

Three New Species of *Cortinarius* From Kashmir Himalayan Coniferous Forests Based On Morphological And Molecular Evidence

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Abstract

Cortinarius is the largest genus of mushroom forming fungi with several subgenera having ectomycorrhizal associations with coniferous trees and other plants. In view of limited studies on this speciose genus from the Himalayan region, a morpho-molecular phylogenetic approach was employed to study this taxon. Phylogenetic analysis and Bayesian inference of nuc rDNA internal transcribed spacer region ITS1-5.8S-ITS2 (ITS barcode) separated these sequences along with the identical sequences from Gene bank into three distinct clads with high bootstrap values suggesting the possibility of new taxa. The new species were found to possess some diagnostic features that separated them from other closely related species in each section. Based on our study in the Kashmir Himalayan forests, we report three new species of *Cortinarius* from the Indian subcontinent. The identified species, *C. cibum*, *C. neocephalixus*, and *C. nigricans* belong to subgenera *Myxacium*, *Phlegmacium* and *Telamonia*. A taxonomic description of morphological characters is also provided for each new taxon. This study marks the beginning in studying the *Cortinarius* genus in Kashmir Himalaya using a combination of nuc rDNA ITS barcode approach together with morphological characters and microscopic spore analysis. The current study will help in filling the knowledge gaps in the study of *Cortinarius* and will further enrich the public DNA database for ease of comparative studies throughout the world.

Introduction

Cortinarius (Pers.) Gray is one of the largest genera in the order Agaricales, with reportedly 2250 species distributed throughout the world (He et al. 2019). The species of this genus form obligate ectomycorrhizal associations with diverse seed plants across various ecosystems (Garnica et al. 2016). The genus can be easily distinguished from other genera on the basis of presence of Cortina in young fruiting bodies and large number of rusty brown spores which provide the gills a brown color at maturity (Peintner et al. 2001). However, considerable phenotypic plasticity and convergence in morphological traits displayed by the species of this genus pose considerable difficulty in correct identification of several species based on conventional morphological approach (Garnica et al. 2016; Badotti et al. 2017).

Most of the *Cortinarius* species have been described from Europe and North America, while only few have been reported from other regions particularly from Asia (Peintner et al. 2004; Frøslev et al. 2005; Garnica et al. 2005; Frøslev et al. 2007; Teasdale et al. 2013; Garnica et al. 2016). The diversity of this genus from the Indian subcontinent remains poorly understood. The ectomycorrhizal *Cortinarius* species viz., *Cortinarius conopileus*, *C. keralensis*, and *C. phlegmophorus* spp. nov. were reported for the first time from south India (Peintner et al. 2003). *Cortinarius* species were also reported in some earlier studies from Kashmir (Abraham 1993). However, only few reports of *Cortinarius* species associated with coniferous forests of Kashmir Himalaya are based on molecular phylogeny (Itoo et al. 2014, 2015).

The Kashmir Himalayan region harbors a rich diversity of ectomycorrhizal fungi forming mutualistic association with the coniferous forest trees (Pande et al. 2004; Itoo et al. 2014). Earlier studies on macrofungal diversity have mostly focused on morphological characters and spore structure for the

identification of fungi (Watling and Abraham 1992; Abraham 1993; Kumar and Sharma 2011; Pala and WANI et al. 2011). Recently, the use of DNA sequences as successful molecular markers has improved the accuracy in identification of fungi. Several DNA barcodes have been employed for the delimitation of various living organisms up to species level. Among them, the nuc rDNA ITS1-5.8S-ITS2 region has been found to be a suitable molecular marker for the correct identification of fungi by the consortium of international barcode (Seifert 2009; Schoch et al. 2012; Schoch et al. 2014). Several studies have found that ITS barcode can prove significantly effective in distinguishing *Cortinarius* species (Garnica et al. 2016; Brandrud et al. 2018). Molecular approach together with morphological analysis can significantly improve the accuracy in identification of such fungi.

In view of paucity of information about the genus *Cortinarius* in the Kashmir Himalaya and absolute reliance on the morphological characters for species identification in earlier studies, extensive surveys in several coniferous forests of this region were undertaken. Further, a combined morphological and molecular approach was employed for characterization of the specimens belonging to genus *Cortinarius* in order to fill the knowledge gap that exists at present regarding the ectomycorrhizal fungi in general and *Cortinarius* in particular.

Materials And Methods

Study area.– The study area included the coniferous forests of Kashmir Himalaya and the areas selected for survey included Sonmarg, Naranag, Yusmarg, Gulmarg, Pehelgam, Sinthan-Daksun, Hirpora, Lolab, Bungus-Nildori and Sadnah, spanning the entire Kashmir Himalayan region. The temperate climate of Kashmir Himalaya allows for dominance of evergreen coniferous forests with four distinct seasons of winter (December to February), spring (March to May), summer (June to August) and autumn (September to November). This Himalayan region receives major part of its precipitation in winter and spring seasons in the form of snow and occasional rains from western disturbances with an average mean maximum and minimum temperatures of Kashmir valley as 19.27 and 7.29 °C, respectively, while as the average rainfall is 84 cm. The temperature starts to gradually rise from the spring season, after which the growth of macrofungal fruiting bodies is expected and collection is started.

Sporocarp collection.– The sporocarps of all the available Basidiomycete fungi commonly called mushrooms were collected from the coniferous forests of Kashmir Himalaya dominated mainly by *Cedrus deodara*, *Pinus wallichiana*, *Abies pindrow*, and *Picea smithiana* along with *Betula utilis* occupying the tree line. With the onset of growing season, regular field surveys were conducted after every fortnight for three consecutive years (2016–2018) during the entire growing season from early March to October. The field surveys and sporocarp collections were carried out following the standard methods referred to as ‘Opportunistic sampling’ (Mueller et al. 2004; Atri 2005; Schmit and Lodge 2005; Halme et al. 2012a, 2012b). The sporocarps were photographed in the field using digital camera and later dug out carefully using a knife and collected in separate bags. Fragile specimens were collected in separate containers to retain their structural integrity. The samples were taken to the laboratory, labelled

and the specimens stored under deep freeze conditions for further molecular investigations. The dried voucher specimens have been deposited in Kashmir University Botanical Garden Herbarium (KASH).

Morphological analysis.– Fresh sporocarps were used for describing macroscopic characters like color, size, shape and detailed anatomical features of various parts. The color of the specimens was ascribed on the basis of color codes Kornerup and Wanscher (1978). The microscopic characters were described from thin sections of tissue mounted in water, 5% KOH, Melzer's reagent, and Congo red using Light microscope with 100X resolution and fitted with Amscope camera (Garnica et al. 2003; Niskanen et al. 2012). The spore measurements and morphology was based on several mature spores observed under light microscope. The Pileipellis structure was studied from sections cut halfway through the Pileus. A key to the new *Cortinarius* species from Kashmir Himalaya was developed based on the diagnostic characters. In this regard, we have referred to the morphology of *Cortinarius* taxa published by (Peintner et al. 2001, 2004).

Molecular analysis.–

DNA extraction. DNA was extracted from fresh sporocarp specimens using standard cetyltrimethylammonium bromide (CTAB)-chloroform method with few modifications (Rogers and Bendich 1994; Porebski et al. 1997). For this, a small piece of fresh pileus was taken and ground into fine powder in liquid nitrogen using pestle and motor. The powdered material was transferred to 2 ml centrifuge tubes and 1ml of pre-warmed CTAB buffer (1M Tris HCl pH 8.0, 5M NaCl, 0.5M EDTA PH 8.0, CTAB, 2% β -mercaptoethanol) was added to it. The centrifuge tubes were incubated at 65 °C for 1 hour with periodic inversion. This was followed by centrifugation of these tubes at 12,000 rpm for 10–15 minutes in order to remove the cell debris. The supernatant from each of these tubes was transferred to new 1.5ml centrifuge tube and C.I mix (chloroform: isoamyl-alcohol in the ratio of 24:1) was added after which the tubes were centrifuged again at 12,000 rpm. This step was repeated twice and in between the RNA'ase treatment was given to the content in the tubes and incubated for 15 minutes. The upper layer was again separated, treated with 1 ml isopropanol and kept overnight at -20 °C. Next day, the tubes were centrifuged again at 10,000 rpm for 10–20 minutes. The supernatant was discarded and the pellet was washed twice with 70% ice cold ethanol and spinned at 8,000 rpm for 5 minutes. The pellet was dried for 30 minutes in an incubator, re-dissolved in elution buffer, and stored at -20 °C. The purified DNA was checked on 1% Agarose gel by staining with ethidium bromide and concentration was measured using Nano-Drop spectrophotometer.

PCR amplification and purification. The isolated DNA was used as template and complete sequences of ITS1, 5.8S rRNA gene and ITS2 with partial sequences of 18S and 28S rRNA gene were amplified by Polymerase chain reaction in Applied Biosystems 2720 Thermal Cycler using four previously reported and widely used primers ITS1/ITS4, ITS1F/ITS4B (Gardes and Bruns, 1993). The 25 μ L reaction mixture for PCR amplification contained 1 μ L template DNA, 3 μ L PCR buffer, 2.5 μ L of 2mM dNTP's, 2 μ L of each primer, 1.5 μ L of Mgcl₂, 1 μ L of DMSO and 0.4 μ L of Taq DNA polymerase. Amplifications were performed in a thermal cycler with an initial denaturation step of 94 °C for 5 minutes followed by 35 cycles of 94 °C

for 1 minute, 52 °C for 30 seconds, and 72 °C for 1 minute and a final extension of 72 °C for 10 minutes. PCR products were loaded on 1.5 % agarose gel, separated by electrophoresis (90 V, 180 mA, 50 min.), stained with ethidium bromide, and viewed in a gel documentation system.

The PCR products were purified using GenElute™ PCR Clean-Up Kit (Sigma) according to manufacturer's protocol and the DNA concentration was confirmed using NanoDrop (Spectrophotometer). The purified PCR products of the ITS amplified region were directly sequenced in both directions using the ITS1 and ITS4, ITS1F and ITS4B pair of amplification primers in Sci-Genome and Xcelris labs). Chromatograms were analyzed using chromas version 2.33 (Technelysium Pty Ltd).

Phylogenetic analysis. –

Sequence alignment. A BLAST (Basic Local Alignment Search Tool) search was carried out for the sequences using the National Center for Biotechnology Information (NCBI) USA (<https://www.ncbi.nlm.nih.gov/>) and UNITE fungal database (<https://unite.ut.ee/>). The sequences were matched in terms of the widely used $\geq 97\%$ sequence similarity cut-off point for fungal species delimitation (Nilsson et al. 2008; Hibbett et al. 2011). In case of *Cortinarius* genus, the sequences that matched correctly having percentage identification $> 99\%$ with the sequences from database and showing consistency downstream, were assigned a species name with certainty while those with percentage identification $< 99\%$ were assigned a generic name (Garnica et al. 2016). The sequences were later deposited in NCBI database with accession numbers listed in Table 1. The first hit on the blast results list was assumed to represent the best match, the next fully identified, and insufficiently identified matches were used to check for consistency. The specimens that showed ambiguity due to low sequence similarity were further confirmed using UNITE, and Mycobank database (<http://www.mycobank.org/>). The species names were further confirmed from Index Fungorum (<http://www.indexfungorum.org>).

Table 1
ITS sequences used in the phylogenetic analyses.

Sequence name	Voucher	Accession No.	Geographic origin	Reference
Cortinarius sp.nov	K.HIM.1.1	MZ203578	Kashmir Himalaya, India	New species
Cortinarius sp.nov	K.HIM.1.2	MZ203579	Kashmir Himalaya, India	New species
Cortinarius sp.nov	K.HIM.1.3	MZ203580	Kashmir Himalaya, India	New species
Cortinarius sp.	ANT275-HRL2130	MN992357.1	Canada	Unpublished
Cortinarius aff. pallidifolius	ANT273-HRL2129	MN992334.1	Canada	Unpublished
Cortinarius olidus	KS C01159	KJ421068.1	Germany	(Garnica et al. 2016)
Cortinarius cephalixus	TUB 011444	AY174784.1	Germany	(Garnica et al. 2003)
Cortinarius sp. 'squameopercomis'	TEB397-16	MK358111.1	Hungary	(Soop et al. 2019)
Cortinarius pseudocephalixus	IK98-1842	KF732634.1	Sweden	(Liimatainen et al. 2014)
Cortinarius aff. pallidifolius	ANT082-QFB28694	MN992333.1	Canada: Quebec	Unpublished
Cortinarius herculeus	TUB 019805	KJ421098.1	Germany	Unpublished
Cortinarius olidoamarus	TUB 019787	KJ421042.1	Germany	Unpublished
Cortinarius misermonitii	IK872172	KF732622.1	Spain	(Liimatainen et al. 2014)
Cortinarius sp.nov	K.HIM.2.1	MZ203581	Kashmir Himalaya, India	New species
Cortinarius sp.nov	K.HIM.2.2	MZ203582	Kashmir Himalaya, India	New species
Cortinarius sp.	OTA:60173	MN846506.1	New Zealand	Unpublished
Cortinarius sp.	OTA:60187	MN846508.1	New Zealand	Unpublished
Cortinarius sp.	OTA:60313	MN846514.1	New Zealand	Unpublished

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Sequence name	Voucher	Accession No.	Geographic origin	Reference
Cortinarius sp.	OTA:61935	MN846523.1	New Zealand	Unpublished
Cortinarius sp. PDD 103885	PDD:103885	KF727393.1	New Zealand	Unpublished
Cortinarius vibratilis	F16046	FJ157098.1	Canada	(Harrower et al. 2011)
Cortinarius sp.	CM13_090	KY774102.1	New Caledonia	Unpublished
Cortinarius sp.	FC1752	MG553130.1	Australia	Unpublished
Cortinarius croceocristallinus	PAM08082212	JQ749630.1	France	Unpublished
Cortinarius vibratilis	DAVFP 26240	EU821696.1	Canada	(Harrower et al. 2011)
Cortinarius croceocaeruleus	TUB 011833	AY669590.1	Germany	(Garnica et al. 2005)
Cortinarius sp.	PERTH:06641814 FC748	MG553035.1	Australia	Unpublished
Cortinarius sp	K.HIM.3.1	MZ203583	Kashmir Himalaya, India	Unpublished
Cortinarius sp	K.HIM.3.2	MZ203584	Kashmir Himalaya, India	Unpublished
Cortinarius varius	TUB 011392	AY174792.1	Germany	(Garnica et al. 2003)
Cortinarius sp.	Daniel 1	FJ717603.1	Canada	(Harrower et al. 2011)
Cortinarius variosimilis	VMS1	FJ717598.1	Canada	(Harrower et al. 2011)
Cortinarius variosimilis	VMS26	FJ717596.1.	Canada	(Harrower et al. 2011)
Cortinarius sp.	061708_2015_LMF1B	KY510816.1	USA: WA, Yakima	Unpublished
Cortinarius variosimilis	F16580	GQ159915.1	Canada	(Harrower et al. 2011)
Cortinarius varius	TUB 019715	KJ421143.1	Germany	(Garnica et al. 2016)

Sequence name	Voucher	Accession No.	Geographic origin	Reference
<i>Cortinarius caesiostamineus</i>	TUB 019702	KJ421179.1	Germany	(Garnica et al. 2016)
<i>Cortinarius varius</i>	TUB 019761	KJ421000.1	Germany	(Garnica et al. 2016)
<i>Cortinarius sp.nov</i>	K.HIM.4.1	MZ203585	Kashmir Himalaya, India	New species
<i>Cortinarius sp.nov</i>	K.HIM.4.2	MZ203586	Kashmir Himalaya, India	New species
<i>Cortinarius sp.nov</i>	K.HIM.4.3	MZ203587	Kashmir Himalaya, India	New species
<i>Cortinarius brunneovernus</i>	PNWKC-07-125-12	KC608577.1	USA: Washington	(Liimatainen et al. 2014)
<i>Cortinarius brunneovernus</i>	DM05-14	KC608580.1	USA: Washington	(Liimatainen et al. 2014)
<i>Cortinarius brunneovernus</i>	JLF8693	MW341327.1	USA: Jackson County	Unpublished
<i>Cortinarius brunneovernus</i>	JLF8670	MW341323.1	USA: Jackson County	Unpublished
<i>Cortinarius brunneovernus</i>	K.HIM.KU.8.1	MW040382.1	Kashmir Himalaya	Unpublished
<i>Cortinarius brunneovernus</i>	K.HIM.KU.8	MW040381.1	Kashmir Himalaya	Unpublished
<i>Cortinarius brunneovernus</i>	WTU:J.F. Ammirati 13331	NR_131826.1	USA: Washington	(Liimatainen et al. 2014)
<i>Cortinarius subcaesiobrunneus</i>	HMJAU:44434	MK234565.1	China	(Xie et al. 2020)
<i>Cortinarius brunneovernus</i>	110501-1	KC608579.1	USA: Washington	(Liimatainen et al. 2014)
<i>Hebeloma circinans</i>		AF124699.1	Netherlands	(Aanen et al. 2000)
<i>Hebeloma fastibile</i>	IB 19940036	AF325643.1	USA	(Peintner et al. 2001)

Data analysis. An ITS data set comprising newly generated sequences and identical sequences retrieved from the fungal database were used in the analysis (Table 1). The sequences of genus *Hebeloma* were selected as outgroups following (Danks et al. 2010; Pastor et al. 2019). DNA sequences that were of insufficient quality or < 350 base pairs in length were discarded from the current analysis. The initial sequence alignment was performed for all the sequences using Muscle program of ClustalW (<http://www.clustal.org/>) (Sievers et al. 2011) and the alignment was further refined with BioEdit 7.0.9 (Hall 1999, 2004). Maximum likelihood (ML) analysis was performed using phyML and MEGA6 (Tamura et al. 2013) and Branch support was assessed with Bootstrap analysis run with 1000 replicates (Felsenstein 1985). Bayesian inference (BI) analysis was performed using MrBayes 3.2.2 (Ronquist et al. 2012).

A threshold dissimilarity value of 0.5% for species hypothesis was used to assess the diversity of *Cortinarius* in order to improve the accuracy and ease of comparison (Kõljalg et al. 2013). A value of 1% is considered significant to distinguish species of *Cortinarius* using ITS region (Garnica et al., 2016).

Results

Phylogenetic analysis.– The ITS sequences belonging to the *Cortinarius* species along with the homologous sequences obtained from Genebank database comprised the final dataset of 52 sequences, that were subjected to phylogenetic analysis, in which sequences of two *Hebeloma* species were selected as an out-group (Table 1). These included ITS sequences representing *Cortinarius* species from Europe, N. America, South America and Asia with original sequences ranging from 600 to 700 base pairs. The best fit model for the dataset was chosen as GTR + 1 using jModelTest program. A Phylogenetic tree depicting the phylogenetic position of different species was generated using Maximum Likelihood method in phyML and MEGA X software, which generated comparatively similar trees with high statistical support values as shown (Fig. 1). The ITS sequences were found to be highly species specific in case of *Cortinarius* species, but in some of the cases, the NJ trees constructed from these sequences did not share similar topology possibly because of dissimilarities in length of amplified fragment used in the study. The inter-specific and intra-specific distances were calculated and were found to be ideal for the DNA barcodes with the interspecific distances exceeding the intraspecific distances. The Bayesian inference values calculated for the aligned sequences were > 0.95 which was in conformity with the obtained results as shown in the Phylogram.

The resulting Phylogram of the ITS sequences of *Cortinarius* grouped them into several clearly distinct clads with moderate to high bootstrap values. However, the basal relationships of the clads were poorly resolved possibly because of the low resolution capacity of ITS sequences in determining the basal relationships within *Cortinarius*. The sequences of three new specimens clustered individually into separate clads from the other sequences obtained from the gene bank with high statistical support values thus conforming the identity of new taxa (Fig. 1). This approach differentiated three novel species belonging to *Cortinarius* genus.

Taxonomy

Cortinarius cibum **S.S. Ahmed and Z.A. Reshi, sp. nov.**

Diagnosis. Pileus conical to hemispherical and slimy with lustrous brown color and light pink gills. Universal veil sparsely present. Basidiospores ellipsoid and irregularly ornamented. Found mostly on wet ground surrounded by Sphagnum grass. The ITS sequence differs from other sequences by at least 10 insertions and 13 substitutions.

Holotype. India. Union Territory of Jammu and Kashmir, District Kupwara, Town Handwara, Mawer valley, Bungus-Nildori, Coniferous forest (Dominated by *Abies pindrow*), 34°27' 19" N, 74°23' 48" E, Altitude 2715m, 6 July 2017, S.S. Ahmed, Genbank acc. No. MZ203581.

Etymology. The name refers to its brown meat color of pileus.

Description. Pileus 2.2–3.7 cm in diam., conical when young, campanulate with slight dome in center at maturity, lustrous brown (6D3–6) with slightly paler margins and dark at the center, glutinous, glabrous with smooth surface, margins entire when young, discontinuous breaking into three or more parts at maturity. Lamellae adnexed, subdistant to moderately spaced, light pink (4D3–5), pale yellow (2C3–4) when young, turning brown at maturity, margins entire, smooth. Stipe 4.3–6.2 cm long, 1.7–2.4 cm thick at apex to base, cylindrical to slightly bulbous and tapering at base, pale white to greyish brown (6C3–5) when young turning pale yellow at maturity, surface with white fibrils all over. Universal veil absent in mature sporocarps. Context greyish white to pale yellow. Odor indistinct, taste bitter. Exsiccata brown (5F8) to black brown (7F5) in color.

Basidiospores $6.2\text{--}7.3 \times 5.3\text{--}4.9 \mu\text{m}$, $Q = 1.11\text{--}1.37$, $X = 6.3\text{--}7.6 \times 6.4\text{--}6.9 \mu\text{m}$, $Q = 1.44\text{--}1.65$ (30 spores), ellipsoid, slightly moderately verrucose. Basidia 4-spored, cylindrical to clavate, $23\text{--}44 \times 6\text{--}11 \mu\text{m}$, thin-walled, slightly hyaline to olivaceous brown in 5% KOH. Lamellar edges sterile, sterile cells cylindrical to clavate, $13\text{--}21 \times 4.1\text{--}8.6 \mu\text{m}$, thin-walled, hyaline in 5% KOH. Lamellar trama hyphae irregular, smooth, pale olivaceous to light yellow in 5% KOH. Pileipellis–epicutis hyphae cylindrical to elongated, $2\text{--}5 \mu\text{m}$ wide, pale yellow to olivaceous brown in 5% KOH, smooth, hypocutis well developed, hyphae $11\text{--}29 \mu\text{m}$ wide, smooth, sub-cellular, thin-walled, hyaline in 5% KOH. Pileus trama hyphae thin-walled, smooth, hyaline to slightly olivaceous brown in 5% KOH. Clamp connections present.

ITS sequence. The ITS sequence of two specimens of *C. cibum* are 650–680 bp long (3 collections, Table 1). All the three sequences (MZ203581, MZ203582 and MW547496) are identical with no indels. The ITS sequence of *C. cibum* (Holotype) differs from other sequences in the section *Vibratilis* by at least 13 substitutions and 10 insertions.

Ecology and distribution. Mycorrhizal with conifers; mostly growing scattered and very rarely sighted, found in summer months, growing in the mid altitudinal gradients of Kashmir Himalaya.

Additional specimens examined. India. Union Territory of Jammu and Kashmir, District Kupwara, Town Handwara, Mawer valley, Bungus-Nildori, Coniferous forest (Dominated by *Abies pindrow* with scattered *Pinus* sp.), 34°35' 44" N, 74°32' 11" E, Altitude 2732m, 6 July 2017, S.S. Ahmed, Genebank acc. No. MZ203581; 34°38' 25" N, 74°24' 16" E, Altitude 2635m, 6 July 2017, S.S. Ahmed Genebank acc. No. MZ203582; 34°35' 22" N, 74°42' 33" E, Altitude 2629m, 6 July 2017, S.S. Ahmed Genebank acc. No. MW547496.

Comments. *Cortinarius cibum* can be easily distinguished from other species by the distinct lustrous surface and deep brown color of pileus. The gills are pink in young specimens and spores are much smaller in size compared to other species. The ITS sequence shows similarity of less than 92 percent from other closely related sequences and separates out as a separate cluster in the phylogenetic analysis.

Cortinarius neocephalixus. **S.S. Ahmed and Z.A. Reshi, sp. nov.**

Diagnosis. Pileus 3.6–5.4cm in Diam., glutinous, glabrous with brownish universal veil remnants scattered over the surface. Lamellae moderately crowded, pale yellow. Stipe clavate, grayish, tapering towards the end. Basidiospores $8.7\text{--}10.3 \times 5.5\text{--}6.4 \mu\text{m}$, Amygdaliform. The ITS sequences differ from other species by at least 11 substitutions and 7 indel positions.

Holotype. India. Union Territory of Jammu and Kashmir, District Kupwara, Town Handwara, Mawer valley, Bungus-Nildori, Coniferous forest (Dominated by *Abies pindrow*), 34°33' 35" N, 74°11' 10" E, Altitude 2635m, 5 July 2017, S.S. Ahmed, Genebank acc. No. MZ203580

Etymology. The name refers to its affinity with *C. cephalixus*.

Description. Pileus 3.6–5.4 cm in diam., hemispherical to convex when young, becoming broadly convex at maturity, ochraceous yellow (4B7–8), reddish brown (9E6–8) at the centre, pale yellow (4A4) towards margins, glutinous, glabrous, with very small scales in the center of the pileus, margins smooth and incurved. Universal veil remnants usually abundant, forming brown, loose scales or patches on the upper surface of pileus. Lamellae 6–9 mm broad, adnate, moderately crowded, pale yellow to grayish white (4A2) when young, turning brown (5F8) at maturity, crenulate margins. Stipe 3.5–5.4 long, 0.7–.09 cm thick at apex, 1.5–2.5 cm thick near base, base cylindrical to clavate, thick girdled to fibrillose of yellow-brown (6C–4) to more rarely whitish (4A3) veil, color grayish white (2A2), ochraceous white (2A1–2) towards base, often completely brownish in lower part from veil remnants; Universal veil prominent, ochraceous brown (9F6) to rather dark brown (8F6–8), rendering the lower part of stipe distinctly girdled-floccose, sometimes more fibrillose-peronate. Context white, more greyish in stipe apex when young. Odor and taste not distinct, yeast like. Reaction to 3% KOH– negative to light brown at pileus, stipe and base. Exsiccata brown (6E5) to dark brown (6F6) in color.

Basidiospores $7.9\text{--}10.3 \times 5.5\text{--}6.4 \mu\text{m}$, $Q = 1.31\text{--}1.67$, $X = 9.2\text{--}9.8 \times 6.3\text{--}6.6 \mu\text{m}$, $Q = 1.37\text{--}1.48$ (30 spores, 3 collections). Amygdaliform, distinctly and usually fairly densely verrucose. Basidia 4-spored,

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cylindrical to clavate, 24–46 × 5–9 µm, moderately thin-walled, yellowish brown to olivaceous brown in 5% KOH. Lamellar edge sterile, sterile cells cylindrical-clavate, 9–22 × 3–8 µm, thin-walled and slightly hyaline in 5% KOH. Lamellar trama hyphae regular, smooth, olivaceous in 5% KOH. Universal veil having thin-walled hyphae, often with well-developed, yellow-brown, encrusted, parietal and intracellular pigment, hyaline to olivaceous yellow in 5% KOH. Gelatinous layer composed of various hyphal strata; Pileipellis duplex, with a distinctly subcellular hypoderm, elements often irregular, almost isodiametric, and imbedded in a brown, amber-like matrix, basal epicutis often with distinct, brown, encrusted pigment.

ITS sequence. The ITS sequence of *C. neocephalixus* are 590–690 bp long (3 collections, Table 1). All the three sequences (MZ203578, MZ203579 and MZ203580) are identical with no indels. The ITS sequence of *C. neocephalixus* (Holotype) differs from other sequences in the section *Phlegmacium* by at least 11 substitutions and 7 indel positions.

Ecology and distribution. Mycorrhizal with conifers, mostly growing scattered and rare, found in summer and early fall, growing in the mid elevational gradients of Kashmir Himalaya.

Additional specimens examined. India. Union Territory of Jammu and Kashmir, District Kupwara, Town Handwara, Mawer valley, Bungus-Nildori, Coniferous forest (Dominated by *Abies pindrow*), 34°34' 39" N, 74°13' 12" E, Altitude 2622m, 5 July 2017, S.S. Ahmed, Genebank acc. No. MZ203578; 33°28' 22" N, 74°18' 16" E, Altitude 2645m, 6 July 2017, S.S. Ahmed Genebank acc. No. MZ203579; 34°26' 25" N, 73°28' 11" E, Altitude 2638m, 6 July 2017, S.S. Ahmed Genebank acc. No. MZ203580.

Comments. Morphologically, *Cortinarius neocephalixus* shares close affinity with *Cortinarius cephalixus* but a closer examination of various structures reveals contrasting differences like the glabrous pileus with scattered universal veil remnants, short pear shaped stipe and the size and structure of basidiospore. Molecular phylogenetic analysis also revealed that the ITS sequences of *C. neocephalixus* showed similarity of less than 97 percent with other closely related sequences and clustered separately in the Phylogram with high bootstrap values.

Cortinarius nigricans **S.S. Ahmed and Z.A. Reshi, sp. nov.**

Diagnosis. Pileus 4.3–6.9 cm in diam. Surface fibrillose, having radiating fibrils from the centre with strongly incurved margins, dark brown in color. Lamella adnate, close to subdistant. Stipe with slight subapical bulb with spongy tissue surrounding the base and a pointed end. Basidiospores 6.6–9.3 × 6.1–9.8 µm, ellipsoid-subamygdaloid. The ITS sequence of the Holotype differs from other closely related species in the section by at least 8 substitutions and 6 indels.

Holotype. India. Union Territory of Jammu and Kashmir, District Kupwara, Town Handwara, Mawer valley, Bungus-Nildori, Coniferous forest (Dominated by *Abies pindrow*), 34°38' 25" N, 72°28' 14" E, Altitude 2475m, 20 July 2018, S.S. Ahmed, Genebank acc. No. MZ203585

Etymology. The name refers to its morphological affinity with *C. brunneus*.

Description. Pileus 4.3–6.9 cm in diam., umbonate to broadly umbonate, becoming uplifted to undulate when mature sporocarps, with incurved to decurved edges, surface finely innately and radially fibrillose, reddish brown (5D5–6) to dark brown all over (5E7–8), surface moderately hygrophanous. Universal veil remnants very sparsely present on pileus or entirely absent in mature sporocarps. Lamellae adnate, moderately dense, close to subdistant, 6–8 mm broad; thick, brown, turning dark brown (5D4–6) at maturity. Stipe 3.8–4.2 × 1.1–2.1 cm thick at apex; cylindrical to subbulbous, with Ocher ring-like velum zones around the base, at first whitish to pallid silky fibrillose with watery brown streaks, soon developing brownish tones and darker brown colors all over the surface. Universal veil persistent, thin, sheathing the base of young stipe. The lower part of basal bulb surrounded by thick network of grayish-white mass of hyphae. Context in pileus and stipe uniformly dark brown, hygrophanous. Odor and taste not distinct, earthy. Reaction to KOH–Negative reaction of pileus, stipe and base. Exsiccata dark grayish brown (10YR 4–2) to brown (10YR 4–3).

Basidiospores 7.2–8.7 μm × 5.4–6.7 μm , $Q = 1.21–1.42$, $X = 8.3–8.8\mu\text{m} \times 6.3–6.6\mu\text{m}$, $Q = 1.32–1.37$, subglobose to broadly ellipsoid, moderately to strongly verrucose, strongly ornamented at the apex, dextrinoid. Basidia 4–spored, clavate, 33–37 × 9–11 μm , hyaline having yellow granulose contents in 5% KOH. Lamellar trama hyphae irregular, smooth-walled, olivaceous to olivaceous brown in 5% KOH. Pileipellis duplex, pale olivaceous yellowish brown, epicutis thin to moderately thick, hyphae 2.5–10 μm wide, with yellowish brown granular contents or sometimes hyaline, smooth. Hypoderm distinct, thick, elements 25–55 μm × 15–25 μm , hyaline or walls with brown pigment, smooth.

ITS sequence. The ITS sequence of *C. nigricans* are 560–600 bp long (3 collections, Table 1). All the three sequences (MZ203585, MZ203586, and MZ203587) are identical with no indels. The ITS sequence of *C. nigricans* (Holotype) differs from other sequences in the section *Telamonia* by at least 8 substitutions and 6 indels.

Ecology and distribution. Mycorrhizal with conifers; mostly growing scattered, found in summer, growing in the mid altitudinal gradients of Kashmir Himalaya.

Additional specimens examined. India. Union Territory of Jammu and Kashmir, District Kupwara, Town Handwara, Mawer valley, Bungus-Nildori, Coniferous forest (Dominated by *Abies pindrow*), 33°34' 26" N, 74°27' 21" E, Altitude 2721m, 20 July 2018, S.S. Ahmed, Genebank acc. No. MZ203585; 33°34' 23" N, 74°17' 15" E, Altitude 2643m, 20 July 2018, S.S. Ahmed Genebank acc. No. MZ203586; 34°22' 25" N, 74°28' 17" E, Altitude 2676m, 20 July 2018, S.S. Ahmed Genebank acc. No. MZ203587.

Comments. *Cortinarius nigricans* is morphologically similar to *C. brunneus* with slight differences in the color and shape of stipe and a white cottony mass around its base. However, the Molecular analysis of ITS sequences of *C. nigricans* reveals a distinct difference from other related species by clustering separately in Phylogram and shares a similarity of only 96 percent with those sequences.

KEY TO NEW CORTINARIUS SPECIES OF KASHMIR HIMALAYA AND MORPHOLOGICALLY SIMILAR

1. Pileus and sometimes stipe also viscid, at least in young specimens..... 2
- 1☒. Pileus and stipe dry, hygrophanous, stem peronate or annulate from the remnants of the veil in addition to the cortina (*Telamonia*) 5
2. Pileus viscid, stipe dry, gills bluish grey to violet, or with violet edges..... (*Phlegmacium*) 3
- 2☒. Pileus and stipe viscid, taste bitter, spores less than long, punctate to almost smooth, if subglobose, smaller (*Myxacium*) 4
3. Pileus viscid, glabrous, orange brown, yellow brown towards disk margins, slightly granulose with very small scales towards the center of the pileus, Lamellae pale ochraceous to pale argillaceous, adnate to emarginate, crowded, edges uneven. Stipe clavate, apex whitish, pallid, Universal veil persistent below cortina forming thick yellow brown floccose ring-zone near apex. Spores marbled to finely verrucose, slender, almond shaped, $8.8-11.7 \times 5-5.9 \mu\text{m}$*Cortinarius cephalixus*
- 3☒. Pileus glutinous, glabrous, ochraceous yellow, darker towards centre, with brownish universal veil remnants scattered over the surface and towards margins. Lamellae pale yellow-greyish white, adnate, moderately crowded. Stipe clavate, pale-grayish white, tapering towards the end with brown veil remnants scattered over the surface. Basidiospores amygdaliform, distinctly and usually fairly densely verrucose, $7.9-10.3 \times 5.5-6.4 \mu\text{m}$*Cortinarius neocephalixus*
4. Pileus yellow to orange yellow, slimy when fresh with pale and entire margins. Stipe cylindrical to clavate and slightly tapering at base. Spores ellipsoid, verrucose. Universal veil white surrounding the base of stipe. Taste of flesh and cuticle very bitter, spores ellipsoid small at most $8-10 \mu\text{m}$ long.....*Cortinarius vibratilis*
- 4☒. Pileus conical when young, lustrous brown glutinous, glabrous, margins entire when young, discontinuous breaking into three or more parts at maturity. Stipe cylindrical to slightly bulbous and tapering at base. Universal veil absent in mature sporocarps. Taste of flesh bitter. Spores ellipsoid, slightly moderately verrucose $6-7 \mu\text{m}$...*Cortinarius cibum*
5. Pileus hygrophanous and not viscid, surface finely innately rivulose, margin light brown to brownish tan. Lamellae subdistant to distant, adnexed with decurrent line to deeply adnexed. Stipe clavate to subbulbous. Universal veil: whitish, thinly sheathing stipe at first. Spores subglobose, ellipsoid to broadly ovoid, thick-walled, moderately to strongly verrucose, somewhat more strongly ornamented at the apex, strongly dextrinoid.....*Cortinarius brunneovernus*
- 5☒. Pileus slightly hygrophanous, surface innately and radially fibrillose, entirely dark brown. Lamellae adnate, close to subdistant. Stipe cylindrical to subbulbous, with Ocher ring-like velum zones around the base. Universal veil persistent, thin, sheathing the base of young stipe. The lower part of basal bulb

surrounded by thick network of grayish-white mass of hyphae. Spores subglobose to broadly ellipsoid, strongly ornamented.....*Cortinarius nigricans*

Discussion

Cortinarius is a relatively large and species rich genus of Agaricales with most of the species described from Europe and North America (Garnica et al. 2003; Liimatainen et al. 2014). The diversity exploration studies of this genus in Asian region have only recently been initiated, particularly in China, while only few studies have been carried out in Indian sub-continent (Xie et al. 2020). The Kashmir Himalayan region has a temperate climate that shares similar affinities with the temperate regions of Europe and N. America, supporting a rich diversity of flora with which diverse fungi form several associations. Some of the fungi reported earlier from this region are also present in the European and N. American forests having similar ITS sequence homologies (Itoo et al. 2015). These species form mycorrhizal associations with conifers as reported in studies from Europe and N. America. The *Cortinarius* species were reported for the first time from south India (Peintner et al. 2003). So far only few studies have been conducted on *Cortinarius* genus associated with the conifers in Himalayan region. Further studies are required in order to assess the diversity of this genus in Himalaya.

The present study mainly focused on the diversity of *Cortinarius* species present in the coniferous forests of Kashmir Himalaya. Morphological observations of several specimens of each species placed them in particular taxonomic groups, but revealed some contrasting characters that separated these taxa from earlier recorded species. This was followed by molecular characterization using rDNA ITS barcode that revealed many differences at several DNA loci and showed lower similarity values with already known sequences in Genebank. Thus, using a combination of morphological characters, spore analysis and molecular phylogenetic analysis, four novel taxa were identified. Among the studied species, two belonged to subgenus *Phlegmacium*, which is a diverse group distributed throughout the Northern Hemisphere. Another species belonged to subgenus *Myxacium* having several species recorded from Europe and N. America, while one species belonged to subgenus *Telamonia*. The *Cortinarius* genus has been further subdivided into several sections and subsections by several authors (Garnica et al. 2003); but the delimitation of such groups remains an active area of debate, hence has been excluded from the current study. The ITS analysis employed in the current study was able to delimit the species more accurately which further confirmed the reliability of ITS as a suitable barcode for *Cortinarius* species as found in previous studies (Liimatainen et al. 2014; Garnica et al. 2016). All the four taxa that were discovered from the Indian subcontinent were novel species reported for the first time, thus confirming the knowledge gaps and the need for further taxonomic studies on this mycorrhizal genus. Thus, further studies focusing on the diversity of *Cortinarius* genus in Kashmir Himalaya will help us in understanding the mycorrhizal associations formed by these species with conifers. This study will partially fill the knowledge gap and will further help in identifying the environmental unknowns obtained from Metagenomic studies of environmental samples by sequence similarities in public data bases. The molecular studies on this genus will further enrich the public DNA databases thus helping in comparing

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Declarations

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Availability of data and material: The ITS data is available in the Genbank public database and the specimens have been deposited in the KASH herbarium.

Code availability: Not applicable.

Authors' contributions

All the authors have contributed in the study and in the preparation of the manuscript.

Mr. Sheikh Sajad Ahmed has worked on the laboratory analysis, initial preparation and further drafting of final manuscript.

Prof. Zafar A. Reshi has supervised the whole process from conceptualization, methodology, and laboratory analysis to review and editing the final manuscript.

Ms. Bushra Jan has contributed to the taxonomic study of the specimens and commented on various sections of the previous versions of draft.

Prof. Khurshid I. Andrabi has supervised the laboratory analysis of applied molecular methods.

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Figures

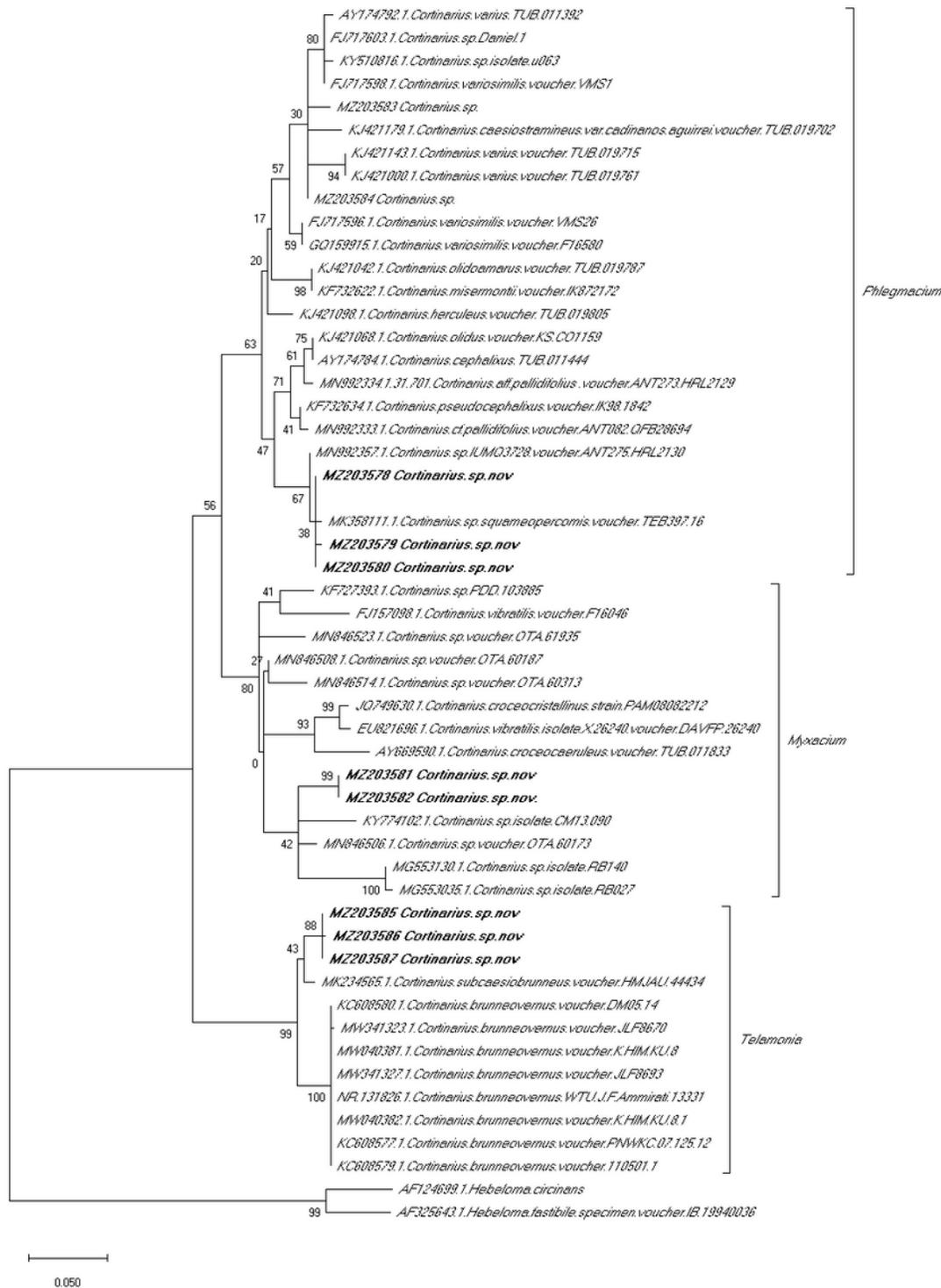


Figure 1

Maximum likelihood tree inferred from ITS sequences. The tree is rooted with *Hebeloma* species treated as outgroup. Newly reported species are shown in Bold. The ML bootstrap values are shown on each branch. The Bayesian posterior probabilities were calculated and found to be significant.



Figure 2

a, b. Sporocarp of *Cortinarius cibum*.

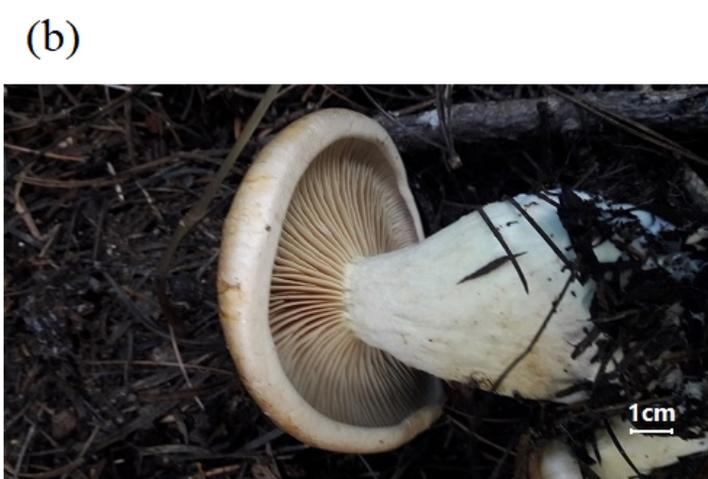
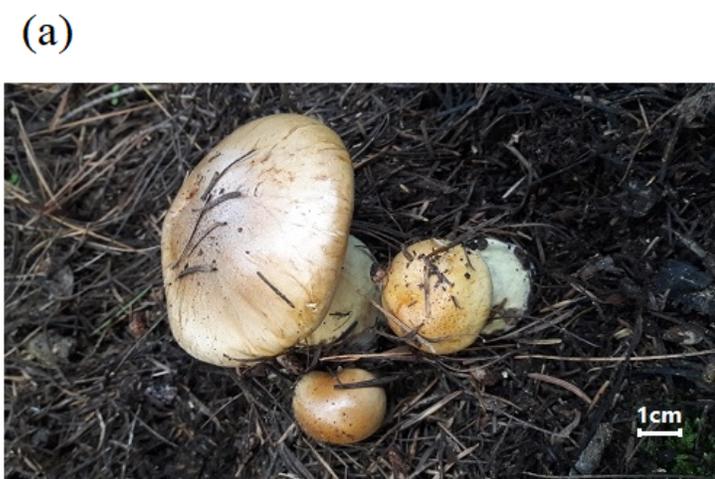


Figure 3

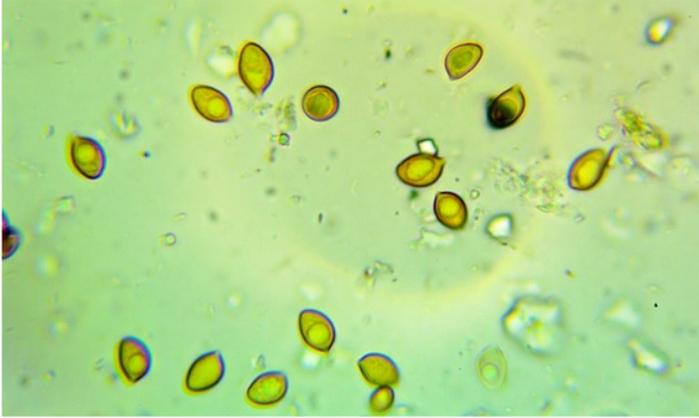
a, b. Sporocarp of *Cortinarius neocephalix*



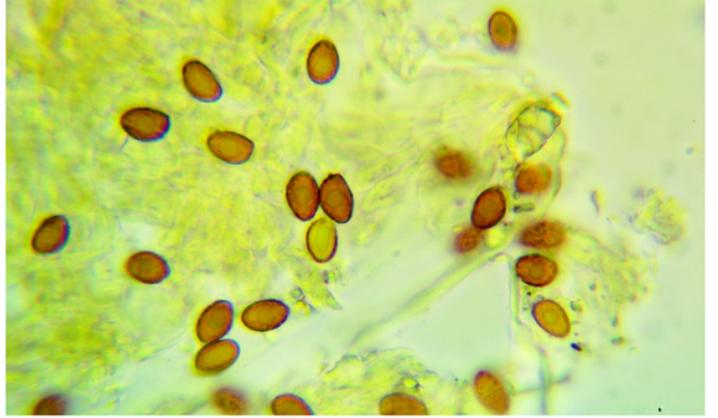
Figure 4

a, b. Sporocarp of *Cortinarius nigricans*.

(a)



(b)



(c)

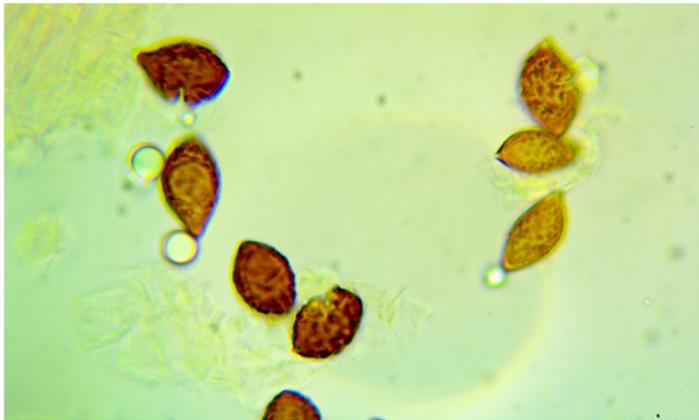


Figure 5

a–c. Microscopic structure of spores.