

Morphological and Molecular Insights into Two Russulaceous Mushrooms from Jammu and Kashmir, India

Komal Verma, Ayushi Mahajan, Sandhya Rajput, and Yash Pal Sharma*

Department of Botany, University of Jammu, Jammu - 180 006, Jammu and Kashmir, India.

*Corresponding author Email: yashdbm3@yahoo.co.in

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ABSTRACT

In this study, two species belonging to the family *Russulaceae*, *Russula aurantioflava* and *Lactarius kesiya*, are documented. *Russula aurantioflava* is reported as a new addition to the Indian mycobiota, while *Lactarius kesiya* is recorded for the first time from Jammu and Kashmir, India. Comprehensive macro- and micromorphological descriptions, along with illustrations and molecular analyses based on nrITS sequences, are provided.

Keywords: Macrofungi, nrITS, New record, Phylogeny, Regional records

INTRODUCTION

The *Russulaceae* Losty, family, belonging to the order *Russulales*, comprises a well-established group of ectomycorrhizal fungi that form a symbiotic relationship with a diverse array of host plants across both temperate and tropical forest ecosystems (Verbeken and Nuytinck, 2013; Looney *et al.*, 2018). This family has a widespread global distribution and includes more than 3,800 recognized species (Wijayawardene *et al.*, 2022). Molecular phylogenetic studies have consistently confirmed the monophyly of *Russulaceae*, which includes four agaricoid genera: *Russula* Pers., *Lactarius* Pers., *Lactifluus* (Pers.) Roussel, and *Multifurca* Buyck & Hofstetter. Among these, *Russula* is the most species-rich genus, which surpasses 2,000 species worldwide (Adamčík *et al.*, 2019; Liu *et al.*, 2024), followed by *Lactarius*, with over 670 species (Verma *et al.*, 2024; Bánki *et al.*, 2024), and *Lactifluus* with more than 230 species (De Crop *et al.*, 2021; Ghosh *et al.*, 2022; Xu *et al.*, 2023). In India, over 180 species of *Russula*, more than 100 species of *Lactarius*, and approximately 33 species of *Lactifluus* have been recorded so far (Das *et al.*, 2020; Ghosh *et al.*, 2022; Verma *et al.*, 2024).

Russula and *Lactarius* collectively comprise nearly 80% of the *Russulaceae* family, underscoring their dominant presence within this macro-fungal lineage. Species within *Russula* are characterized by their brightly coloured, fragile fruiting bodies, amyloid spore ornamentation, brittle context enriched with sphaerocytes, and the presence of gloeoplerous elements in various fruiting structures

(Ghosh *et al.*, 2023). In contrast, members of *Lactarius* are distinguished by their production of latex, as well as features such as an azonate to strongly zonate pileus, glutinous to dry surface texture, bearded to glabrous margins, absence of thick-walled elements in pileipellis and stipitipellis, amyloid ornamented basidiospores, sphaerocytes in the context, hymenial pseudocystidia, and lactiferous hyphae (Heilmann-Clausen *et al.*, 1998; Verma *et al.*, 2024).

During macrofungal surveys conducted in various regions of Jammu and Kashmir, India, multiple specimens of russulaceous mushrooms were collected from coniferous forests. Detailed morphological assessments, supplemented by molecular analyses, confirmed that *Russula aurantioflava* has been described here as a new record for the Indian mycobiota while *Lactarius kesiya* is reported for the first time from Jammu and Kashmir, India. This study presents comprehensive macro- and micromorphological descriptions, accompanied by illustrations and a phylogenetic analysis based on nrITS sequences.

MATERIALS AND METHODS

Macro- and Micromorphology

Fresh basidiomes were collected from the field and photographed using a Nikon D5300 camera. Macromorphological characteristics, along with habitat details and associated host information, were documented directly from fresh specimens.

The terminology for macromorphological features follows Vellinga (1988), while color descriptions were assigned according to the system proposed by Kornerup and Wanscher (1978). Chemical spot tests were conducted on the pileus surface, stipe surface, and context of fresh basidiomes using 10% KOH, FeSO₄, phenol, and guaiacol. For micromorphological analysis, freehand sections were prepared from dried specimens and mounted in 5% KOH, 1% Phloxine, or 1% Congo Red. Observations were performed using an Olympus CH20i compound microscope. Micromorphological structures were illustrated with the aid of a Camera Lucida at 2000× magnification, while photomicrographs were obtained using a digital camera mounted on an Olympus CX33 microscope. Basidiospore measurements were taken from 40 spores per specimen using Melzer's reagent and are presented as minimum-mean-maximum length × minimum-mean-maximum width. The Q value denotes the length-to-width ratio, while Qm represents the mean Q value across all observed basidiospores. Furthermore, scanning electron microscope (SEM) imaging of basidiospores was conducted on dried specimens to examine spore ornamentation patterns, utilizing a JOEL JSM-IT-300 model. All collected specimens have been deposited in the Herbarium of the Department of Botany, University of Jammu (acronym HBJU).

DNA Extraction, PCR Amplification, and Sequencing

Genomic DNA was extracted from 100 mg of dried basidiocarps using the Plant II Kit. The internal transcribed spacer (ITS) regions of nuclear ribosomal DNA were amplified using the ITS1 and ITS4 primers (White *et al.*, 1990). PCR amplification was performed in a 20 µl reaction mixture containing 1X Phire PCR buffer (with 1.5 mM MgCl₂), 0.2 mM of each dNTP, 1 µl DNA, 0.2 µl Phire Hotstart II DNA polymerase, 0.1 mg/ml BSA, 3% DMSO, 0.5M Betaine, and 5 pM of both forward and reverse primers. The PCR reactions were conducted using a GeneAmp PCR System 9700 (Applied Biosystems) with the following thermal cycling conditions: initial denaturation at 96 °C for 2 minutes, followed by 30 cycles of 30 seconds at 96 °C, 40 seconds at 50 °C, and a final extension at 60 °C for 4 minutes. The amplified PCR products were purified using the QIAquick Gel Extraction Kit (QIAGEN,

Germany) and subsequently sequenced using Sanger sequencing on ABI3730xl DNA Analyzer (Applied Biosystems, USA) with the same primers used for amplification. The obtained ITS sequences were deposited in GenBank, and accession numbers were assigned accordingly.

Phylogenetic analysis

Phylogenetic analysis based on nrITS sequences was carried out to establish the phylogenetic placement of our species. The data set used to analyze the phylogeny consisted of nrITS sequence of our collection, reference sequences acquired from nBLAST search against GenBank (Altschul *et al.*, 1997; Clark *et al.*, 2016) and relevant published phylogenies (Bera and Das, 2021; Adamcik *et al.*, 2019). A total, 27 nrITS sequences of *Lactarius* were used in the dataset of *L. kesiya* while 55 nrITS sequences of *Russula* were used in the dataset of *R. aurantioflava*. Sequences were aligned using MAFFT v.7 (Katoh and Standley, 2013). Phylogenetic analysis was undertaken based on maximum likelihood (ML) criteria in raxml- GUI ver. 2.0 (Edler *et al.*, 2021). One thousand bootstrap replicates were analysed to obtain nodal support values.

RESULTS

Phylogenetic inferences

Phylogenetic analyses have confirmed the identification of the current specimens as *Russula aurantioflava* and *Lactarius kesiya*. The sequences derived from Indian collection of *Russula aurantioflava* (GenBank OQ372974, OQ372985) were placed within the *R. aurantioflava* clade consisting of the sequences from Pakistan (GenBank MN130069, MN130070, MN130071) and supported by a robust bootstrap support of 98%. Moreover, the sequence derived from Indian collection of *Lactarius kesiya* (GenBank PV105568) was placed within the *L. kesiya* clade consisting of the sequences from Vietnam (GenBank KR025615, KR025614) and India (GenBank MW075677, MW075682), and supported by a robust bootstrap support of 100%. These molecular evidences, corroborated by congruent macro- and micromorphological characteristics, confirms the identity of present species (Figures 1 and 2).

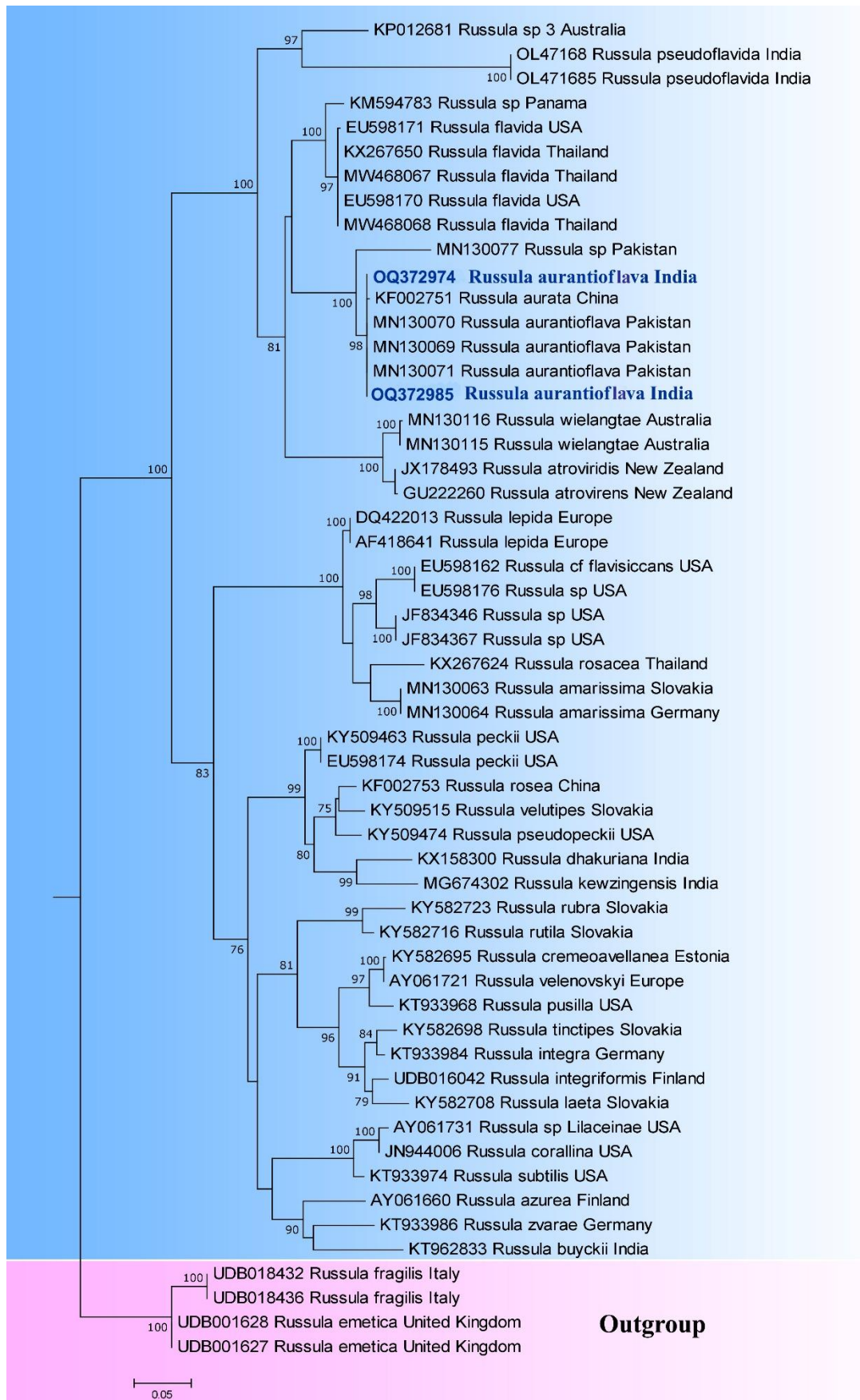


Figure 1: Phylogram generated from Maximum Likelihood approach (ML) in RAxML v7.2.6. Bootstrap support values (>50%) obtained from Maximum Likelihood (ML) analysis are shown above or below the branches at nodes. The Indian specimens of *Russula aurantioflava* are shown in blue and bold in the phylogenetic tree

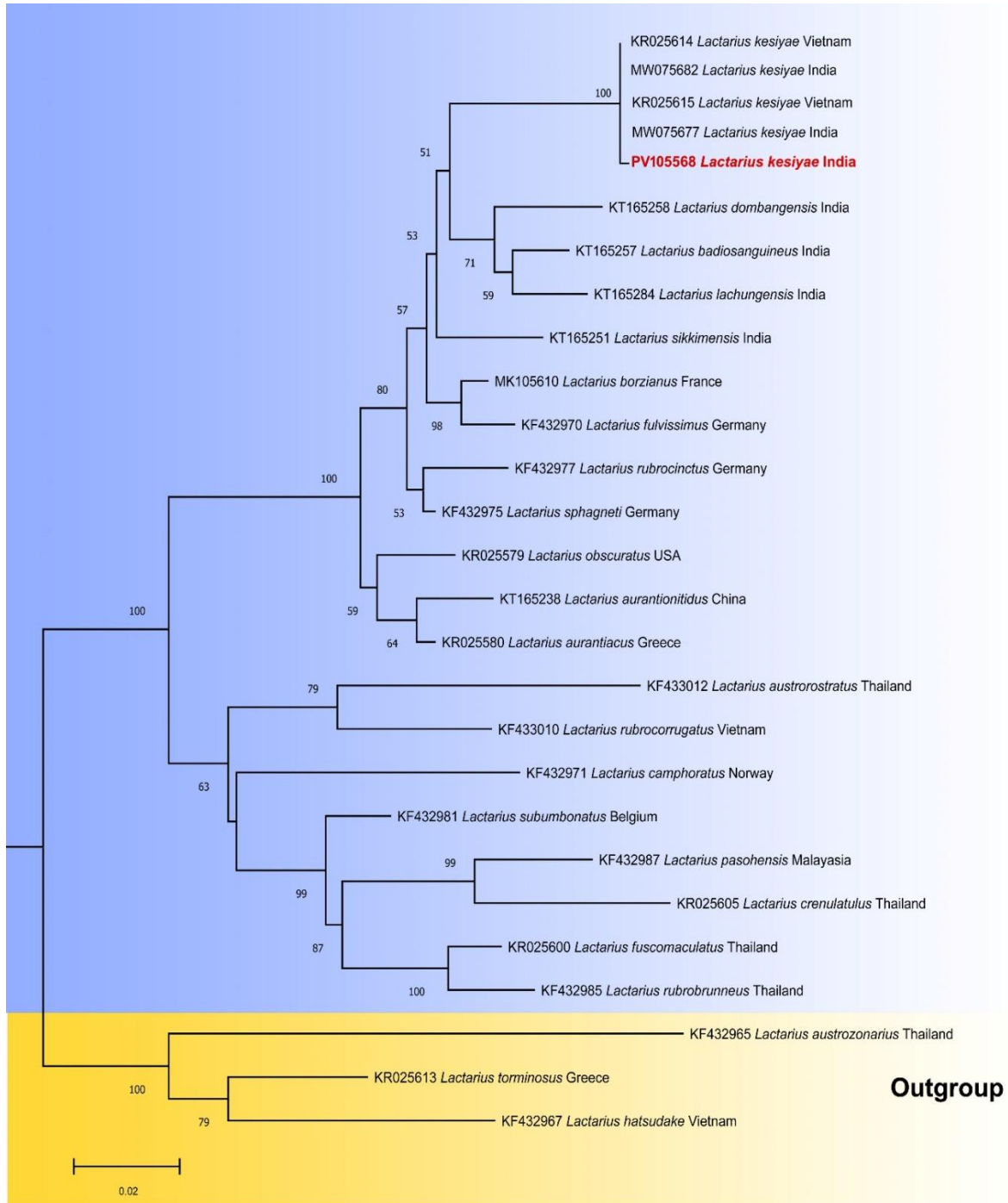


Figure 2: Phylogram generated from Maximum Likelihood approach (ML) in RAXML v7.2.6. Bootstrap support values (>50%) obtained from Maximum Likelihood (ML) analysis are shown above or below the branches at nodes. *Lactarius kesiyae* is shown in red and bold in the phylogenetic tree.

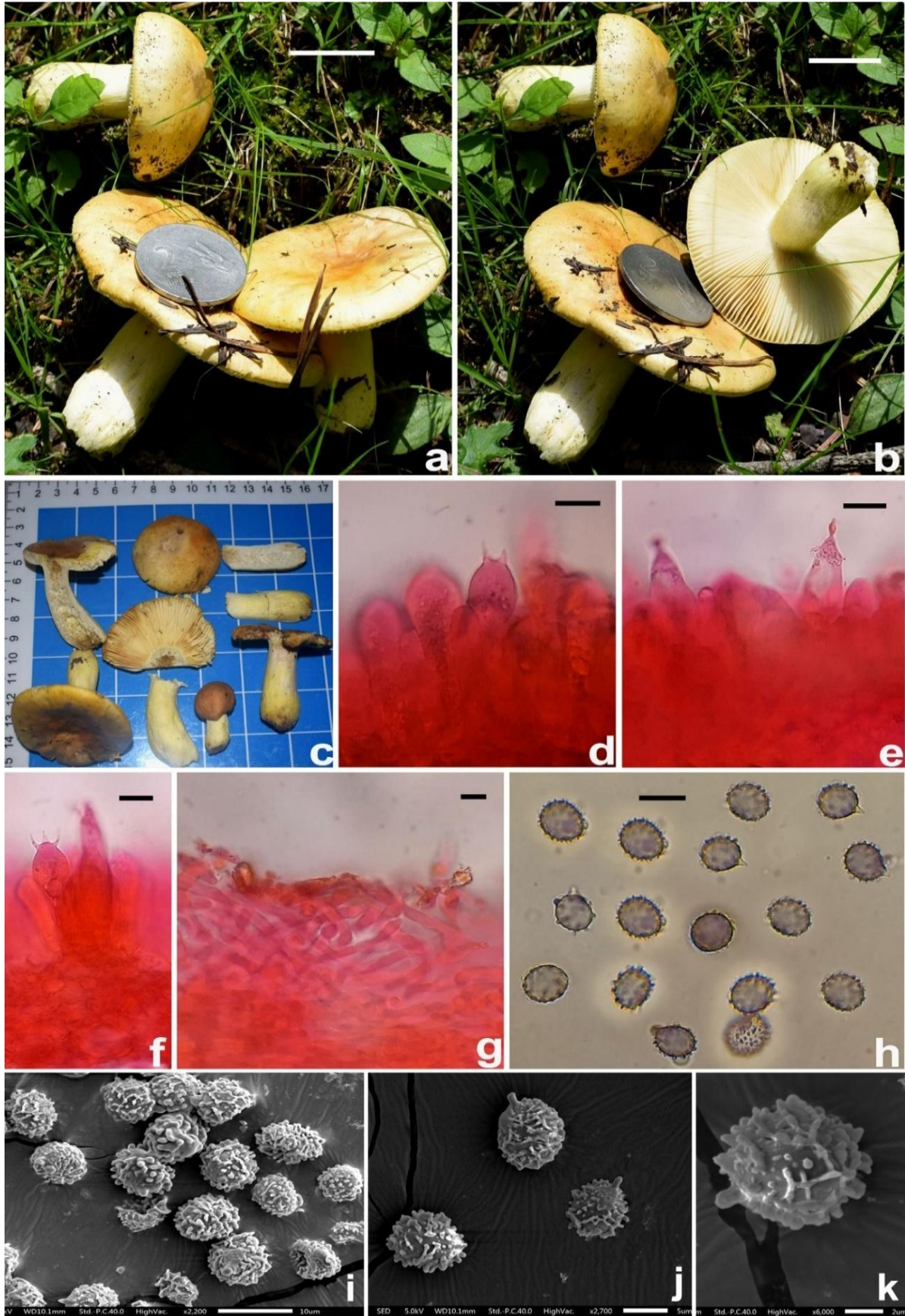
Taxonomy

Russula aurantioflava Kiran & Khalid, *Fungal Diversity* 99: 396 (2019) **Figures 3 and 4.**

GenBank: OQ372974, OQ372985

Description: Basidiomata small to medium sized. Pileus 52–68 mm broad, initially hemispherical to convex, becoming planoconvex to applanate with slight depression in the centre, margin initially entire becoming crenate with age, smooth to faded

striations present when mature, dry, smooth, shiny, become viscid when moist, pale yellow (4A3) to deep yellow (4A6), golden yellow (4C6) to greyish yellow (4B6) in the centre, light yellow (4A5) to pale yellow (4A3) towards margin. Lamellae 2–4 mm broad, adnexed to adnate, crowded 11–13 L+I/cm, pale yellow (4A3), entire, concolorous edges. Stipe 30–40 × 15–18 mm, equal, cylindrical, solid, central, longitudinally striate, yellow white (2A2) to vivid yellow (2A8).



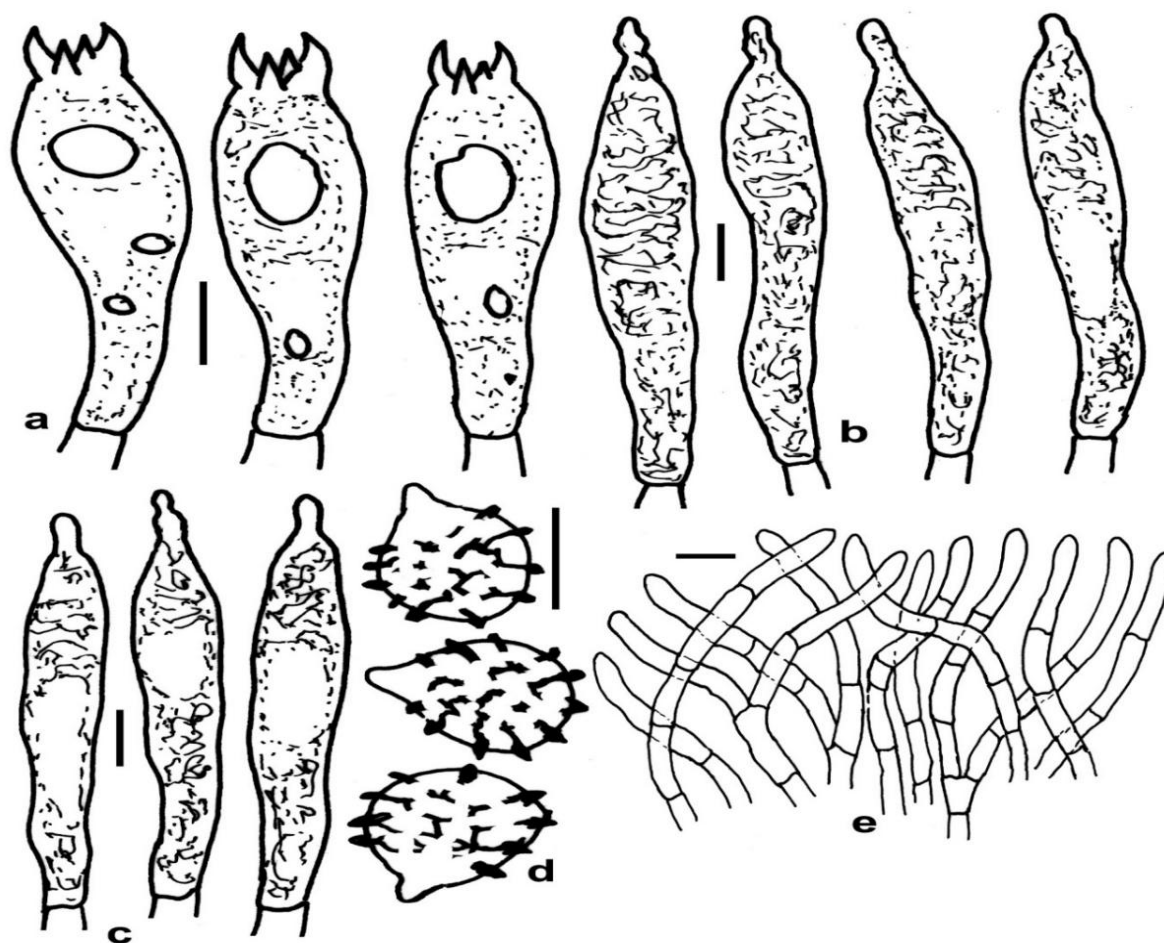


Figure 4: Line drawings of *Russula aurantioflava*. a, Basidia; b, Pleurocystidia; c, Cheilocystidia; d, Basidiospores; e, Transverse section through pileipellis. Scale bars: a–e = 10 µm.

Context 3–5 mm thick at mid pileus, yellowish white (2A2), solid to stuffed at stipe, compact, brittle, immediately changing to reddish brown (8D6) with guaiacol. Pileus surface changing to slight brownish with KOH and reddish brown (8D6) with guaiacol. Odour insignificant. Taste mild.

Basidiospores $8.0\text{--}9.7\text{--}11.5 \times 7.5\text{--}8.2\text{--}9.0 \mu\text{m}$, $Q=1.06\text{--}1.18\text{--}1.27$ ($n=40$), subglobose to broadly ellipsoid, amyloid ornamentation, consists of isolated warts or spines and fine lines or ridges connected together to form an incomplete reticulum, sometimes seem as small crests, suprahilar plage amyloid. Basidia $45\text{--}65 \times 10\text{--}14 \mu\text{m}$, subclavate to clavate, 4-spored; sterigmata $3\text{--}6 \mu\text{m}$ long. Pleurocystidia $58\text{--}80 \times 9\text{--}12 \mu\text{m}$, emergent about $28 \mu\text{m}$ subclavate to clavate, lanceolate, with moniliform, mucronate, capitate and obtuse or acute apices, granular to crystalline dense content. Lamellar edges fertile with basidia, basidioles and cheilocystidia. Cheilocystidia $32\text{--}54 \times 7\text{--}10 \mu\text{m}$, sub-cylindrical to subclavate, mucronate, capitate and obtuse or acute apices, granular to crystalline dense content. Pileipellis

$100\text{--}150 \mu\text{m}$ thick, ixotrichoderm type, mainly consists of branched, loose hyphal terminations, suprapellis $60\text{--}75 \mu\text{m}$ thick, mainly consists of subulate, cylindrical and apically constricted terminal cells $20\text{--}35 \times 7\text{--}15 \mu\text{m}$, subpellis $40\text{--}75 \mu\text{m}$ thick, consists of irregular and near horizontally oriented, $2.5\text{--}4.5 \mu\text{m}$ wide hyphae. Pileocystidia absent.

Habit and habitat: Solitary to scattered, gregarious, occurring under *Pinus wallichiana* and *Abies pindrow* in a mixed coniferous forest.

Distribution: Previously reported from Pakistan (Adamcik *et al.*, 2019).

Specimen examined: India, Jammu and Kashmir, Jammu, Kishtwar, Chingam, 3297 m.a.s.l altitude, N $33^{\circ}34'.057''$ E $75^{\circ}33'.562''$, 16 July 2021. K. Verma and Y. P. Sharma. Chingam, 3112 m.a.s.l altitude, N $33^{\circ}32'.558''$ E $75^{\circ}33'.341''$, 18 July 2022. K. Verma and Y. P. Sharma, HBJU99.

Notes: *Russula aurantioflava*, which was originally reported from Pakistan was characterized by medium-sized, convex, becoming applanate when

old, lemon to golden yellow coloured pileus with darker orange centre, vivid yellow lamellae edges and stipe, large basidiospores, with warts linked in the forms of chains, strongly gelatinized, pileipellis with loose hyphal terminations of two types; apically attenuated with longer terminal cells and apically obtuse with shorter ones, without defined pileocystidia and its occurrence under *Pinus wallichiana* and *Abies pindrow* (Adamcik *et al.*, 2019). Our collection shows similar characters with the holotype reported from Pakistan, and nrITS-based phylogenetic analyses also validate the identification of the current species as *Russula aurantioflava* (Figure 1).

Lactarius kesiya Verbeken & K.D. Hyde
Phytotaxa 207: 229 (2015) **Figures 5 and 6.**

Genbank: PV105568

Description: Basidiomata small to medium sized. Pileus 23–48 mm broad, convex when young with small umbo, becoming planoconvex to applanate to uplifting with age, sometime umbo remains;

surface smooth when young become wrinkled with age, viscid, moist in young specimens, yellowish grey (3B2) with slight light orange (5A4) at the centre, pale yellow (4A3) to light yellow (4A4) towards margin; margin entire, decurved, becoming striate with maturity, greyish orange (5B4) spots are prominent in the matured basidiomata. Lamellae 5–8mm in width, crowded (13–16/cm), adnate to subdecurrent, edge entire, pale yellow (2A2) to yellowish grey (2B2) when young, becoming light orange (5A4) or darker with maturity, lamellulae numerous 4–6 series. Stipe 25–40 × 4–10 mm, cylindrical, central to eccentric, slightly tapered toward base, surface viscid, light orange to pale orange (5A3), gradually becoming darker towards base, hollow. Context 5–8 mm in pileus, pale yellow (1A2), stuffed to hollow in stipe, immediately turning red to dark red in guaiacol. Latex watery white, moderate. Taste bitter, Odour fruity.

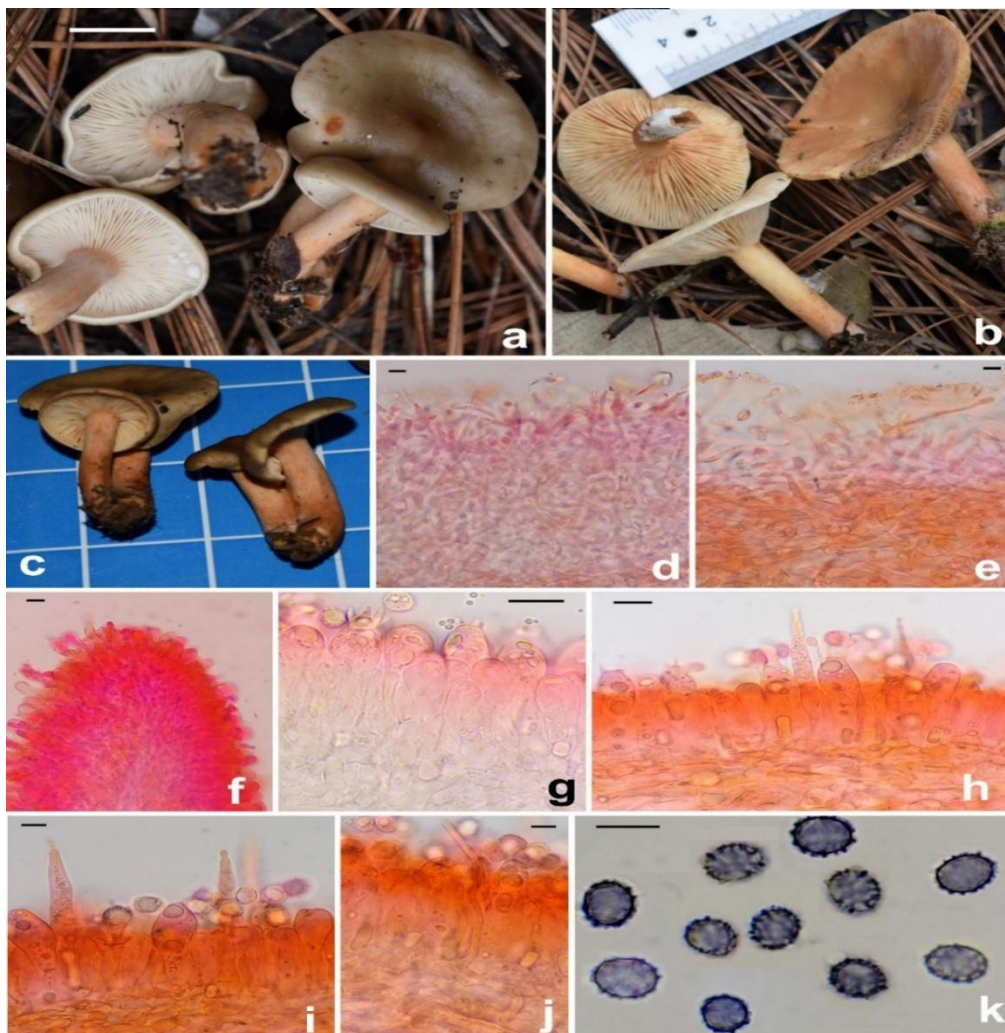


Figure 5: *Lactarius kesiya*. a–b. Basidiomata in the field; c. Basidiomata in the base camp; d & e. Transverse section through pileipellis; f. Cheilomacrocystidia; g & h. Basidia; i. Pleuromacrocystidia; j. Pleuropseudocystidia; k. Basidiospores under light microscope. Scale bars: a= 20 mm; d – k=10 µm.

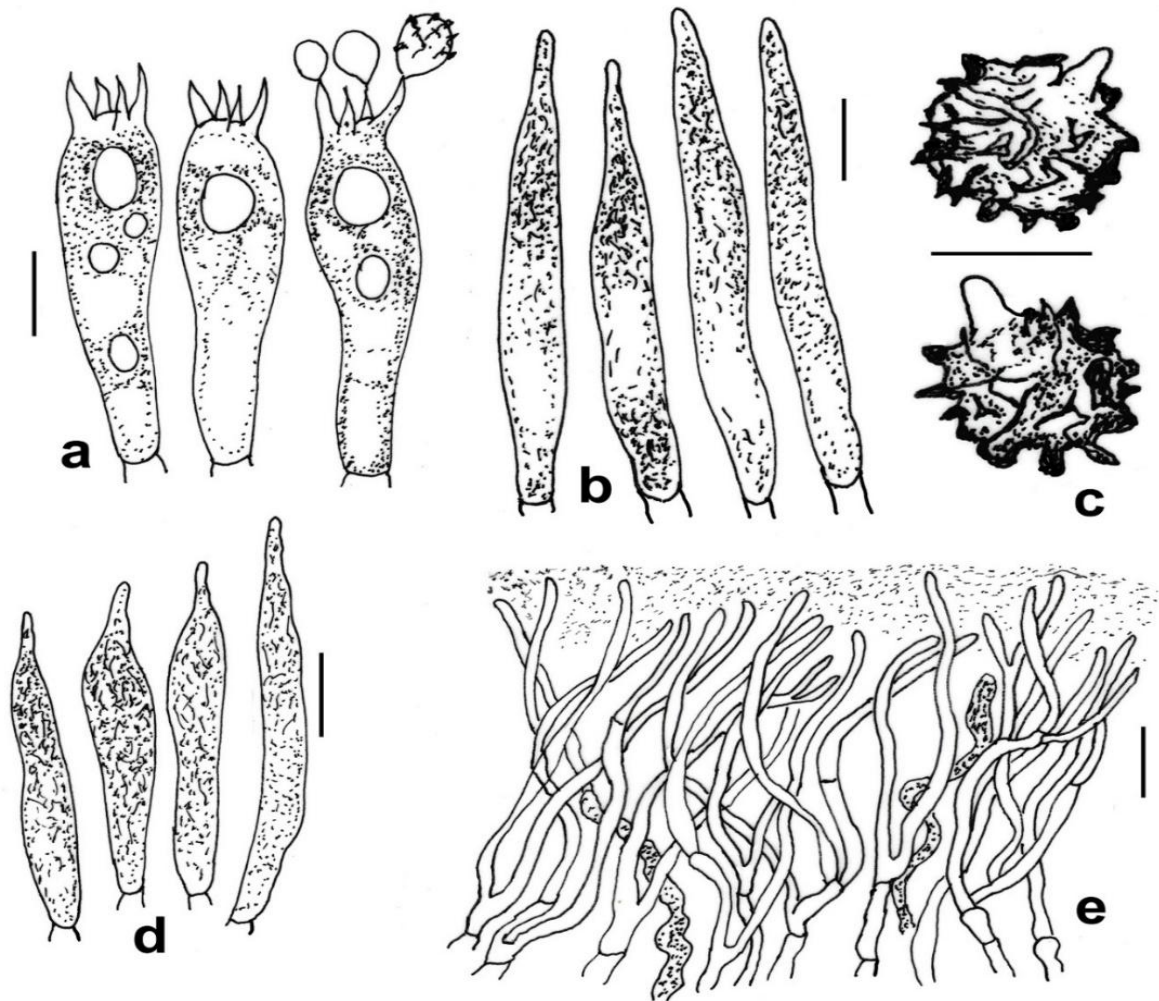


Figure 6: Line drawings of *Lactarius kesiya*. a. Basidia; b. Pleuromacrocystidia; c. Basidiospores; d. Cheilomacrocystidia; e. Transverse section through pileipellis. Scale bars: a-e =10 µm.

Basidiospores $6.4\text{--}7.5\text{--}8.5 \times 5.5\text{--}6.5\text{--}7.0$ µm, $Q=1.08\text{--}1.15\text{--}1.26$ ($n=40$), subglobose to broadly ellipsoid, sometimes ellipsoid, amyloid ornamentations up to 1.2 µm high, consist of ridges and isolated warts, forming an incomplete to almost complete reticulum, plagiately amyloid. Basidia $35\text{--}48 \times 8\text{--}10.5$ µm, subclavate, 4 spored, sterigmata $4\text{--}6$ µm long. Pleuromacrocystidia $55\text{--}85.6 \times 6.5\text{--}9.5$ µm, abundant, emergent up to 38.5 µm, subcylindrical with fusiform to subfusoid, mucronate, subcapitate apices, thin-walled; granular to fibrous content dense. Pleuropseudocystidia $3.5\text{--}5.5$ µm wide, abundant, mostly embedded in hymenium, cylindrical, tortuous, with rounded apex. Lamellar edge fertile with basidioles, basidia and cystidia. Cheilomacrocystidia $35.3\text{--}55 \times 5\text{--}6.5$ µm rare, emergent up to 25 µm, subcylindrical with subfusoid apex round to mucronate, thin-walled. Lactiferous hyphae up to 7 µm wide, abundant in hymenophoral trama. Pileipellis up to 130 µm thick, an ixotrichoderm, suprapellis composed of

erect to repent hyphae, hyphae $3\text{--}4.5$ µm wide. Stipitipellis ixocutis up to 45 µm thick, composed of interwoven, septate hyphae.

Habit and habitat: Solitary to scattered, common, under *Pinus roxburghii* in sub-temperate coniferous forest.

Distribution: Originally reported from Vietnam (Wisitrassameewong *et al.*, 2015), recently reported from Arunachal Pradesh, India (Bera and Das, 2021).

Specimens examined: India, Jammu and Kashmir, Jammu, Udhampur, Majalta, Samanabanj, 1507 m.a.s.l. altitude, $N32^{\circ}41'4.86''$ $E 75^{\circ}24'2.26''$, 30 July 2020. K. Verma and Y.P. Sharma. Jammu, Udhampur, Choremotu 1732 m.a.s.l altitude, $N 32^{\circ}43'4.30''$ $E 75^{\circ}25'8.53''$, 2 August 2020. K. Verma and Y. P. Sharma, HBJU64.

Notes: *Lactarius kesiya* is a quite distinct species within *Lactarius* subg. *Russularia* due to the

presence of a viscid to sticky pileus due to which microscopically it is reflected as an ixotrichoderm type of pileipellis. This species was originally described from Vietnam by Wisitrassameewong *et al.* (2015). The main distinguishing characters of *L. kesiyae* are pale brownish grey to pale orange basidiomes, watery white latex, pale cream to cream lamellae turning greyish orange on bruising. Microscopically, it is characterized by suglobose to broadly ellipsoid basidiospores ($6.3\text{--}7.8 \times 5.7\text{--}6.8 \mu\text{m}$), with up to $1.2 \mu\text{m}$ high ornamentations; abundant pleuromacrocystidia, non-emergent pseudocystidia and ixocutis to ixotrichoderm type of pileipellis. Our specimen exhibits close resemblance with Indian specimen described from Arunachal Pradesh (Bera and Das, 2021) and with Vietnamese holotype. However, in present specimen, slightly larger basidiospores were observed but other characters like ixotrichoderm type of pileipellis and its occurrence under *Pinus* sp. are similar to the holotype. Moreover, nrITS-based phylogenetic analyses also validate the identification of the current species as *Lactarius kesiyae* (Figure 2).

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