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Diversity of the genus *Suillus* Gray from coniferous forests of the northwestern Himalayas, India: Taxonomy, ecology and some new records

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ABSTRACT

The coniferous forests of the northwestern Himalayan region of India are rich in mycodiversity and were explored for *Suillus* diversity during the present study. We documented seven *Suillus species viz. S. sibiricus, S. granulatus, S. flavidus, S. placidus S. triacicularis, S. himalayensis* and *S. indicus* from this region. All these species were examined morphologically and identified molecularly by sequencing the internal transcribed spacer region of basidiocarps. Out of seven species identified, two taxa namely, *S. flavidus* and *S. placidus* are new records from India, whereas *S. sibiricus* and *S. granulatus* are re-reported. *Suillus flavidus, S. placidus, S. sibiricus* and *S. granulatus* are described taxonomically as well as molecularly for the first time from India. Ecological data regarding occurrence, abundance, distribution and association of these *Suillus* species is also discussed and suggested that *S. sibiricus* is the most frequently encountered and widely distributed species in the northwestern Himalayan region of India. In addition, a key to the northwestern Himalayan species of the genus *Suillus* is also provided.

Key words: *Suillus*, distribution, association, ITS, phylogeny, taxonomy

INTRODUCTION

Suillus Gray is a genus of basidiomycetous fungi belonging to the family *Suillaceae* and order *Boletales*. Before 1997, the genus *Suillus* was placed under the family *Boletaceae* of order *Boletales*. On the basis of chemotaxonomic findings (Besl and Bresinsky, 1997), the genus *Suillus* was carved out from the family *Boletaceae* and now placed in the newly formed family *Suillaceae*. About 50 species of *Suillus* have been reported from different parts of the world (Kirk *et al*., 2008). Among them majority of the species are described by Smith and Thiers (1964; 1971); Corner (1972) and Thiers (1976; 1979). Thereafter, diversity and phylogeny of *Suillus* species have been studied from many parts of the world (Kretzer *et al*., 1996; Kretzer and Bruns, 1997; Wu *et al*., 2000; Manian *et al*., 2001; Beatriz *et al*., 2006; Feng *et al*., 2008; Bruns *et al*., 2010; Min *et al*., 2014; Sarwar and Khalid, 2014). Kretzer *et al*. (1996) derived the ITS sequences from 47 isolates belonging to 38 recognized species of the genus *Suillus* from America, Canada, Europe and Asia. They also revealed that the generic and species concepts of *Suillus*, *Gastrosuillus*, *Boletinus* and *Fuscoboletinus* should be re-evaluated and suggested the collapsing of genera *Boletinus* and *Fuscoboletinus* into the genus *Suillus*. Later on, the genus *Gastrosuillus* was also merged into *Suillus* on the basis of ITS data (Kretzer and Bruns, 1997). The phylogenetic relationship between 14 eastern Asian and 22 eastern North American S*uillus* species has been determined by Wu *et al*. (2000) and interrelationship among 34 common European *Suillus* isolates representing 8 species was studied by Manian *et al*. (2001). Moreover, 19 different isolates representing 7 *Suillus* species were recorded from Mediterranean area of central Spain (Beatriz *et al*., 2006). In addition, genetic diversity of 27 *Suillus* strains isolated from Inner Mongolia has been documented (Feng *et al*., 2008). A new *Suillus* species, *S. quiescens* T.D. Bruns & Vellinga, was described from California and Oregon by Bruns *et al*. (2010). Min *et al*. (2014) re-evaluated the *Suillus* diversity from South Korea and recorded 8 distinct *Suillus* species from this country. Sarwar and Khalid (2014) have also reported 12 *Suillus* species from coniferous forests of Pakistan. Recently, a world-wide key to the genus *Suillus* was given by Klofac (2013).

Suillus species are important ectomycorrhizal symbionts of coniferous trees in temperate, boreal and Mediterranean ecosystems (Singer, 1986; Kretzer *et al*., 1996; Wu *et al*., 2000). They exhibit a high degree of host specificity toward conifers. They are common root symbionts of the family *Pinaceae* (Smith and Thiers, 1964; Kretzer *et al*., 1996; Wu *et al*., 2000) and coexist with the natural occurrence of *Pinaceae*. In India, the northwestern Himalayan region is a natural abode of large number of conifer plants, which mainly includes *Pinus roxburghii*, *Pinus wallichiana*, *Pinus girardiana*, *Cedrus deodara*, *Abies pindrow* and *Picea smithiana*. Very little is known about *Suillus* diversity from this region. Although, two species of this particular genus (*S. sibiricus* (Singer) Singer and *S. granulatus* (L.) Roussel) have been reported from the northwestern Himalayan region of India (Sagar and Lakhanpal, 2005; Dar *et al*., 2010) but both these species have not been described taxonomically as well as molecularly. Recently, we have reported three new *Suillus* species namely, ***S. triacicularis*** B. Verma & M.S. Reddy**, *S. himalayensis*** B. Verma & M.S. Reddy and ***S. indicus*** B. Verma & M.S. Reddy from this region (Verma and Reddy, 2014a; b; 2015). In this paper, we report seven *Suillus* species collected during monsoon season (2009-2013) in the states of northwestern Himalayan region of India. Among these two are new records (*S. flavidus* (Fr.) Singer and *S. placidus* (Bonord.) Singer) and two are re-reports (*S. sibiricus* and *S. granulatus*) from this region, which are described taxonomically as well as molecularly for the first time.

MATERIAL AND METHODS

Collection area and sampling

In the present study, different conifer forests of the northwestern Himalayan terrain **(Fig. 1)** of India were surveyed in order to explore *Suillus* diversity of this region. The region is situated between 28°43'-37°05'N latitude and 72°31'-81°03'E longitude covering a geographical area of 3,

Fig. 1 Map of the northwestern Himalayan terrain of India, showing different districts of three states (Jammu and Kashmir, Himachal Pradesh and Uttarakhand) from where *Suillus* specimens were collected.

31, 392 sq km, which constitute about 10% of the country's total geographical area. The area mainly comprises of three states namely, Jammu and Kashmir (J.&K.), Himachal Pradesh (H.P.) and Uttarakhand (U.K.). For the collection of *Suillus* basidiocarps, frequent excursions were undertaken to various localities of the northwestern Himalayas during monsoon season (July-September) from 2009-2013. Although, attempts were made to explore or cover the maximum area of the region but main districts from where *Suillus* specimens were found include, Anantnag, Ganderbal, Kulgam and Udhampur in J.&K.; Chamba, Kangra, Kinnaur, Kullu, Mandi, Shimla and Sirmaur in H.P. and Chamoli, Dehradun, Nainital, Tehri Garhwal and Uttarkashi in U.K. **(Fig. 1)**. Standard methodology employed for the collection and preservation of basidiocarps followed is after Atri *et al*. (2005). Usually, data regarding the habit, habitat and ecological parameters of the specimens, such as altitude, soil type, forest type and probable association were recorded on the field key (Atri *et al.*, 2005).

Morphological observations and molecular phylogenetic analysis

The basidiocarps of collected specimens were examined in accordance to the traditional taxonomic methodology given by Corner (1972). Color codes were taken from Kornerup and Wanscher (1978). To study anatomical features, microscopic mounts of dried specimens were revived either in water or in 3% KOH (w/v) and observed at 1000× magnification under an Olympus light microscope (Olympus, Japan) with the aid of a mirror type camera lucida. The spore measurements exclude the length of apiculus and the basidium length excludes the length of sterigmata. Quotient value (Q=L/W) was calculated considering the mean value of length and width of 20 basidiospores. Voucher specimens have been deposited in the Herbarium of the Botany Department (PUN), Punjabi University, Patiala, India.

Genomic DNA was extracted from dried basidiocarps as per the protocol described by Zhou *et al*. (1999). The internal transcribed spacer (ITS) region of nrRNA was amplified and sequenced as described in a previous publication (Verma and Reddy, 2015). All the sequences obtained in the present study have been deposited in GenBank. A dataset was assembled for the phylogenetic analysis by selecting published ITS sequences of *Suillus* species from GenBank based on the nucleotide BLAST search algorithm and the outcomes of recent phylogenetic study on *Suillus* (Bruns *et al.*, 2010). The ITS dataset consisted of 77 *Suillus* taxa (21 from the present study) representing homologous ITS sequences of 28 related *Suillus* species. *Rhizopogon subcaerulescens* (M91613) was included as an outgroup taxon for rooting purpose. The alignment processing was done using MAFFT ver. 7.0 (Katoh and Standley, 2013) and edited with BioEdit 5.0.6 (Hall, 1999). The alignment resulted in a data matrix comprising 751 characters including gaps. Phylogenetic analysis was conducted with MrBayes v.3.2.2 (Ronquist *et al.*, 2012) with two parallel runs each consisting of four incrementally heated Monte Carlo Markov Chains. The analysis was run using Metropolis-coupled Markov Chains Monte Carlo search algorithm over 1,000,000 generations and the convergence of Bayesian analysis was observed by examination of the standard deviation of split frequencies <0.01. The sample frequency was set to 100, resulting in an overall sampling of 10,000 trees and the first 2,500 trees were discarded as "burn-in" (0.25). For the remaining 7500 trees, a majority rule consensus tree was computed to obtain estimates for Bayesian posterior probabilities (BPP).

RESULTS

Taxonomy

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

Figs. 2(A-E) & 3(A-E)

Macrocharacters: Basidiocarps 4.0-9.0 cm in height. Pileus 3.5-10.0 cm broad, convex, expanding to plane with slight umbo in few specimens, flattened with age; margin regular and incurved in young specimens and upturned with age; appendiculate with white (1A1) cottony pileal veil, collapsing with age and finally evanescent; pileus surface viscid to glutinous, white (1A1), yellowish white to light yellow (1A2-1A5), brownish yellow (5C7-5C8) or dingy olive yellow in age, cuticle fully peeling, umbo absent; scales either absent or if present then streaked to appressed fibrillose, ranging from reddish brown through light brown to dark brown in color, distributed throughout the pileal surface or towards periphery. Pileus context pale yellow (1A3/2A3/3A3), cinnamon brown on bruising, mostly up to 12 mm thick (15 mm in few specimens); odor not distinctive, taste mild. Tubes 5-10(15) mm deep, decurrent, radial, crowded, fully peeling, pale yellow to yellow (2A3/3A32A7/3A7) with age, staining cinnamon brown on bruising; pore mouths angular, large, 0.5-2.0 mm broad. Spore deposit olive brown to light brown (6D6-6D8). Stipe central, 3.0-8.0 cm long, 6-12 mm thick

Fig. 2 Basidiocarps of *Suillus* species: **A-E.** *S. Sibiricus*; **F-I.** *S. granulatus*; **J-L.** *Suillus flavidus*; **M-O.** *S. placidus*. Scale bars: AO=2 cm

(rarely 15 mm), tubular and equal in diameter, covered throughout with glandular dots darkening in mature specimens, concolorous with tubes; base whitish (1A1), soon becoming vinaceous to reddish, staining dull brown when handled; prominent annulus present in few specimens, mostly veil remnants attached to the stipe; mycelium at base white. Stipe context solid, pale yellow (1A3/2A3/3A3), changing cinnamon brown when bruised.

Microcharacters: Spores 7.2-10.8 × 3.0-4.6 µm, oblong to narrowly cylindric in shape (Q= 2.3), yellowish in KOH, dark yellowish in Melzer's reagent, smooth. Basidia 4 spored, 18.5-28.6 × 4.3-7.2 µm, clavate, hyaline in KOH, yellowish in Melzer's reagent; sterigmata 2.3-3.8 µm high.. Pleurocystidia in bundles, individual cystidia 30.0-70.0 × 4.0-9.0 µm, cylindric to narrowly clavate with blunt rounded ends, often crooked and contains brownish contents inside. Cheilocystidia similar to pleurocystidia. Caulocystidia 35.0-80.0(95.0) × 4.0-8.0 µm, with brown coagulated contents inside, rest similar to pleurocystidia. Tube trama gelatinous, made up of divergent hyaline hyphae. Clamp connections absent.

Chemical color reactions: Pileal flesh: 2.5% KOH-reddish, 10% $FeSO_4$-yellowish green, 14% ammonia- reddish brown then to black, conc. HNO_3- no color reaction. Pileus cuticle: 2.5% KOH-light brown then to blue black, 10% $FeSO_4$-blackish brown, 14% ammonia- brown then to black brown, conc. HNO_3- no color reaction.

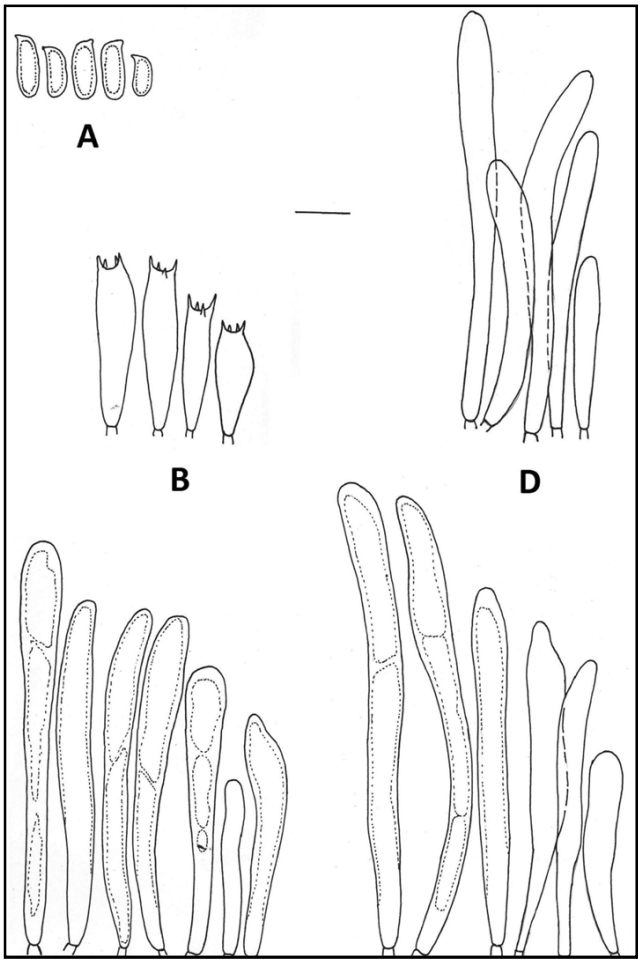

Fig. 3 Microcharacters of *Suillus sibiricus*: **A.** Basidiospores; **B.** Basidia; **C.** Pleurocystidia; **D.** Cheilocystidia; **E.** Caulocystidia. Scale bar: A-E=10 µm

Habitat and distribution: Solitary to scattered and gregarious under coniferous trees of *Pinus wallichiana, Cedrus deodara* and *Picea smithiana*. Associated mainly with *Pinus wallichiana*, very few records (PUN 6587 and PUN 6572) found with *Cedrus deodara* and only one specimen (PUN 6569) found with *Picea smithiana*. The basidiocarps of *Suillus sibiricus* are distributed throughout the northwestern Himalayas ranging from J.&K. to U.K. and were noticed to be frequently occurring and most dominant in the northwestern Himalayas.

Specimens examined: INDIA. Jammu and Kashmir: Ganderbal, Mamar, 1916 m, 2nd August 2009, B. Verma (PUN 5520; GenBank Accession KM 882913); Kulgam, Aharbal, 2266 m, 5th August 2009, B. Verma (PUN 5522; GenBank Accession KM 882918); Anantnag, Pahalgam, 2740 m, 20th July 2010, B. Verma (PUN 5524). Himachal Pradesh: Shimla, Kharapathar, 2673 m, 25th July 2010, B. Verma (PUN 6575; GenBank Accession KM 882915); Shimla, Rohru, Shallan, 1900 m, 26th July 2011, B. Verma (PUN 6577; GenBank Accession KM 882914); Mandi, Karsog, Seri, 2065 m, 6th August 2011, B. Verma (PUN 6579; GenBank Accession KM 882919); Kullu, Banjar, Jibhi, 2300 m, 25th July 2012, B.

Verma (PUN 6581); Kullu, Banogi, 1340 m, 26th July 2012, B. Verma (PUN 6582); Chamba, Bharmour, 2133 m, 3rd August 2012, B. Verma (PUN 6583); Sirmaur, Shillai, 1900 m, 28th July 2013, B. Verma (PUN 6586); Sirmaur, Haripurdhar, 2500 m, 31st July 2013, B. Verma (PUN 6587); Sirmaur, Tarna, 1969 m, 2nd August 2013, B. Verma (PUN 6588). Uttarakhand: Chamoli, Joshimath, 1890 m, 4th August 2010, B. Verma (PUN 5529); Nainital, Mukteshwar, 2290 m, 21st August 2011, B. Verma (PUN 5532; GenBank Accession KM 882917); Uttarkashi, Janki Chatti, 2650 m, 11th August 2012, B. Verma (PUN 6569; GenBank Accession KM 882916); Uttarkashi, Harsil, 2620 m, 13th August 2012, B. Verma (PUN 6570); Tehri Garhwal, Chandrabadni, 2277 m, 18th August 2013, B. Verma (PUN 6572).

Remarks: The specimens collected from the northwestern Himalayas closely resemble to *S. sibiricus* (Singer) Singer (Smith and Thiers, 1964). Morphologically, the basidiocarps are very similar to *S. himalayensis* B. Verma & M.S. Reddy and *S. flavidus* (Fr.) Singer. It differs from that of *S. himalayensis* by the pileus rather dingy olive yellow, flesh and tubes changing to dull cinnamon on bruising, tubes 5-15 mm, slight umbo present occasionally, and the basidiospores being less cylindrical (Q=2.3). The second closest species is *S. flavidus*, but can be distinguished by the pileal surface that is floccose to squamulose in *S. sibiricus* and glabrous in *S. flavidus*. Smith and Thiers (1964) observed it growing gregarious under *Pinus monticola*. The populations from northwestern Himalayas have been recorded mainly from *P. wallichiana* forests. Occasional specimens have been found in association with *Cedrus deodara* and *Picea smithiana*.

2. *Suillus granulatus* (L.) Roussel, *Flore du Calvados et terrains adjacents, composée suivant la méthode de Jussieu*: **34** (1806).

Figs. 2(F-I) & 4(A-E)

Macrocharacters: Basidiocarps 3.0-10.0 cm in height. Pileus 1.5-9.5 cm broad, convex becoming broadly convex with age; margin regular to irregular; pileal veil absent; pileus surface dry to moist, viscid only when wet, yellowish brown (5D8) to light brown (6D4-6D5) to cinnamon brown (6D6), glabrous or streaked or spotted with brownish ground color, young specimens pallid or grayish orange (5B3-5B4), cuticle fully peeling, umbo and scales absent. Pileus context yellowish white (1A2) to pale yellow (1A3), not staining when bruised, up to 25 mm thick, watery greenish immediately above the tubes; odor not distinctive, taste mild. Tubes 2-7 mm deep, adnate to sub-decurrent, close, fully peeling, yellowish white (1A2) to pale yellow (1A3) when young, soon yellow (2A6-2A7) becoming olive yellow (2C8-2D8) with age, unchanging on bruising, spotted light brown to brown with age; pore mouths angular, small, 1-3 per mm, often with cloudy droplets when young. Spore deposit light brown (dull cinnamon, 6D8). Stipe central, 2.5-7.0 cm long, 4-12 mm thick (up to 15 mm at top), tubular and equal in diameter, tapering at base in few specimens, covered with pinkish tan to vinaceous brown glandular dots or smears throughout, color white (1A1) throughout in young specimens, becoming pale yellow (1A3) at top with maturity;

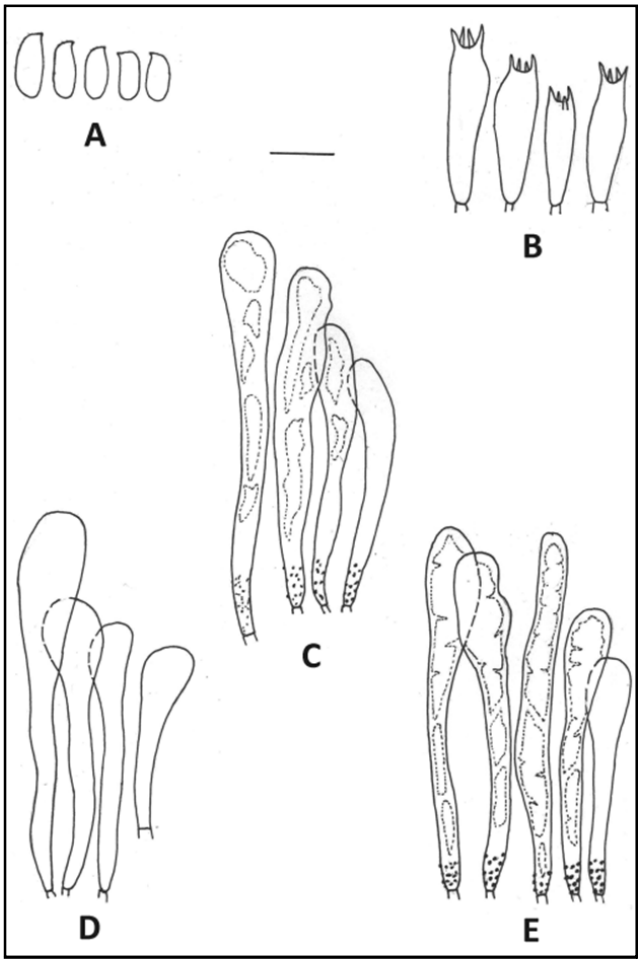

Fig. 4 Microcharacters of *Suillus granulatus*: **A.** Basidiospores; **B.** Basidia; **C.** Pleurocystidia; **D.** Cheilocystidia; **E.** Caulocystidia. Scale bar: A-E=10 µm

annulus absent; mycelium at the base white. Stipe context soft, unchanging, white at first and then becoming pale yellow (1A3) with age.

Microcharacters: Spores 7.0-9.5 × 2.3-3.8 µm (Q=2.8), cylindrical in shape, yellowish in KOH and Melzer's reagent, smooth. Basidia 16.0-24.0(30) × 4.6-6.2 µm, sub-cylindrical to clavate, 4 spored, hyaline in KOH, yellowish in Melzer's reagent; sterigmata 2.3-3.1 µm high. Pleurocystidia 38.0-62.0 × 5.0-8.0 µm, clavate, in fascicles incrusted with brown particles at the base, hyaline with brown coagulated contents in KOH, pale yellowish with brownish contents in Melzer' reagent. Cheilocystidia 28.0-58.0 × 6.0-9.2 µm, mostly in fascicles, clavate, hyaline in KOH, pale yellowish in Melzer's reagent, uniformly distributed brown content inside. Caulocystidia 37.0-56.0 × 6.0-9.0 µm, clavate or cylindrical, fasciculate, content hyaline with brown coagulated material, base incrusted with brown particles. Trama gelatinous and divergent. Clamp connections absent.

Chemical color reactions: Pileal flesh: 2.5% KOH- reddish, 10% $FeSO_4$- grayish green, 14% ammonia- reddish then to reddish brown, conc. HNO_3- no color reaction. Pileus cuticle:

2.5% KOH- light brown then to blue black, 10% $FeSO_4$- olive gray, 14% ammonia- brown then to black brown, conc. HNO_3- no color reaction.

Habitat and distribution: Solitary, scattered to gregarious on humicolous soil associated mostly with *Pinus wallichiana* trees, but a few specimens (PUN 5536 and PUN 6585) were also recorded with *Cedrus deodara* trees. *Suillus granulatus* is also distributed throughout the northwestern Himalayas ranging from J.&K. to U.K. and represents the second most abundant *Suillus* species in the northwestern Himalayas after *S. sibiricus*. Its distribution ranges from 1500 m to 3000 m.

Specimens examined: INDIA. Jammu and Kashmir: Kulgam, Aharbal, 2266 m, 5th August 2009, B. Verma (PUN 5521); Anantnag, Kukernag, 2000 m, 21st July 2010, B. Verma (PUN 5525; GenBank Accession KM 882912). Himachal Pradesh: Shimla, Kumarsain, Oddi, 1850 m, 26th July 2011, B. Verma (PUN 5533; GenBank Accession KM 882909); Kullu, Anni, Dagsari, 2350 m, 25th July 2012, B. Verma (PUN 5536); Chamba, Tissa, 1870 m, 5th August 2012, B. Verma (PUN 5539); Kinnaur, Kalpa, 2960 m, 18th July 2013, B. Verma (PUN 6585; GenBank Accession KM 882911). Uttarakhand: Uttarkashi, Barkot, 1524 m, 29th July 2010, B. Verma (PUN 5526); Chamoli, Naagnath, 1615 m, 2nd August 2010, B. Verma (PUN 5528); Chamoli, Gwaldam, 1708 m, 6th August 2010, B. Verma (PUN 5530; GenBank Accession KM 882910); Tehri Garhwal, Dhanaulti, 2286 m, 22nd August 2013, B. Verma (PUN 6573).

Remarks: *S. granulatus* basidiocarps collected from the northwestern Himalayas resemble well with those described by Smith and Thiers (1964) and Corner (1972). However, they differ by having few larger basidia up to 30 µm in length. Smith and Thiers (1964) found it from *Pinus strobus* forest and Corner (1972) recorded it from mixed conifer forest growing under white pines. The present specimens are collected mostly from pure *P. wallichiana* forests and rarely from *Cedrus deodara* forests. Yellowish brown to light brown and cinnamon brown pileus and unchanging pileal context distinguish it from *Suillus placidus* (Bonord.) Singer, which has white to pale yellow pileus and vinaceous staining pileal context.

3. *Suillus flavidus* (Fr.) Singer, *Farlowia* **2**: 260 (1945).

Figs. 2(J-L) & 5(A-E)

Macrocharacters: Basidiocarps 5.0-12.0 cm in height. Pileus 3.0-8.0 cm broad, convex, expanding to plane with slight umbo in few specimens at maturity, flattened with age; margin regular and slightly upturned with age; appendiculate with white (1A1) cottony pileal veil; pileus surface viscid to glutinous, glabrous with patches of gluten when dried, yellow (2A4-2A6) to yellowish brown (5C7-5C8) in age, cuticle fully peeling. Pileus context pale yellow (2A3), soft, browning when cut, up to 10 mm thick; odor not distinctive, taste mild. Tubes 3-9 mm deep, adnate to sub-decurrent, radial, crowded, fully peeling, yellow (3A6-3A7), slowly staining cinnamon brown to pinkish brown on bruising; pore mouths angular to irregular, large, 1-2 mm broad. Spore print light brown (6D8). Stipe central, solid, 3-7 cm long, 5-12 mm thick, tubular and equal in diameter, color pale yellow (2A3) to yellow (2A5), soon becoming pinkish red or reddish with age, covered with pallid to pale yellowish glandular dots, darkening when handled or with age; whitish (1A1) thick band of annulus present, staining brownish on handling; mycelium at the base white. Stipe context solid, pale yellow (2A3), and changing cinnamon brown when bruised.

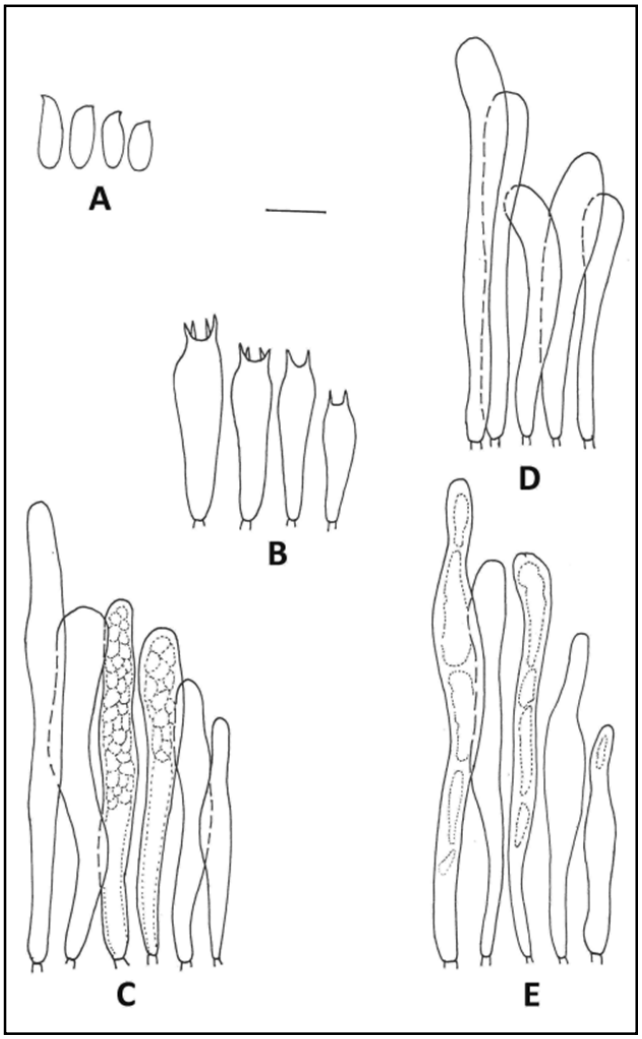

Fig. 5 Microcharacters of *Suillus flavidus*: **A.** Basidiospores; **B.** Basidia; **C.** Pleurocystidia; **D.** Cheilocystidia; **E.** Caulocystidia. Scale bar: A-E=10 µm

Microcharacters: Spores cylindrical in shape, smooth, 7.5-10.8 × 3.0-3.6 µm (Q=2.7) in size, yellowish in KOH and yellowish brown in Melzer's reagent. Basidia 18.0-28.0 × 5.0-8.0 µm, clavate, hyaline in KOH, yellowish brown in Melzer's reagent, 1-4 spored; sterigmata 1.5-3.1 µm high. Pleurocystidia 38.0-70.0 × 5.0-8.0 µm, scattered to fasciculate with brown incrusting particles around the base, clavate to cylindrical, content hyaline to yellowish brown. Cheilocystidia 38.0-62.0 × 6.0-8.0 µm, abundant with uniform brown content, shape similar to pleurocystidia. Caulocystidia 35.0-75.0 × 6.0-8.0 µm, in fascicles, surrounded by brown incrusting particles at the base of cystidia. Tube trama gelatinous and divergent. Clamp connections absent.

Chemical color reactions: Pileal flesh: 2.5% KOH- reddish brown, 10% $FeSO_4$- yellowish green, 14% ammonia- reddish brown, conc. HNO_3- no color reaction. Pileus cuticle: 2.5% KOH- light brown then to blue black, 10% $FeSO_4$- blackish brown, 14% ammonia- brown then to brownish black, conc. HNO_3- no color reaction.

Habitat and distribution: Found scattered in pure forests of *Pinus wallichiana* on humicolous soil. Only two specimens were collected one each from Baghi (latitude 31°14'41"N and longitude 77°32'51"E) sub locality of district Shimla and Chindi (latitude 31°22'16"N and longitude 77°10'28"E) sub locality of district Mandi, Himachal Pradesh, India.

Specimens examined: INDIA. Himachal Pradesh: Shimla, Baghi, 2685 m, 25th July 2010, B. Verma (PUN 6574; GenBank Accession KM 882920); Mandi, Karsog, Chindi, 1825 m, 6th August 2011, B. Verma (PUN 6580).

Remarks: The basidiocarps of *Suillus flavidus* recorded from the northwestern Himalayas resembles well with those described from Pakistan by Sarwar *et al*. (2012) in association with *Pinus wallichiana*. The species is characterized by its yellow to yellowish brown glabrous pileus with patches of gluten, pallid to pale yellowish glandular dots on the stipe and a whitish thick annulus staining brownish on handling. Morphologically the species is close to *Suillus sibiricus* (Singer) Singer, but can be differentiated by its glabrous pileal surface as compared to floccose or squamulose in *S. sibiricus*.

4. *Suillus placidus* (Bonord.) Singer, *Farlowia* **2**(1): 42 (1945).

Figs. 2(M-O) & 6(A-E)

Macrocharacters: Basidiocarps 4.0-12.0 cm in height. Pileus 3.0-8.5 cm broad, broadly convex becoming plane with age; margin regular, inrolled when young; pileal veil absent; pileus surface viscid to glutinous, glabrous, white (1A1) when young becoming pale yellow (1A2-1A3) in age, cuticle fully peeling, umbo and scales absent. Pileus context white (1A1) to yellowish white (1A2), pale yellow (1A3) adjacent to tubes, slowly turning vinaceous on bruising, up to 14 mm thick; odor not distinctive, taste mild. Tubes 4-10 mm deep, adnate to decurrent, radial, crowded, fully peeling, pale yellow (1A3) at maturity, unchanging on bruising; pore mouths angular, 0.5-2.0 mm broad, often with pinkish droplets of exudates and with pinkish glandular dots. Spore deposit dull cinnamon (6D6). Stipe central, 4-12 cm long, 3-10 mm thick, tubular and equal in diameter, covered with vinaceous brown glandular dots or smears throughout, color white (1A1), pale yellow (1A3) at top in mature specimens; veil or annulus absent during all stages of development; mycelium at the base white. Stipe context soft, white, becoming pale yellow with age, slowly turning vinaceous at the base when bruised.

Microcharacters: Spores 7.0-9.5 × 2.8-3.5 µm (Q=2.24) in size, oblong to narrowly cylindrical in shape, smooth, greenish hyaline in KOH, pale yellow in Melzer's reagent. Basidia 18.0-28.0 × 5.5-8.0 µm, clavate, 4 spored, hyaline in KOH and yellowish in Melzer's reagent; sterigmata 1.5-3.1 µm high. Pleurocystidia abundant, 30.0-70.0 × 5-9 µm, sub-cylindrical to clavate , scattered, but typically in fascicles, hyaline in KOH, pale yellowish in Melzer' reagent. Cheilocystidia 30.0-85.0 × 5.0-14.0 µm, in fascicles, clavate to cylindrical with inflated ends, brownish in KOH and light brown in Melzer's reagent. Caulocystidia 30.0-70.0 × 6.0-18.0 µm, found mostly in bundles with capitate ends, brown in KOH. Brown incrusting particles found at the base of each cystidia. Trama gelatinous and divergent. Clamp connections absent.

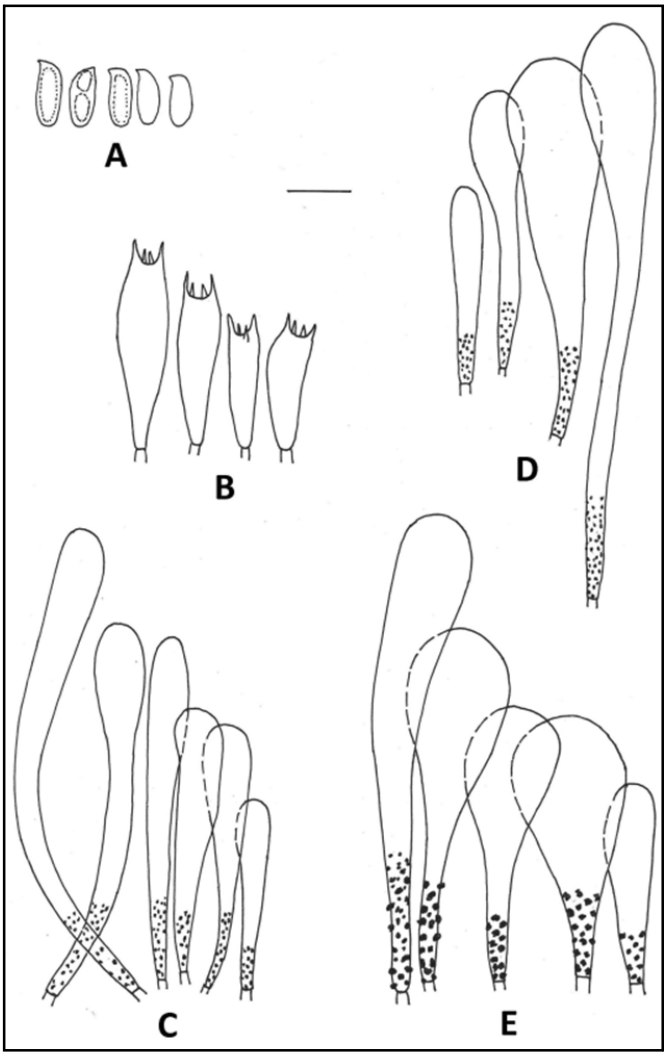

Fig. 6 Microcharacters of *Suillus placidus*: **A.** Basidiospores; **B.** Basidia; **C.** Pleurocystidia; **D.** Cheilocystidia; **E.** Caulocystidia. Scale bar: AE=10 µm

Chemical color reactions: Pileal flesh: 2.5% KOH- pink then to brownish lavender, 10% $FeSO_4$- gray or olive brown, 14% ammonia- pink becoming blue gray, conc. HNO_3- no color reaction. Pileus cuticle: 2.5% KOH- pink becoming brownish black, 10% $FeSO_4$- brown to blackish brown,14% ammonia- pink becoming red or finally black, conc. HNO_3- no color reaction.

Habitat and distribution: Solitary to scattered or gregarious on humicolous soil under *Pinus wallichiana* trees. Only two specimens were collected one each from Theog (latitude

31°7'12"N and longitude 77°21'0"E), a sub locality of district Shimla, H.P. and Tehri (latitude 30°22'48"N and longitude 78°28'48"E), a sub locality of district Tehri Garhwal, U.K., India.

Specimens examined: INDIA. Himachal Pradesh: Shimla, Theog, 1965 m, 10th July 2013, B. Verma (PUN 6584; GenBank Accession KM 882921). Uttarakhand: Tehri Garhwal, Tehri, 1750 m, 16th August 2012, B. Verma (PUN 6571).

Remarks: The specimens collected from the northwestern Himalayas closely resemble to those described by Smith and Thiers (1964) and Corner (1972). They found it growing gregarious under *P. strobus* trees whereas, the present specimens were collected in association with *P. wallichiana* trees. It is characterized by pure white pileus when young, becoming pale yellow with age and with vinaceous brown glandular dots or smears throughout the stipe surface. It is close to *Suillus granulatus* (L.) Roussel, but can be distinguished by white to pale yellow pileus and vinaceous staining of pileal context on bruising. On the other hand, *S. granulatus* have yellowish brown to light brown and cinnamon brown pileus and unchanging pileal context.

5. *Suillus triacicularis* B. Verma & M.S. Reddy, *Phytotaxa* **162**(3): 160 (2014).

Specimens examined: INDIA. Jammu and Kashmir: Udhampur, Kud, 1855 m, 18th July 2010, B. Verma (PUN 5523; GenBank Accession KM 882908). Himachal Pradesh: Mandi, Karsog, 1404 m, 5th August 2011, B. Verma (PUN 5534, Holotype; GenBank Accession KF 977188); Kangra, Palampur, 1472 m, 29th July 2012, B. Verma (PUN 5538; GenBank Accession KF 977189). Uttarakhand: Dehradun, Mussoorie, 1825 m, 30th July 2010, B. Verma (PUN 5527; GenBank Accession KM 882907); Nainital, 2084 m, 20th August 2011, B. Verma (PUN 5531).

Habitat and distribution: *Suillus triacicularis* is found fruiting solitary, scattered or gregarious under *Pinus roxburghii* trees during the monsoon season. Its distribution ranges with the natural distribution of *P. roxburghii* in the northwestern Himalayas. The occurrence of *Suillus triacicularis* is rare and the species is seldom found in pure stands of *P. roxburghii* in this geographical region. No records were found from other pine forests during our 5 years of excursions to these forests from 2009 to 2013.

Remarks: Morphologically, *S. triacicularis* resembles *S. granulatus* (L.) Roussel and *S. collinitus* (Fr.) Kuntze. The absence of watery green context above the tubes during any stage of development distinguishes it from *S. granulatus*. The species looks very similar to pallid variants of *S. granulatus* when young, but differs by the absence of cloudy droplets over the tubes. Moreover, the yellow to reddish or orange-yellow pileus at maturity separates it from *S. granulatus*, which has a cinnamon brown pileus at maturity. *S. collinitus*, which is very similar to *S. granulatus*, can be distinguished by its pinkish mycelia at the stipe base as compared to pure white mycelia in *S. granulatus* and *S. triacicularis*.

6. *Suillus himalayensis* B. Verma & M. S. Reddy, *Nova Hedwigia* **99**: 543 (2014).

Specimens examined: INDIA. Himachal Pradesh: Mandi, Barot, Lachkandi, 1829 m, 7th August 2011, B. Verma (PUN 5535, Holotype; GenBank Accession KF 699850); Mandi, Barot, Tikkan, 1829 m, 28th July 2012, B. Verma (PUN 5537; GenBank Accession KJ 472765).

Habitat and distribution: The species was found solitary to scattered on humicolous soil under young to moderate aged *Pinus wallichiana* trees along with the population of *Suillus sibiricus* throughout the Barot valley in the district Mandi of H.P., India. The vegetation of the Barot valley is dominated by *Cedrus deodara* and *Quercus* spp. with some randomly distributed stands of mixed and pure *Pinus wallichiana* patches along the bank of the river Uhl.

Remarks: Morphologically, the species come close to *Suillus americanus* (Peck) Snell and *S. sibiricus* (Singer) Singer. Till date, there is no record for *S. americanus* from India and *S. sibiricus* is the abundantly fruiting and most dominating *Suillus* species associated with *Pinus wallichiana* in the northwestern Himalayas. *Suillus americanus* is the nearest relative of *S. himalayensis*. The species varies in possessing the yellow white to pale yellow pileus with light greenish tinge, brownish squamules, white pileal veil, upturned margin and plane apex as compared to the bright yellow pileus surface, orange to reddish squamules, yellowish pileal veil, incurved margin and occasional presence of low obtuse umbo in *S. americanus.* Also, the species differs as its flesh changes to grayish green and tubes to olive brown color on bruising in contrast to vinaceous brown in *S. americanus*. Microscopically, the less broaden cystidial elements (3.8-6.2 μm) than *S. americanus* (7-12 μm) is the main distinguishing characteristic of the present specimens. Basidia are larger and basidiospores more cylindrical (Q=3.0) than *S. americanus* (Q=2.5). The second nearest species is *S. sibiricus*, but it differs from that of *S. himalayensis* by the pileus rather dingy olive yellow, flesh and tubes changing to dull cinnamon on bruising, tubes 5-15 mm, slight umbo present occasionally, and the basidiospores being less cylindrical (Q=2.3).

7. *Suillus indicus* B. Verma & M.S. Reddy, *Mycology* **6**(1): 37 (2015).

Specimens examined: INDIA. Himachal Pradesh: Shimla, Narkanda, 2621 m, 27th July 2010, B. Verma (PUN 6576, Holotype; GenBank Accession KJ 675502); Shimla, Kandyali, 2450 m, 29th July 2011, B. Verma (PUN 6578; GenBank Accession KJ 675500).

Habitat and distribution: Solitary on humicolous soil under *Cedrus deodara* trees in mixed forest of *Pinus wallichiana* and *Cedrus deodara*. Only two specimens were collected from Narkanda (latitude 31°16'12"N and longitude 77°27'0"E) and Kandyali (latitude 31°13'29"N and longitude 77°26'4"E), sub localities of district Shimla, H.P., India. The vegetation of the collection sites is dominated by randomly distributed pure and mixed stands of *Pinus wallichiana* and *Cedrus deodara*.

Remarks: *Suillus indicus* is close to *S. decipiens* (Peck) Kuntze but differs considerably identifying it as a separate species. The presence of umbo, less numerous/prominent fibrillose squamules over the pileal surface and absence of squamules over the stipe differentiate it from *S. decipiens*. Anatomically, the occasional presence of 2-spored basidia and complete absence of caulocystidia distinguish the species from *S. decipiens*. *S. spraguei* (Berk. & Curt.) Kuntze is the next closest species, which is commonly referred to as *Suillus pictus* A.H. Sm. & Thiers although the name *S. spraguei* is used for one or even several disjunct species of *S. pictus* in Asia (Wu *et al.*, 2000; Burchhardt *et al.*, 2011; Klofac, 2013). When fresh, the pileus of *S. spraguei* fresh specimens is much redder than *S. decipiens* and *S. indicus* but the faded specimens strongly resemble *S. decipiens*. In contrast to *S. indicus*, *S. spraguei* also bear fibrillose squamules on the stipe.

Key to the Indian species of *Suillus* investigated from the northwestern Himalayas

1. Glandular dots absent on the stipe surface ..... ***S. indicus***
1. *Glandular dots present on the stipe surface **2**
2. A well developed veil or false veil found in young specimens either leaving an annular zone or true annulus on the stipe, or otherwise adhered to the pileal margin at maturity ……**3**
2. Pileal veil and annulus absent during all stages of the development ……..**5**
3. Pileus yellow white to pale yellow with light greenish tinge; cystidial elements less broaden (3.8-6.2 µm)……***S. himalayensis***
3. *Pileus not as above, cystidial elements comparatively broader……. …**4**
4. Pileus yellow to yellowish brown, usually umbonate, glabrous; stipe with distinct annulus …… ***S. flavidus***
4. *Pileus white, yellowish white through light yellow to brownish yellow or dingy olive yellow in age, usually plane, rarely obtusely umbonate, mostly covered with appressed fibrillose scales; either prominent annulus present or otherwise veil remnants attached to the stipe…… ***S. sibiricus***
5. Associated exclusively with *Pinus roxburghii* …… … ***S. triacicularis***
5. *Associated with other conifers ……**6**
6. Pileus white when young, slowly becoming pale yellow with age; pileal context not watery greenish above the tubes; associated only with *Pinus wallichiana* …… ***S. placidus***
6. *Pileus pallid or grayish orange when young, becoming yellowish brown to light brown or cinnamon brown with age; pileal context watery greenish immediately above the tubes; associated either with *Pinus wallichiana* or with *Cedrus deodara* ……***S. granulatus***

Phylogenetic inference

A phylogenetic analysis was performed to determine the evolutionary relationships. The Bayesian analysis of selected *Suillus* species clustered these *Suillus* species mainly into two different groups namely, *Suillus* group I and *Suillus* group II (**Fig. 7**). The ITS sequences of *S. himalayensis*, *S. triacicularis*, and *S. indicus* obtained in the present study formed a well-supported monophyletic lineage when compared with ITS sequences of other related *Suillus* species. Bayesian consensus showed splits between *S. himalayensis* and a well-resolved clade containing *S. americanus*. Similarly, split between *S. triacicularis* and a well-resolved clade containing *S. collinitus* and *S. granulatus* was also observed. *Suillus indicus* also formed an independent clade in the group. Posterior probability percentage values support these species as separate clades in the group. As revealed morphologically, molecular analysis also identified these species as distinct *Suillus* species within the genus *Suillus*. The ITS sequences of *S. sibiricus*, *S. flavidus*, *S. placidus* and *S. granulatus* are grouped with their respective homologous sequences available in the NCBI database, which further confirm their identification based upon the molecular analysis.

Most of the *Suillus* species formed well-supported independent clades with a few exceptions. A few pairs of *Suillus* species (*S. cothurnatus* and *S. subluteus*, *S. pseudobrevipes* and *S. volcanalis*, *S. glandulosipes* and *S. neoalbidipes*, *S. brevipes* and *S. weaverae*) are not distinguishable by the ITS locus. This might be due to over-description (synonymy) or to the lack of ITS divergence among sibling species. Thus, there are limitations of using ITS locus for species-level determinations in the genus *Suillus*. On the contrary, the ITS sequences of *S. granulatus* derived from Europe (L54076, AY898617, DQ068968, AJ272409, AJ272408 & AJ272410) and Asia (L54121, KM882909, KM882910, KM882911 & KM882912) are polyphyletic and sub-divided into two different subclades **(Fig. 7)**, probably suggesting that a cryptic speciation process has taken place between these isolates. Heterogeneity among *S. granulatus* isolates on the basis of molecular data has been also observed by Kretzer *et al*. (1996), Manian *et al*. (2001), and Bruns *et al*. (2010) and it seems that isolates reported as *S. granulatus* from Europe, Asia, and North America might represent at least two different taxa. Similarly, the ITS sequences of *Suillus spraguei* derived from American (M91617 and AF166524) and Chinese (AF166518 and AF166520) collections are also paraphyletic and sub-divided into two different subclades. Chinese *S. spraguei* isolates were found to be sister to American *S. decipiens* isolates (L54079 and AF166508) rather than American *S. spraguei* isolates. On the basis of ITS data, Wu *et al*. (2000) also observed similar heterogeneity among the Chinese and American *S. spraguei* isolates.

Occurrence and ecology

Various localities of the northwestern Himalayan region of India were explored to collect the basidiocarps of *Suillus* species. In total 40 specimens were collected from the northwestern

Fig. 7 Phylogenetic tree inferred from the bayesian analysis of the ITS region of *Suillus* species with *Rhizopogon subcaerulescens* as an out-group taxon. Bayesian posterior probability percentages (above 50%) are shown at the nodes of the tree. The scale represents the substitution rate. Bold indicates sequences derived in the present study.

Himalayas, out of which 6 were found from J.&K., 22 from H.P. and 12 from U.K. It was found that their occurrence prevails from 1300 m [mid hill sub-montane zone (1000-2000 m)] to 2800 m [high hill wet temperate zone (2000-3000 m)]. Generally, they start appearing after first showers of monsoon in the first half of July and flourish well during July-August and even up to first half of September, depending upon the duration of monsoon season. During our collection trips to the northwestern Himalayas, *Suillus* species were found mostly in mid monsoon season (15th July-15th August).

The present study identified seven different *Suillus* species from the northwestern Himalayan region of India, based on morpho-anatomical descriptions and molecular analysis of the collected basidiocarps. Ecological data regarding abundance and distribution suggested that *S. sibiricus* is the most frequently encountered and widely distributed *Suillus* species followed by *S. granulatus* and *S. triacicularis*. *Suillus sibiricus*, *S. granulatus* and *S. triacicularis* were found distributed throughout the northwestern Himalayan region of India ranging from J.&K. to U.K., whereas only two specimens were recorded each for *S. himalayensis*, *S. indicus*, *S. flavidus* and *S. placidus*. *Suillus himalayensis* and *S. indicus* were observed only from the Barot Valley of Mandi district and Narkanda range of Shimla district of H.P., respectively, although probability of their occurrence from other localities can't be denied. *Suillus flavidus* was found only in H.P. as compared to *S. placidus*, which was collected both from H.P. and U.K. The field experience further suggested that *Suillus* species are mainly associated with the members of family *Pinaceae*. Most of them were found associated with *Pinus wallichiana*, but some also with *P. roxburghii*, *Cedrus deodara* and *Picea smithiana* trees. *Suillus sibiricus* and *S. granulatus* were found mainly with *Pinus wallichiana* and rarely with *Cedrus deodara*. In addition, one specimen of *Suillus sibiricus* (PUN 6569) was also found with *Picea smithiana*. *Suillus himalayensis*, *S. flavidus* and *S. placidus* were found only in pure *Pinus wallichiana* forest, whereas *S. indicus* specimens were found from the mixed forests of *Cedrus deodara* and *Pinus wallichiana*. *Suillus triacicularis* was found fruiting exclusively in association with *Pinus roxburghii* trees.

DISCUSSION

Several morphological features are useful to distinguish the species of genus *Suillus*. These include presence or absence of

veil, annulus and glandular dots on the basidiocarps, pileal color, pileal surface, pileal context and host association, as revealed by our own and other studies (Smith and Thiers, 1964; 1971; Corner, 1972; Thiers, 1976; 1979; Wu *et al.*, 2000; Klofac, 2013; Min *et al.*, 2014; Sarwar and Khalid, 2014). The present study demonstrates that seven species of *Suillus* are distributed in the northwestern Himalayan region of India and all these species are morphologically well delimited.

We have also summarized the ecological distribution of all the seven species collected from this region. *Suillus sibiricus* and *S. granulatus* are by far the two most common species in northwestern Himalayas. The former is widely distributed and frequently encountered. *Pinus wallichiana* was found most common host plant for a number of *Suillus* species (*S. sibiricus, S. granulatus, S. placidus, S. himalayensis* and *S. flavidus*), whereas only one *Suillus* species was found associated both with *Pinus roxburghii* (*Suillus triacicularis*) and *Picea smithiana* (*Suillus sibiricus*). *Cedrus deodara* forests seem to be colonized rarely by *Suillus* species and are represented only by very few specimens of *S. sibiricus, S. granulatus* and *S. indicus*. The specimens of *S. indicus* were collected from underneath deodar trees in the mixed forests of *P. wallichiana* and *C. deodara* suggesting its putative association with *C. deodara*, although its association with *P. wallichiana* cannot be denied. In fact, host shifts amongst basidiomycetous fungi are considered to be major driving forces in their evolutionary processes (Refrégier *et al.*, 2008; Li *et al.*, 2009; Rochet *et al.*, 2011). No *Suillus* specimens were found from *Abies pindrow* forests during our forest surveys from 2009 to 2013.

Bayesian analysis of ITS region of selected *Suillus* specimens revealed that *Suillus* species are clustered into two different groups, *viz. Suillus* group I and *Suillus* group II **(Fig. 7).** According to Smith and Thiers (1964) and Klofac (2013), these groups can be morphologically described on the basis of a few leading morphological characteristics of the *Suillus* species such as, presence or absence of veil and/or annulus on the basidiocarps. All the species in *Suillus* group I, except *S. placidus*, comprises mainly of the species possessing a well developed veil or false veil in young specimens either leaving an annular zone or true annulus on the stipe, or otherwise adhered to the pileal margin at maturity. Contrastingly, the *Suillus* group II comprises of the species in which either only annulus is absent or otherwise both annulus and veil are absent on the basidiocarps. Despite the fact that *S. placidus* is phylogenetically related to *Suillus* group I, morphologically it resembles *Suillus* group II due to absence of veil and annulus on basidiocarps during any stage of development (Singer, 1945; Smith and Thiers, 1964). Thus, the *Suillus* species under consideration are phylogenetically grouped on the basis of their leading morphological features with few exceptions.

In summary, the present work enhanced our knowledge about diversity, phylogeny, ecology and biogeography of *Suillus* species occurring in the northwestern Himalayan region of India. In addition, the ITS sequences of Indian *Suillus* species acquired in the present study have provided a phylogenetic framework for biogeographical analysis of the genus *Suillus*.

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