect to other *Suillus* spp. Tree inferred by maximum likelihood analysis. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The numbers against branches indicate the percentage at which a given branch was supported in 1000 bootstrap replications

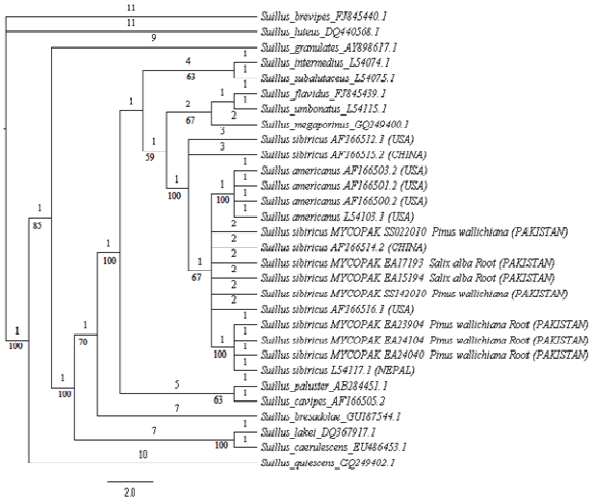

Fig. 11: Bootstrap 50% majority–rule consensus tree indicating phylogenetic position of *Suillus sibiricus* and its ectomycorrhizae with respect to other *Suillus* spp. Cladogram based on parsimony analysis of rDNA–ITS region of different species of *Suillus*. MP tree generated by parsimony analysis of rDNA–ITS with 5.8s gene. The number above branches refer to number of changes, those below to Bootstrap values. The accession numbers of analyzed sequences are sown after each taxon name

Smell mild; Taste mild

Edibility edible, but soft textured

Chemical reactions context stains slightly greenish in

characters and 0.0% genetic divergence with the same. These values are well supportive to confirm our sequence as *S. c.f. granulatus.*

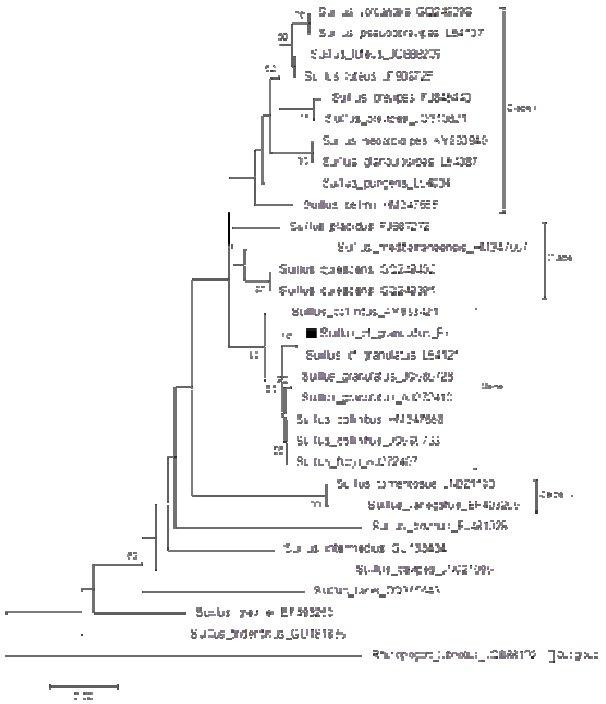

Fig. 8: Phylogenetic position of *Suillus c.f. granulatus* from Pakistan with respect to other *Boletus* spp. Tree inferred by maximum likelihood analysis based on rDNA sequences, including 5.8S and ITS2 (Ln likelihood = –13434.09). *Rhizopogon luteolus* was used as outgroup. The numbers against branches indicate the percentage (>50%) at which a given branch was supported in 1000 bootstrap replications. GenBank accession number are given at the end of species names. ■ indicate species reported from Pakistan

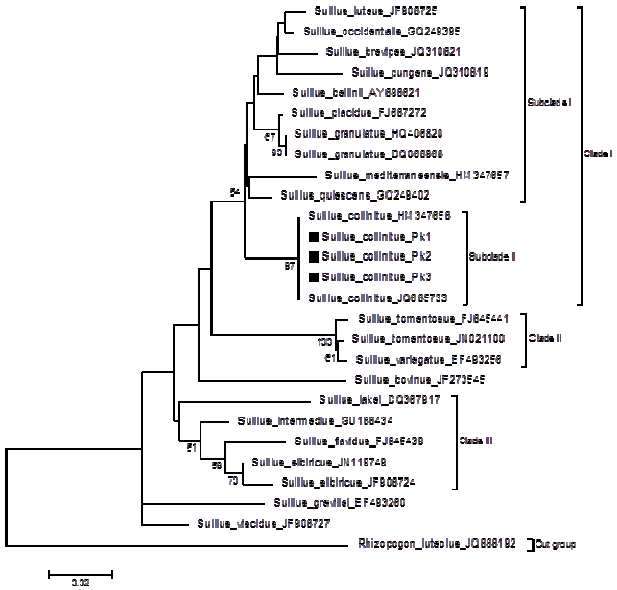

Fig. 9: Phylogenetic position of *Suillus collinitus* with respect to other *Suillus* spp. Tree inferred by maximum likelihood analysis based on rDNA sequences, including 5.8S and ITS2 (Ln likelihood = –47481.29). *Rhizopogon luteolus* was used as outgroup. The numbers against branches indicate the percentage (>50%) at which a given branch was supported in 1000 bootstrap replications. GenBank accession number are given at the end of species names. ■ indicate species reported from Pakistan

Molecular analysis of *S. collinitus* was carried out with 3 different fruiting bodies by using 5.8S and ITS2 part of nrDNA region. All these showed more than 97% similarity with *S. collinitus* (HM347658) and (JQ685733) during BLAST. During phylogenetic analysis 27 sequences containing 412 genetic characters were used in the final aligned datasheet. These sequences contained 270 conserved sites, 118 variable sites and 64 parsimony informative sites. The aligned data was analyzed by maximum lkelihood using, MEGA 5.0. All characters were equally weighted and unordered. *S. collinitus* sequences from Pakistan form a clade with *S. collinitus* (HM347658) and (JQ685733) retrieved from GenBank (Fig. 9). *S. collinitus* sequences from Pakistan shared above 99% genetic characters with other with 0.0–0.3% genetic divergence with each other and with *S. collinitus* (HQ406820) and *S. collinitus* (HM347658) these shared above 99% genetic characters with genetic divergence 0.0–0.3%. Phylogenetically these sequences have been confirmed *S. collinitus.*

When ITS–rDNA sequence of *Suillus flavidus* from Pakistan was submitted for similarity in GenBank, it was identified as *uillus flavidus* with 98% maximum identity and 100% query coverage with *S. flavidus* (FJ845439) from Canada. The phylogenetic analysis included 25 sequences belonging to 13 species. For phylogenetic analysis, a total of 426 genetic characters were used in an aligned datasheet. These sequences contained 330 conserved sites, 91 variable sites, 66 parsimony informative sites. The phylogram based on maximum likelihood criterion represented by 2 major clades. Clade I is formed by 11 sequences and their clustering is not highly resolved (42% bootstrapping). Topologically, *S. flavidus* occupied the top position in the phylogram. This species is represented by 9 sequences, 6 from sporocarps and 3 from *P. wallichiana* ectomycorrhizal roots, from Pakistan and 2 sequences retrieved from GenBank. Sequences from Pakistan shared 100% of their genetic characters (rDNA–ITS sequences) with each other and shared about 98% with *S. flavidus* (FJ845439). It shared 92.1% of its genetic characters with *S. lakei* (Murrill) A.H. Sm. and Thiers (DQ367912), and 91.6% with *S. caerulescens* A.H. Sm. and Thiers (EU486453). Genetic divergence was also measured for *S. flavidus* with all the sequences included in the analysis. No genetic divergence was found among the rDNA–ITS of *S. flavidus* from Pakistan (Fig. 10). There was little genetic divergence (0.5–2.5) compared with *S. flavidus* (FJ845439).

The sequences of *S. sibiricus* from Pakistan showed 99% similarity with isolates of *S. sibiricus* from China and America, confirming the morphological identification. The phylogenetic analysis for *S. sibiricus* was carried out using parsimony as optimality criterion. The sequences included

in this analysis had around 659 genetic 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 and 26 variable sites. 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 6457051 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 indicating three major clades and one independent clade.

A Maximum Parsimony consensus tree was constructed exclusively for *Suillus* species from geographically different localities specially from Eastern Asia (China and Nepal), Eastern North America and from Pakistan to resolve exact identification. The cladogram represents (Fig. 11) a major polytomous clade formed by *S. americanus* (Peck) Snell and *S. sibiricus* species. 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.

The Maximum Parsimony analysis resulted in a major polytomous clade comprising sixteen isolates of *S. americanus* and *S. sibiricus*. All these species are monophyletic along with *S. flavidus* (FJ845439), *S. megaporinus* Snell & E.A. Dick (GQ249400) and *S. umbonatus* Dick & Snell (L541115). *S. sibiricus* has been published by the author in ICMBMP7.

Discussion

Suillus is an important ectomycorrhizal bolete characterized by a slimy pileus, stipe with glandular dots and ring, wide pore openings, smooth spores and usually associated with conifers (Bessette *et al*., 2000; Kuo, 2004). Many scientists have done molecular and Phylogenetic analyses of *Suillus* species. Kretzer *et al*. (1996) analyzed 38 sequences of *Suillus* species for phylogenetic and taxonomic studies. Wu *et al*. (2000) discussed the bio-geographic pattern and phylogenetic relationship of *Suillus* species. Manian *et al*. (2001) investigated the genetic diversity and relationships between *Suillus* species based on ribosomal DNA sequences. *S. quiescens* T.D. Bruns and Vellinga was first time reported and described morpho-anatomically and molecularly by Bruns *et al*. (2010).

From Pakistan nine (9) species of *Suillus* such as *Suillus bovinus*, *S. grevillei*, *S. granulatus*, *S. luteus*, *S. placidus*, *S. sibiricus*, *S. tomentosus* and *S. viscidus* have already been reported (Ahmad, 1962; Shibata, 1992; Murakami, 1993; Iqbal and Khalid, 1996; Razaq, 2007; Niazi, 2008; Sultana *et al*., 2011). *S. sibiricus* (Singer) Singer was also analyzed phylogenetically from Pakistan (Sarwar *et al*., 2011).

The present investigation explores the status of *Suillus* from the high mountains of Pakistan. *S. brevipes* and *S. flavidus* were compared with closely related species. *S. brevipes* from Pakistan has maximum similarity with *S. brevipes* reported from other countries due to convex to hemispheric pileus, chocolate brown smooth, shiny pileus surface, whitish to light yellowish context with no colour change upon bruising, clavate stipe without ring and glandular dots, whitish to light yellowish pore surface and smooth spores. *S. brevipes* is similar with *S. albidipes* (Peck) Singer, *S. c.f. granulatus* and *S. pallidiceps* A.H. Sm. and Thiers due to convex pileus, no ring on stipe and smooth spores. The major differences between these species is that *S. c.f. granulatus* has a shorter stipe, and distinctly raised granules on the stipe while *S. brevipes* has a smooth, white stipe. Similarly *S. brevipes* is differentiated from *S. albidipes* by having stipe without glandular dots and larger spores in former. Major difference of *S. brevipes* from *S. pallidiceps* is white to pale yellow pileus of latter, while *S. brevipes* has chocolate brown (Thiers, 1975; Bessette *et al*., 2000; Santana *et al*., 2007; Bruns *et al*., 2010).

S. flavidus is characterized by a convex to hemispheric pileus with some reddish brown spots on the margin and small hanging veil remnants. Cap color is yellowish with prominent ring on stipe and smooth spores which range in color from light to dark brown. *S. flavidus* resembles *S. lakei* but the pileus surface of the latter is covered with dull reddish brown small scales without glandular dots on stipe. *S. flavidus* also resembles to *S. caerulescens* A.H. Sm and Thiers but *S. caerulescens* does not have glandular dots on stipe. *S. flavidus* is similar to *S. grevillei* (Klotzsch) Singer but *S. grevillei* has a reticulated stipe and glandular dots characteristic of *S. flavidus* (Thiers, 1975; Bessette *et al*., 2000) are absent.

S. collinitus, S. c.f. granulatus, S. sibiricus and *S. tomentosus* were characterized morpho-anatomically and these shares many characters but can be differentiated from each other due to some major differences. *S. sibiricus* can be differentiated due to its ring on the stipe which is absent in other three. *S. sibiricus* is often confused with the 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 other feature that separates these two species is their geographical distribution (Wu *et al*., 2000). *S. collinitus* can be identified due to its pinkish basal mycelia. *S. c.f. granulatus* has glandular dots on upper half of the stipe. The context and pore surface in *S. tomentosus* stains blue upon exposing which is distinguishing character of this species (Thiers, 1975; Bessette *et al*., 2000; Santana *et al*., 2007).

In conclusion, according to the results of this study many trees like *Abies*, *Cedrus*, *Pinus*, *Populus*, *Quercus* and other coniferous trees are dependent upon mycorrhizal fungi for their survival. Knowledge of the mycorrhizal symbionts like *Suillus* species give us a better understanding of the ecology of these important timber trees. Also understanding the host preference of *Suillus* species has aided local people in locating a new source of edible species of this mushroom which in turn benefits the local economy. Species documented from this study compared with those from other areas of the world give us a better understanding of biogeography patterns and address questions concerning species dispersal. Phylogenetic studies of this genus are currently available only for a limited number of species from other parts of the world. Phylogenetic studies of Asian *Suillus* species give us a better understanding of the evolution of this genus on a worldwide scale, and aid in the biogeographic analysis. The present work is the first molecular analysis of this genus in Pakistan.

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References

Ahmad, S., 1962. Further contributions to the fungi of Pakistan. II. *Biologia*, 8: 123–150

Bessette, A., W.C. Roody and A.R. Bessette, 2000. *North American Boletes: a Color Guide to the Fleshy Pored Mushrooms*, p: 396. Syracuse University Press, New York, USA

Brundrett, M., N. Bougher, B. Dell, T. Grove and N. Malajczuk, 1996. *Working with Mycorrhizas in Forestry and Agriculture,* p: 374. ACIAR Monograph 32 ACIAR, Canberra, Australia

Bruns, T.D., L.C. Grusiba, J.M. Trappe, J.F. Kerekes and E.C. Vellinga, 2010. *Suillus quiescens*, a new species commonly found in the spore bank in California and Oregon. *Mycologia,* 102: 438–446

Dentinger, B.T.M., S. Margaritescu and J.M. Moncalvo, 2010. Rapid and reliable high–throughput methods of DNA extraction for use in barcoding and molecular systematic of mushrooms. *Mol. Ecol. Resour.*, 10: 628–633

Hussain, S.S., 1995. *Pakistan Manual of Plant Ecology*, pp: 161–162. Mirror Press Ltd., Karachi, Pakistan

Iqbal, S.H. and A.N. Khalid, 1996. Material for the fungus flora of Pakistan. I. Check list of Agarics, their distribution and association with the surrounding vegetation. *Sci. Int.*, 8: 51–64

Kirk, P.M., P.F. Cannon, D.W. Minter and J.A. Stalpers, 2008. *Dictionary of the Fungi*, 10th edition. CABI, Wallingford, UK

Kretzer, A.M., Y. Li, T. Szaro and T.D. Bmns, 1996. Internal transcribed spacer sequences from 38 recognized species of *Suillus* sensu lato: phylogenetic and taxonomic implications. *Mycologia*, 88: 776–785

Kuo, M., 2004. *The Genus Suillus.* Retrieved from the *MushroomExpert.Com* Web site: http://www.mushroomexpert.com/*Suillus*.html

Manian, S., S. Sreenivasaprasad, G.D. Bending and P.R. Mills, 2001. Genetic diversity and interrelationships among common European *Suillus* species based on ribosomal DNA sequences. *FEMS Microbiol. Lett.,* 204: 117–121

Murakami, Y., 1993. Larger fungi from Pakistan. *In*: Cryptogamic Flora of Pakistan, Vol. 2, pp: 105–147. Nakaike, T. and S. Malik (eds.). Pakistan Science Foundation, Islamabad, Pakistan

Niazi, A.R., 2008. Biodiversity of Ectomycorrhizas in Conifers from Himalayan Moist Temperate Forests of Pakistan. *Ph.D. Thesis*, Department of Botany, University of Punjab, Lahore, Pakistan

Razaq, A., 2007. Taxonomic Studies on Basidiomycota from Northern Areas of Pakistan. *Ph.D. thesis*, University of Karachi, Karachi, Pakistan

Santana, B.O., D.J. Lodge, T.J. Baroni and E.E. Both, 2007. Boletes from Belize and the Dominican Republic. *Fungal Diversity,* 27: 247–416

Sarwar, S., M. Hanif, A.N. Khalid and J. Guinberteau, 2011. Diversity of Boletes in Pakistan, focus on *Suillus brevipes* and *Suillus sibiricus*. *In*: *Proceedings of the Seventh International Conference on Mushroom Biology and Mushroom Products*, Vol. 1, pp: 123–133. Savoie, J.M., M.O. Foulongne, M. Largeteau and G. Barroso (eds.). INRA, France. http://www.wsmbmp.org/Previous_Conference_7.html

Shibata, H., 1992. Higher Basidiomycetes from Pakistan. *In*: *Cryptogamic Flora of Pakistan*, Vol. 1, p: 145. Nakaike, T. and S. Malik (eds.). Pakistan Science Foundation, Islamabad, Pakistan

Sultana, K., C.A. Rauf, A. Riaz, F. Naz, G. Irshad and M.I. Hauqe, 2011. Checklist of agarics of Kaghan valley. *Pak. J. Bot.,* 43: 1777–1787

Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei, and S. Kumar, 2011. MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance and Maximum Parsimony Methods. *Mol. Biol. Evol.,* 28: 1731–1739

Thiers, H.D., 1975. *California Mushrooms*. A field guide to the boletes. Hafner Press, New York, USA

White, T.J., T.D. Bruns, S. Lee and J. Taylor, 1990. *Amplification and Direct Sequencing of Fungal Ribosomal RNA Genes for Phylogenetics*, pp: 315–322. PCR Protocol, a Guide to Methods and Applications. Academic Press, San Diego, California, USA

Wu, Q.X., G.M. Mueller, F.M. Lutzoni, Y.Q. Huang and S.Y. Guo, 2000. Phylogenetic and biogeographical relationships of eastern Asia and eastern North American disjunct *Suillus* species (fungi) as inferred from nuclear ribosomal RNA ITS sequences. *Mol. Phylogenet. Evol.,* 17: 37–47

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