



## ***Scleroderma nastii* sp. nov., a gasteroid mushroom from Phulchoki hill, Nepal**

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### **Abstract**

A new species of *Scleroderma* is described from temperate Oak forest of Phulchoki hill, at the highest peak (2,762 m) ringing the Kathmandu valley, Nepal. Smooth to velvety, pseudostipitate basidiome, smaller and sub-reticulate basidiospores in comparison to its close relatives, are key diagnostic features of this newly discovered mushroom. The novelty of the taxon was also confirmed based on phylogenetic analysis using the data sets of the internal transcribed spacer region (ITS) of nuclear rDNA sequences along with the analysis of scanning electron micrographs of its basidiospores. A comprehensive description, illustrations, and comparisons with phenetically similar species are provided in this study. Additional surveys are needed to verify the species diversity and clarify their geographic distribution.

**Key words** – Ectomycorrhiza – Lalitpur – Oak forest – Phylogeny – Pseudostipe

### **Introduction**

The genus *Scleroderma* Pers. (Basidiomycota) was described by Persoon in 1801 (Rusevska et al. 2014). It shows a cosmopolitan distribution from temperate to tropical regions forming ectomycorrhizal associations with a wide range of forest trees (Kumla et al. 2013). The Index Fungorum online database (<http://www.indexfungorum.org/Names/Names.asp>; accessed 07 April 2020) lists 184 *Scleroderma* species, subspecies, and varieties worldwide. Several species of *Scleroderma* have been proposed based on morphological characteristics of their basidiomes and basidiospores (Guzmán 1970, Sims et al. 1995, Guzmán et al. 2004, Kumla et al. 2013). Recent studies have also used molecular methods to analyze inter- and intraspecific variation in *Scleroderma* species (Phosri et al. 2009, Nouhra et al. 2012, Baseia et al. 2016). The characteristics which have been extensively used to separate the species within the genus are the thickness and the scaliness of the peridium and whether the basidiome is stalked or not, and if the former the structure of the stipe. These characteristics at a later date were combined with the size of the basidiospores and their ornamentation and in the latter case whether the ornamentation is composed of isolated spines or warts or forms a reticulum. The spines may be narrow or broadly based or the reticulum complete and very distinctive or incomplete (Phosri et al. 2009). Basidiospore size and pattern of ornamentation were subsequently used by Guzmán (1970), Sims et al. (1995), and Guzmán et al. (2013) to produce identification keys in global basis. Guzmán et al. (2013) divided

the genus into three sections based on the surface structure of the basidiospores and on the presence or absence of a clamp connection: (1) *Reticulatae*, characterized by reticulated spores, (2) *Scleroderma*, with echinulate spores, and (3) *Sclerangium*, presenting subreticulated spores. Molecular studies, based on comparison of internal transcribed spacer (ITS) nrDNA, confirm this classification (Zhang et al. 2013, Rusevska et al. 2014, Baseia et al. 2016, Crous et al. 2016).

Berkeley (1854, reprint 1969) was first to report six gasteroid fungi including one *Scleroderma* species (*S. nitidum* Berk.) from Nepal. Thereafter, altogether nine species, *S. areolatum* Ehrenb (= *S. lycoperdoides* Schwein), *S. bovista* Fr., *S. cepa* Pers. (= *S. flavidum* Ellis & Everh.), *S. citrinum* Pers. (= *S. vulgare* Horneum), *S. polyrhizum* (J.F. Gmel.) Pers. (= *S. geaster* Fr.), *S. sinnamariense* Mont., *S. texense* Berk. *S. nitidum* Berk, and *S. verrucosum* (Bull.) Pers., have been reported from different parts of the country up to now (Adhikari 2014). During our studies on sequestrate fungi in Kathmandu valley, a new species of *Scleroderma* was found in Phulchoki hill, the highest peak (2,762 m) ringing the valley. A complete description, illustration and phylogenetic analysis based on ITS nuclear rDNA along with the scanning electron micrographs analysis of basidiospores of the species are presented here.

## Materials and Methods

### Morphology

Basidiomata were collected from oak forest of Phulchoki hill, Nepal during the rainy season in July 2012. Fresh specimens were examined and photographed. All descriptions of macro- and microscopic features were obtained from fresh as well as dried materials. Microscopic observations and measurements were made on material mounted in 25% aqueous ammonia. Color terms and notations used in this description are based on Kornerup & Wanscher (1978). Herbarium abbreviations are according to Holmgren & Holmgren (1998). Basidiospore statistics:  $\bar{x}_m$ , the arithmetic mean of the spore length by spore width ( $\pm$  standard deviation); n, number of spores measured; Q, the quotient of spore length and width;  $Q_m$ , the mean of Q-values ( $\pm$  standard deviation). For scanning electronic microscopy (SEM) of spores, specimens were mounted directly on aluminum stubs with a carbon band and subsequently covered with a layer of gold with 15 nm in thickness, using a JEOL Smart Coater. Spores were examined in microscope model JEOL – JSM IT100.

### DNA extraction

Fruit body was cut into small pieces and manually grounded in 1.5 mL microcentrifuge tubes to obtain about 50 mg of dried mushroom. Thereafter 500  $\mu$ l of pre-warmed (at 60°C in water bath) TES lysis buffer (100 mM Tris pH 8.0; 10 mM EDTA pH 8.0; 2% SDS with 10  $\mu$ l proteinase K was added and incubated in 60°C for 1 hr. Then 140  $\mu$ l of 5 M NaCl and 64  $\mu$ l of 10% (w/v) of CTAB were added to the suspension and incubated at 65°C for 10 minutes. The supernatant was taken in new tubes and centrifuged at 14000 rpm for 10 minutes at 4°C. After centrifugation, equal volume (around 500  $\mu$ l) of chloroform:isoamyl alcohol (24:1) was added and again centrifuged at 14000 rpm for 10 minutes. After that 0.6 volume of cold isopropanol and 0.1 volume of 3 M sodium acetate pH 5.2 were added and maintained overnight at -20°C. Next day the mixture was centrifuged at 14000 rpm for 15 minutes at 4°C and pellet was obtained throwing the supernatant. The pellet was washed twice with 70% ethanol and air dried. Then, the obtained DNA pellet was suspended in 100  $\mu$ l of TE with 10 mg/ml RNase and stored at -20°C for further use. Concentration, yield, and quality control indices based on absorbance readings at 230, 260, and 280 nm (A260/280 and A260/230 ratios) were carried out. Genomic DNA was visualized in 1% agarose gel.

### PCR and Sequencing

A pair of primers ITS1 (5'-TCCGTAGGTGAACCTGTCGG-3') and ITS4 (5'-TCCTCCGCTTATTGTATGC-3') was used to amplify the ITS1-5.8S-ITS4 ribosomal DNA

region. PCR reaction was performed in 25  $\mu$ l reaction volume, each containing 25 ng of template DNA, 1.5 U Taq-DNA polymerase, 200  $\mu$ M each of dATP, dTTP, dGTP and dCTP, 1X PCR buffer, 2.5 mM MgCl<sub>2</sub> (Fermentas, Thermo Fisher Scientific) and 0.4  $\mu$ M of each primer. The reaction mixtures were incubated in Applied Biosystems Veriti 96 well thermal cycler. (Thermo Fisher Scientific, USA) programmed with an initial cycle of 3 min at 94°C, 1 min at 50°C and 2 min at 72°C, followed by 39 cycles of 30s at 94°C, 30s at 50°C, and 1.15 min at 72°C. The final extension cycle consisted of 5 min at 72°C and 2 min at 25°C. PCR products were separated via electrophoresis on a 1.5% agarose gel in 1 $\times$  TAE buffer. The bands were stained with ethidium bromide (45 min in 1 $\times$  TAE buffer) and faded in water for 15 min prior to visualization and documented using gel documentation system (Syngene Bioimaging, UK). PCR products were purified and sequenced by a dideoxy-mediated chain termination method (Sanger et al. 1977). The sequencing was done in ABI 3500XL genetic analyzer housed at Nepal Academy of Science and Technology, laboratory. Sequence was assembled and edited using the Codoncode Aligner version 4.1 software. The edited sequence was then used for BLAST search in the GenBank database ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)). The newly generated nucleotide sequence was deposited in GenBank /EMBL/DDBJ under the accession number KJ740390.

### Sequence Divergence and Phylogeny

Sequence divergence and phylogenetic analysis was performed to know the relationship of newly described taxon with other species of *Scleroderma*. Nine different species of *Scleroderma* representing wider geographic regions have been considered to compare with our taxon in the phylogenetic analysis. A total of 33 ITS sequences of varied *Scleroderma* species from different areas including out group were retrieved from GenBank (Table 1). *Pisolithus arhizus* was used as an out-group since it is close relative of *Scleroderma*. Our analysis comprises a set of 34 sequences including out group and our taxon. Sequence matrix of all 34 accessions were aligned using ClustalW in MEGA software version 6 (Tamura et al. 2013) and the resulting alignment was manually adjusted in necessary nucleotide positions. Sequence divergence between *Scleroderma* species was estimated using Kimura 2 Parameter (K2P) in MEGA. The alignment was deposited in TreeBASE (<http://www.treebase.org/>) under the accession URL and number <http://purl.org/phylo/treebase/phyloids/study/TB2:S25819?x-access-code=a01ded10319c27d179bf2b5e4db78f15&format=html>. Maximum parsimony (MP) analysis was performed with the alignment matrix in MEGA 6. All positions containing gaps and missing data were not considered for analyses. The MP tree was obtained using the Tree-Bisection-Regrafting (TBR) algorithm (Nei & Kumar 2000) with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates). The robustness of inferred MP tree topologies was tested by the bootstrap value (Felsenstein 1985) with 1000 replicates.

**Table 1** List of taxa included in the phylogenetic analyses with their herbarium number, country of origin and GenBank accession numbers of internal transcribed spacer (ITS) nuclear rDNA.

Taxon	Voucher Specimen No.	Origin	GenBank accession No.
<i>S. areolatum</i>	ARESCL2	USA	FM213352
	ARESCL3	USA	FM213353
	OSC122632	USA	FM213351
	OSC38819	USA	EU819438
	PGK193	USA	GQ166910
<i>S. bermudense</i>	BZ3961	Belize	EU718118
<i>S. bovista</i>	N.A.	Japan	AB099901
	N.A.	Japan	AB211267
	MCF 01/168	Macedonia	HF933234
	MCF 05/788	Macedonia	HF933235

**Table 1** Continued.

<b>Taxon</b>	<b>Voucher Specimen No.</b>	<b>Origin</b>	<b>GenBank accession No.</b>
<i>S. cepa</i>	MCF 09/11184	Serbia	HF933242
	SOC-541	USA	DQ453694
	HE2907		KC505548
<i>S. citrinum</i>	CEPSCL5	USA	FM213355
	SCL3	UK	FM213333
	SCL7	UK	FM213335
	E00278300	USA	FM213344
	K (M) 53906	UK	EU784414
<b><i>S. nastii</i></b>	<b>NAST-FB11</b>	<b>Nepal</b>	<b>KJ740390</b>
<i>S. nitidum</i>	UFRN-Fungos 2034	Brazil	KU759904
	UFRN-Fungos 2550	Brazil	KU759906
	UFRN-Fungos 2219	Brazil	KU759908
	UFRN-Fungos 2500	Brazil	KU759909
<i>S. polyrhizum</i>	POLSCL1	USA	FM213349
	POLSCL2	USA	FM213350
<i>S. sinnamariense</i>	SCLK4	Thailand	FM213356
	SCLP3	Thailand	FM213357
	SCLY5	Thailand	FM213359
	SC1	Thailand	FM213360
<i>S. verrucosum</i>	MCF 07/7984	Macedonia	HF933232
	MCF 08/10124	Macedonia	HF933233
	MCF 89/4709	Macedonia	HF933237
	MCF 06/7265	Macedonia	HF933241
Outgroup			
<i>Pisolithus arhizus</i>	REG-588	USA	GU187538

N.A.: not available; new species is described in black bold.

## Results

### *Scleroderma nastii* Raut, sp. nov.

Fig. 1

MycoBank number: 835277

Etymology – The species name refers to the acronym of the research organization in Nepal where it was described.

Macroscopic characteristics – Basidiome epigeous, depressed globose to subglobose, 10–13 mm across and 10–20 mm tall including mycelial base, smooth to velvety, slightly areolate, blackish-brown. Peridium 1 mm or < 1 mm thick, pseudostipitate often transversely cracked. Gleba compact, fleshy to dusty, blackish-brown (Fig. 1a).

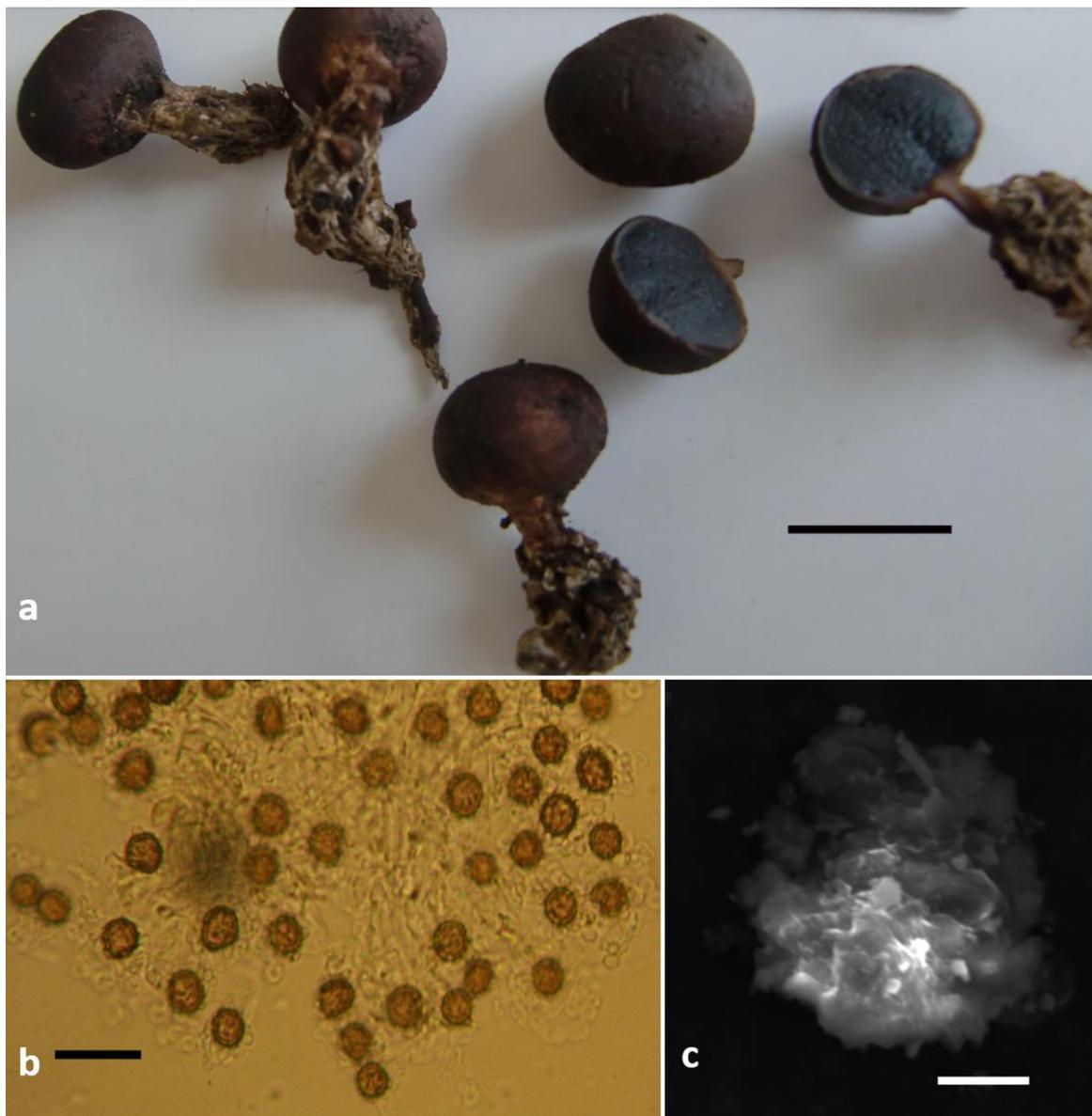
Microscopic characteristics – Basidiospores (7–) 8–9 (–10)  $\mu\text{m}$  without spines; average = 8.3, SD =  $\pm 0.7$ , n=25, globose, yellowish brown, subreticulated under LM (Fig. 1b), irregular reticulum under SEM (Fig. 1c)). Basidia not observed. Clamp connections absent

Habit and Habitat – Solitary to caespitose on soil under leaves and litter of *Quercus* trees in north facing temperate forest.

Known distribution – Only known from the type locality in Nepal.

Material examined – Nepal, Lalitpur, Oak forest in Phulchoki hill, on soil, alt. 2630 m, N27°34.205" E085°24.077", 13 July 2012, J. K. Raut, NAST-FB11 (KATH-FB11, Holotype)

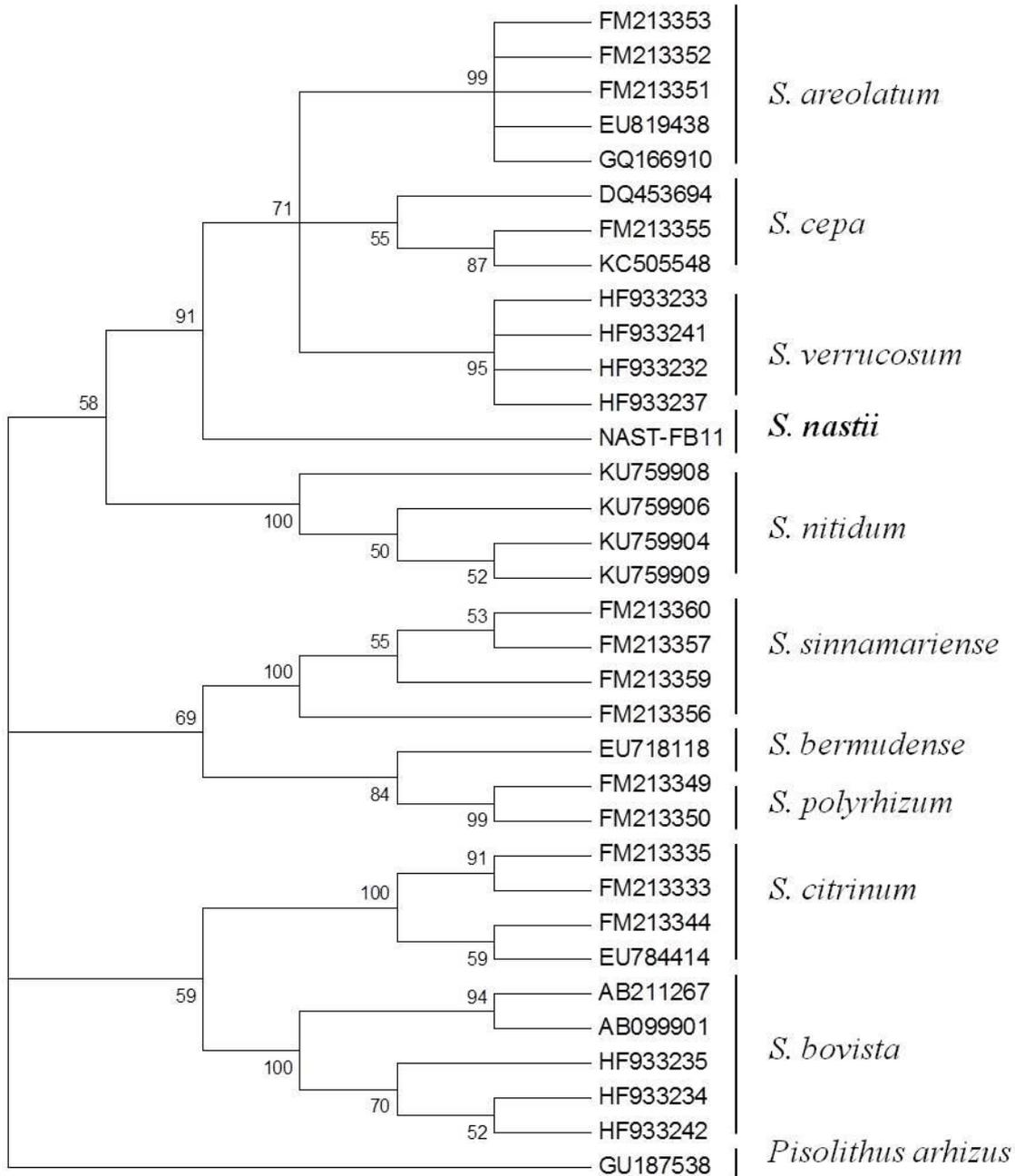
Additional specimen examined – Mexico, Chiapas: near Ocozocuatla, Laguna B lgica Ecological Park, tropical rain forest, 850 m alt., Oct. 12, 1992, E. Palacios & T. Cabrera 2112, *S. mexicana* (holotype, XAL)



**Fig. 1** – Macro- and micro features of *Scleroderma nastii* (holotype) a Basidiomes with opening gleba. b Basidiospores under light microscope (LM). c Basidiospores under scanning electron microscope (SEM). Scale Bars: a = 10 mm, b = 20  $\mu$ m, c = 5  $\mu$ m.



**Fig. 2** – *Scleroderma mexicana* (holotype) a Basidiome. b Basidiome with opening gleba. c Basidiospores under scanning electron microscope (SEM), Scale Bars: a, b = 5 mm, c = 5  $\mu$ m.



**Fig. 3** – Bootstrap consensus tree inferred from 1000 replicates using the Maximum Parsimony method. Numbers on the branches represent bootstrap values. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. Sidebar represents the inferred clades of *Scleroderma* species. *Pisolithus arhizus* was included as out group.

### Species delimitation and phylogeny

Among 33 accessions, the length of the entire ITS region for newly described species, is 676 base pairs. Within the aligned matrix of ITS sequences (including ITS1, 5.8S and ITS4), our species had six unique mutations. Sequence divergence between species based on Kimura 2 Parameter revealed that *Scleroderma* congeners maintained a range of species divergence from 3.08–13.60%. The lowest species divergence was between 3.08–3.94% in *S. areolatum*, *S. cepa* and *S. verrucosum* group. The highest species divergence was between 10.70–13.60% in rest of the other groups (*S. sinnamariense*, *S. bermudense*, *S. polyrhizum*, *S. citrinum* and *S. bovista*) except *S. nitidum*, to which divergence was 8.09%.

The Maximum Parsimony analysis of the dataset results 8 Parsimonious trees with a length of 248 steps. MP inferred a consistency index (CI) = 0.7439, a retention index (RI) = 0.922 and a composite index of 0.640927. In total three moderately supported clades with bootstrap values >58 % were observed. The first clade with bootstrap support value of 69% was contained with *S. sinnamariense*, *S. bermudense*, and *S. polyrhizum*. The second clade consists *S. citrinum* and *S. bovista* with bootstrap support value of 59%. Newly described species *S. nastii* was clustered in the third clade with *S. areolatum*, *S. cepa*, *S. verrucosum* and *S. nitidum*. Within the third clade, *S. nastii*, as shown in the strict consensus tree (Fig. 3), forms strong relation with *S. nitidum* and *S. areolatum*, *S. cepa* and *S. verrucosum* grouped with very strong bootstrap value of >90%.

## Discussion

Morphologically *Scleroderma nastii* is very close to *S. verrucosum*, *S. areolatum*, *S. cepa*, *S. mexicana*, and *S. nitidum* especially in the terms of basidiospores size and stipe. However, it differs from all those by its irregular reticulum spore ornamentation (Table 2). Additionally, it differs from *S. verrucosum*, *S. areolatum* by their verrucose scaly exoperidium and comparatively larger basidiome. *Scleroderma areolatum* is close to *S. verrucosum*; only the size of the basidiospores separates both species and the exoperidium is covered with numerous tiny patches or scales, which in *S. areolatum* are surrounded by a ring zone, giving an areolate structure. Likewise the stipe in *S. nastii* (Fig. 1) is not well formed like in *S. mexicana* (Fig. 2) which is sharply stipitate and reported only from type locality in tropical region with spiny spore ornamentation.

*Scleroderma nitidum* had been interpreted in different ways since it was described by Berkeley (1854) from Nepal. It was considered as an independent taxon by Guzmán in 1967 and later it was synonymized with *S. verrucosum* (Guzmán 1970). Guzmán & Tapia (1995) considered *S. nitidum* as *Veligaster nitidus*, due to a well-developed solid stipe, with darker subgelatinous patches in the upper part, as well as in the base of the globose part of the basidiome which are the typical features of *Veligaster*. After the synonymy of *Veligaster* with *Scleroderma*, *V. nitidus* was synonymized with *S. nitidum* (Guzmán et al. 2013). The type of *S. nitidum* deposited at Royal Botanic Gardens, Kew has sessile and stipitate basidiomata with (8- ) 9-11(-13)  $\mu\text{m}$  diam. which was collected from eastern Nepal, at 3000 m elevation. Guzmán & Ramírez-Guillén (2010) studied this species with basidiospores (7- ) 8-11 (-12) diam. in the Guzmán collection which was from *Quercus* forest in a subtropical region of Nepal at 1000 m elevation.

Moreover, in the sequence divergence estimation and phylogenetic analysis based on ITS nucleotide sequences, *S. nastii* formed a conspicuous and distinct clade among its allies. The tree topology is strongly supported by high bootstrap values (Fig. 3), that indicates *S. nastii* is a distinct taxon. This result was observed in another phylogenetic studies of *Scleroderma* by Baseia et al. (2016) and Crous et al. (2016) as well.

Regarding the ethnomycological importance, *S. cepa*, *S. citrinum*, *S. sinnamariense*, *S. texense* and *S. verrucosum* are reported as edible in Nepal. They are sold in local markets and consumed even as raw by local Tamang ethnic group. They are locally known as various names such as Aalu Chyau, Dalle Chyau, Til Chyau, Paduke Chyau, Kudaki Chyau, Bhudkee Chyau, Patteu Chyau and Shankan Shyamo in the different parts of Nepal (Christensen et al. 2008, Adhikari 2014). However, no any ethnomycological information about this newly discovered *Scleroderma* was recorded during the study.

**Table 2** Distinguishing features of *S. nastii* with other close relatives in the genus.

	Spore size ( $\mu\text{m}$ )	Ornamentation	Eco-region	Basidome	Reference
<i>S. areolatum</i>	(9–) 10–15 (– 18)	Spiny	Temperate	15–30 mm diam, dark brown or blackish scales, sessile or shortly pseudostipitate	Guzmán et al. 2013

**Table 2** Continued.

	Spore size ( $\mu\text{m}$ )	Ornamentation	Eco-region	Basidome	Reference
<i>S. cepa</i>	(7–) 8–13 (–14)	Spiny	Temperate	20–30 (–40) mm, smooth to coarsely cracked, sessile	Guzmán et al. 2013
<i>S. mexicana</i>	(7–) 8–10 (–11)	Spiny	Tropical	10–26 mm diam., smooth to somewhat velvety, stipitate	Guzmán et al. 2013
<i>S. nastii</i>	(7–) 8–9 (–10)	<b>Irregular reticulum</b>	<b>Temperate</b>	<b>10–13 mm across, smooth to somewhat velvety, pseudostipitate</b>	<b>Present work</b>
<i>S. nitidum</i>	(7–) 8–11 (–12)	Spiny	Tropical or Subtropical	(15–) 20–25 (–30) mm diam., verrucose-scaly, sessile or sharply stipitate	Guzmán et al. 2013
<i>S. verrucosum</i>	(8–) 9–12 (–14)	Spiny	Temperate or Subtropical	(20–) 25–30 (–45) mm diam., small dark brown or blackish scales, shortly pseudostipitate	Guzmán et al. 2013

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