Morphological and phylogenetic evidence for two new *Lactarius* species (Russulales, Basidiomycota) from India

Kanad DAS\textsuperscript{a}, Annemieke VERBEKEN\textsuperscript{b}, Dyutiparna CHAKRABORTY\textsuperscript{a}, Rameshwar AVCHAR\textsuperscript{c} & Abhishek BAGHELA\textsuperscript{c}\textsuperscript{*}

\textsuperscript{a}Cryptogamic Unit, Botanical Survey of India, P.O. Botanic Garden, Howrah 711103, India, email:daskanadbsi@gmail.com

\textsuperscript{b}Ghent University, Department of Biology, Research Group Mycology, K.L. Ledeganckstraat 35, BE 9000, Gent, Belgium, email: Mieke.Verbeken@ugent.be

\textsuperscript{c}MACS’ Agharkar Research Institute, Biodiversity and Palaeobiology Group, National Fungal Culture Collection of India (NFCCI), G.G. Agarkar Road, Pune – 411004, India, email: abhishekbaghela@aripune.org

**Abstract** – *Lactarius ferruginascens* sp. nov. and *L. indoaquosus* sp. nov. are described from the East and South districts of Sikkim, a small Himalayan state in India. Macro- and micromorphological descriptions coupled with the illustrations and nrITS-based molecular analyses are given for both species. *Lactarius ferruginascens* is a species in *L.* subg. *Plinthogalus* with striking features such as a sticky pileus surface caused by the ixohymeniderm structure of the pileipellis, watery white latex which is turning rusty brown on the lamellae and the occurrence under *Abies* sp. in the subalpine mixed forest. *Lactarius indoaquosus* is a new representative of *L.* subg. *Russularia* with completely transparent and unchanging latex and is furthermore characterized by basidiospores with a zebroid ornamentation, the absence of hymenial macrocystidia and the occurrence under *Castanopsis* sp. in the temperate broadleaf forest. Both species are illustrated and compared with related taxa.

**INTRODUCTION**

Milkcaps are forming an important group of macrofungi, striking in the field because of the exudation of latex and ecologically indispensable in all ecosystems worldwide as they belong to the largest genera of ectomycorrhizal fungi (EM). The traditional genus *Lactarius* was recently (Buyck et al. 2008) split in two milkcap genera: *Lactarius* (about 450 described species worldwide, 83 reported from India), *Lactifluus* (about 150 described species worldwide, 27 reported from

\* Corresponding author: abhishekbaghela@aripune.org
India) and a mixed genus *Multifurca*. The latter one is a very small genus (7 described species worldwide and only 1 from India), with a remarkable distribution: North and Central America, Asia and Australia, but absent from Europe and Africa (Buyck *et al*. 2008; Atri *et al*. 2016).

Metagenomic studies support the view that the EM genus *Lactarius* is one of the most diverse and abundant genera at high altitudes and latitudes (Geml *et al*. 2009, 2011) forming EM with important plant families such as Fagaceae, Betulaceae or Pinaceae. Since 2008, we carry out an intensive exploration and inventory of macrofungi in Sikkim, a small Indian state bordering Nepal, Bhutan, Tibet and China. Sikkim belongs to the Eastern Himalaya Global Diversity Hotspot (Mittermeier *et al*. 2005) and its location on the border between the Oriental and Palearctic eco-regions, and the variation in elevation (300-8598 m) and climatic regime (tropical to cold desert) provide a wide range of vegetation types, from subtropical to temperate coniferous, broadleaf and mixed forests, alpine forests, shrubs and meadows (Bhupathy *et al*. 2009; Das *et al*. 2010). Before 2008 very little was known about the EM fungi, but since we started our fungal biodiversity study in the area 17 *Lactarius* species (14 new to science) and 10 *Lactifluus* species (5 new to science) were reported from this state (Das & Verbeeken 2011, 2012; Van de Putte *et al*. 2012; Das & Chakraborty 2014; Das *et al*. 2015, 2017; Wisitrassameewong *et al*. 2016). Sixteen of these species are occurring in the subalpine zone (2700-5000 m), whereas eleven occur in the temperate zone (1500-2700 m). In this paper we introduce two more new *Lactarius* species: *L. ferruginascens* growing under *Abies* in the subalpine zone and *L. indoaquosus* growing under *Castanopsis* in the temperate zone (Fig. 1) of East and South districts of Sikkim respectively. Both species are presented with morphological details and molecular data of the nrITS region.

**MATERIAL AND METHODS**

**Collecting and morphology**

Field and macromorphological characters, ecological notes, macrochemical color tests (with the application of 10% KOH, guaiac and FeSO₄) were recorded from young to mature fresh basidiomata in the field and/or in the base camp. Specimens were dried with hot air using a portable aluminium field dryer. Images of fresh basidiomata and microphotographs were captured with Canon SX 120 and Nikon-DS-Ri1 (dedicated to “Nikon Eclipse Ni” compound microscope) cameras respectively. Color codes and terms mostly follow Methuen Handbook of Color (Kornerup & Wanscher 1978). Micromorphological features were recorded with the help of the aforementioned compound microscope from free-hand sections of dry basidiomata mounted in 5% KOH, or stained in a mixture of 5% KOH and phloxin and mounted in 30% glycerol or distilled water. Micromorphological drawings were made with the help of a drawing tube (attached to Nikon-DS-Ni1) at 400× and 1000× magnifications. Basidium length excludes length of sterigmata. Basidiospores were mounted in Melzer’s reagent and spore measurements were recorded in profile view from twenty spores. Spore-size measurements and length/width ratios (Q) are given as: minimum-mean-maximum. Herbarium codes follow Thiers (continuously updated).
DNA isolation, amplification and sequencing

Genomic DNA was extracted from dried herbarium specimens (10-50 mg) using the Fungal gDNA Mini Kit (Xcelris Genomics, Ahmedabad, India). The nuclear ribosomal ITS region was amplified using the primers ITS4 and ITS5 (White et al. 1990). PCR was performed in a 50 μl reaction using 2 μl template DNA (10-20 ng), 0.5 U Taq DNA polymerase (Sigma-Aldrich, India), 5 μl 10× Taq DNA polymerase buffer, 1 μl 200 μM of each dNTP (Sigma-Aldrich, India), 1 μl of each primer ITS4 and ITS5 (10 pmole μL⁻¹), and the remaining volume was made up by H₂O (Sterile Ultra Pure Water, Sigma-Aldrich). Amplification was done using an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) with the following parameters: 5 min step at 95°C, followed by 30 cycles of 1 min at 95°C, 30 s at 55°C, and 1 min at 72°C and a final 7 min extension step at 72°C. The PCR products were purified with an Axygen PCR cleanup kit (Axygen Scientific Inc, CA, USA). Sequencing of the PCR products was accomplished with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA), using the amplification primers. The cycle sequencing products were run on an ABI Avant 3100 automated DNA sequencer (Applied Biosystems, USA). The raw DNA sequencing files were edited and combined using ChromasLite v. 2.01. The final sequence data were deposited in the NCBI nucleotide sequence database (accession numbers: ITS-rDNA for KD 16-051 and KD 16-006 are KY867683 and KY867682 respectively).
Phylogenetic analysis

Phylogenetic analyses based on ITS-rDNA sequence data were carried out to establish the phylogenetic placement of the two new species. Two separate ITS datasets (respective reference sequences and out-groups) were assembled from previous studies on this genus, one on L. subg. Plinthogalus and another one on L. subg. Russularia (Le et al. 2007; Stubbe & Verbeken 2012; Lee et al. 2015; Wisitrassameewong et al. 2015; Wisitrassameewong et al. 2016; Hyde et al. 2016) and from BLAST (Altschul et al. 1997) as suggested by BLAST searches in GenBank (Clark et al. 2016). Alignments were performed using CLUSTAL W (http://www.ebi.ac.uk/clustalw/) and the relationships were inferred by the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura 1980). One-thousand bootstrap replicates were analyzed to obtain nodal support values. L. deliciosus and L. austrotorminosus were chosen as outgroup taxa for the phylogeny of Lactarius ferruginascens sp. nov., similarly L. friabilis, L. fuliginosus, and L. pterosporus were considered as the outgroup taxa for the phylogeny of Lactarius indoaquosus sp. nov. The bootstrap values below 50% were not shown in the phylogenetic trees. The phylogenetic analyses were conducted in MEGA 6.0 (Tamura et al. 2013).

RESULTS

Phylogenetic inference

Our ITS-based phylogenetic analysis (Fig. 2) conducted with 36 nucleotide sequences supports (bootstrap 100%) the monophyly of Lactarius subg. Plinthogalus with L. deliciosus and L. austrotorminosus of L. subg. Lactarius as outgroup taxa. It shows that the sequence derived from KD 16-051 (i.e. Lactarius ferruginascens, GenBank KY867683) is clearly clustered amongst the other species of L. subg. Plinthogalus, but is recovered as a distinctly different taxon on a separate branch in a significantly supported (71% bootstrap) clade being sister to the branch bearing two species, L. lignyotus and L. fallax.

Similarly, our second ITS-based phylogenetic analysis (Fig. 20) undertaken with 56 nucleotide sequences places the taxa of L. subg. Russularia in a single clade (with L. friabilis, L. fuliginosus and L. pterosporus from L. subg. Plinthogalus as outgroup taxa). It shows that KD 16-006 (GenBank KY867682) i.e. L. indoaquosus is clearly nested in L. subg. Russularia but turns out to be an individual species on a separate and well supported (76% bootstrap) branch being sister to the clade containing L. aquosus and L. quietus.

Taxonomy

Lactarius ferruginascens K. Das & Verbeken, sp. nov. Figs 2-19

MycoBank: MB820868.

Diagnosis: Differing from the related species L. lignyotus and L. crenulatus by the presence of a glutinous to sticky pileus surface and an ixohymeniderm structure of the pileipellis.

Two new Lactarius species from India

**Etymology**: Referring to the latex staining reddish brown to rust color on the lamellae.

**Pileus** 18-47 mm diam., at first planoconvex with a central papilla, becoming infundibuliform or uplifted with depressed center and with a small papilla with maturity; surface glutinous, sticky, brown (6E5-8) to rusty brown or chocolate brown to grey; margin slightly incurved when young, decurved at maturity, non-striate, irregularly wavy or undulated to interrupted. **Lamellae** subdecurrent to decurrent, crowded (22-25/cm at pileus margin), never forked, initially white (2A2), gradually pale yellow (4A3) with maturity, with lamellulae in five series; edge entire, greyish brown (6D3) in part. **Stipe** 45-75 × 4-8 mm, central, cylindrical or slightly wider towards base, mostly curved, minutely pruinose, brown (6E4-5), greyish brown (8F3) to dark brown (9F4) or concolorous with the pileus but yellowish white to pale yellow (3A2-3) towards base and white at the extreme base. **Context** white (chalky), hollow or pithy in stipe, changing to orange white (6A2).
with FeSO$_4$, pale yellow (3A3) with KOH and brownish orange (6C4) with guaiac. **Latex** watery white, unchanging when isolated but, becoming brownish red to reddish brown (9C7-9D8) or rusty on cut lamellae after a long exposure, abundant, mild. **Odor** mild, spicy. **Taste** mild. **Spore print** not known.

Two new *Lactarius* species from India

**Basidiospores** 8.7-9.3-10 × 8.1-8.9-9.5 μm, (n = 20, Q = 1.00-1.03-1.09), globose to subglobose; ornamentation amyloid, 2-2.8 μm high, composed of irregular to regular ridges which are aligned or connected and forming a complete reticulum; plage distinct and strongly distally amyloid; under SEM, margin of ridges with irregular spine-like extremities. **Basidia** 35-57 × 12-15 μm, 2- to 4-spored, clavate to subclavate to subventricose; sterigmata 7-9 × 2-3 μm. **Pleuromacrocytidia** absent. **Pleuropseudocystidia** abundant, irregularly cylindrical to tortuous, often branched at apex, thin-walled, mostly not emergent, 3-6 μm wide, with olivaceous or darker content. **Lamellae edge** sterile. **Cheilomacrocytidia** absent. **Cheilopseudocystidia**

present, same as pleuropseudocystidia. **Marginal cells** abundant, multiseptate, with terminal elements 12-32 × 4-11 µm, cylindrical to subclavate or subventricose, thin- to slightly thick-walled, often with brown intracellular pigmentation. **Hymenophoral trama** with lactifers. **Pileipellis** an ixohymeniderm, 70-90 µm thick (including 6-15 µm thick gluten layer); terminal elements cylindrical to subclavate or clavate, 11-37 × 7-18 µm, thin- to slightly thick-walled, with brown intracellular pigmentation. **Stipitipellis** a palisade, 60-75 µm thick, with terminal elements cylindrical to subventricose and thin-walled, with brown intracellular pigmentation. **Stipe trama** with abundant nests of sphaerocytes. **Clamp connections** absent.

**Other collection examined:** INDIA: Sikkim, North district, between Lachung and Yumthang, 27°46′42.4″N, 88°42′47.4″E, 3522 m asl., 27th Aug. 2011, K. Das, KD1 6-119 (CAL).

**Comments:** The combination of dark pigmented, often brown to grey pileus and stipe, basidiospores with high ornamentation, absence of macrocystidia and presence of abundant dark pigmented marginal cells twell for a placement in *L.* subg. Plinthogalus. An ixohymeniderm is exceptional in this group, but also occurs in the African *L. sulcatulus* Verbeken. Morphologically, *L. crenulatus* K. Das & Verbeken, which was also reported from Sikkim, reminds *L. ferruginascens* in the field. The former can be separated by the smaller pileus with absence of glutinous or sticky surface, the smaller stipe (40-57 × 3.5-5 mm), watery white latex drying pinkish on cut lamellae, an ixohymeniderm without glutinous layer as pileipellis structure and the occurrence under *Castanopsis* sp., a broadleaf tree (Das & Verbeken 2012).

Three species from this same subgenus, *L. fumosus* Peck, *L. subvernalis* var. albo-ochraceous Hesler & A.H. Sm., and *L. subvernalis* var. Himalayensis Atri, Siani & M.K. Saini were reported earlier from this subcontinent and appear to be close to *L. ferruginascens* (though for the first two species the conspecificity between North American and Indian taxa have not yet been confirmed through molecular studies). Morphologically, all three species can be distinguished from *L. ferruginascens* as they do not have an ixohymeniderm as a pileipellis, which is also reflected in the macroscopical aspect because they are not glutinous or sticky. Moreover, *L. fumosus* (Rawla 2002) has hyaline cheilocystidia and unbranched filiforme pleurocystidia, whereas both *L. subvernalis* var. albo-ochraceus and *L. subvernalis* var. Himalayensis possess pleuro- as well as cheilomacrocystidia, a rare character in *L.* subg. *Plinthogalus* (Atri et al. 1990; Atri et al. 1993).

*Lactarius lignyotus* Fr. and *L. fallax* A.H. Sm. & Hesler (both showing 97% identity under 100% query coverage) appeared as closest relatives of the new species in our phylogenetic analysis. Both in *L. lignyotus* and *L. fallax*, the injured lamellae turn dirty pinkish to reddish as in our new species, but they are separated from it in the field by the more robust nature of the basidiomata and the dry (not sticky) aspect of the pileus surface (always glutinous and sticky in *L. ferruginascens*) (Heilmann-Clausen et al. 1998; Kränzlın 2005). Micromorphologically, the hymeno-epithelium structure of the pileipellis and the basidiospores with comparatively low ornamentation are distinct for *L. lignyotus*, whereas *L. fallax* has a trichoderm structure as pileipellis and stipitipellis of “vertical-interwoven hyphae” (Hesler & Smith 1979).

**Lactarius indoaquosus** K. Das, Verbeken & A. Baghela, sp. nov.

**Figs 20-38**

*Mycobank*: MB820869.

**Diagnosis:** Distinct from the morphologically similar *L. aquosus* by the larger azonate pileus, absence of pleuromacrocystidia and a hymeniderm to trichopalisade as pileipellis structure.
Two new Lactarius species from India

Holotype: INDIA: Sikkim, South district, Maenam Wild Life Sanctuary, 27°18′44.1″N, 88°21′53.6″E, 2096 m asl., 16th Aug. 2016, K. Das, KD 16-006 (CAL).

Fig. 20. Phylogram generated from ITS-rDNA sequences: The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model [Kimura M. 1980]. The tree with the highest log likelihood (-4211.5117) is shown. Lactarius indoaquosus sp. nov. (KD 16-006) having GenBank accession number KY867682 is shown in red color. The Lactarius friabilis, L. fuliginosus, and L. pterosporus were considered as the out group. Evolutionary analysis was conducted in MEGA6 (Tamura et al. 2013).
**Etymology:** Referring to the locality (indo-) and the completely transparent latex (aquosus).

**Pileus** 25-65 mm diam., convex with slightly depressed center when young, gradually more depressed and often with a small umbo at the center but never

---

Figs 21-32. *Lactarius indoaquosus* sp. nov. (KD 16-006). **21 & 22.** Fresh/dissected basidiomata. **23 & 24.** Transparent (colorless) latex oozing out from the cut lamellae. **25 & 26.** Hymenium layer showing pleuropseudocystidia, basidia & basidioles. **27 & 28.** Radial section through pileipellis. **29 & 30.** Transverse section through stipitpellis. **31 & 32.** Basidiospores under SEM. Scale bars **25, 29 & 31 = 10 µm; 26, 27, 28 & 30 = 10 µm; 32 = 5 µm.**
infundibuliform; margin incurved to decurved but irregularly wavy or lobed; surface smooth, moist, never viscid or sticky, brownish orange, light brown to reddish brown (7-8D6-7). Lamellae adnexed or rarely subdecurrent, crowded (20-25/cm at pileus margin), initially white (1A1), becoming pale orange (5A3) after sometime or with age, with lamellulae in 9-10 series; edge entire, concolorous. Stipe 26-40 × 4-9 mm, mostly eccentric, slender, brittle, cylindrical or gradually tapered towards base;

surface smooth, dry, strigose (hairy) at base, brownish orange to light brown (7C-D5), changing greenish grey to dull green (28D2-3) with guaiac and grey (28D1) with FeSO$_4$. Context pithy in stipe, brownish orange (6C4), slightly darker sometimes after exposure. Latex abundant, watery, colorless (transparent), unchanging when isolated but, slightly yellowish shortly after exposure. Odor not distinctive. Spore print pale cream (3A2).

**Basidiospores** 7.1-7.95-9.0 × 6.8-7.62-8.4 μm, \( [Q = 1.00-1.04-1.09(1.13)] \), globose to subglobose; ornamentation amyloid, 2-2.3 μm high, composed mostly of parallel to subparallel ridges which are aligned and form a zebroid pattern; under SEM with short or medium, irregular to regular ridges which are aligned or connected forming a clear zebroid pattern, with some isolated small warts; plage indistinct and inamylloid. **Basidia** 30-44 × 9-13 μm, 4-spored, subclavate to ventricose; sterigmata 5-8.5 × 1.5-3 μm. **Pleuromacrocytidia** absent. **Pleuropseudocystidia** 5-6 μm wide, cylindrical with suboclavate, subcapitate or irregular apex, sometimes with frequent constrictions towards apex, 5-8 μm emergent. **Lamellae edge** fertile, mostly with basidioles, basidia and cheilopseudocystidia. **Cheilomacrocytidia** absent. **Cheilopseudocystidia** filamentous. **Subhymenium** 18-25 μm thick, pseudoparenchymatous. **Marginal cells** 11-20 × 7-11 μm, mostly clavate to subclavate, often multisepate. **Hymenophoral trama** with lactifiers. **Pileipellis** a hymeniderm to trichopalisade, 130-250 μm thick, composed mostly of hyphal to flattened cells arranged in chains; terminal cells 10-50 × 8-39 μm, cylindrical to appendiculate, pyriform, clavate to conical, thick-walled; wall 1-1.5 μm thick. **Pileus trama** with numerous nests of sphaerocytes. **Stipitipellis** 90-120 μm thick, a trichopalisade, composed of erect, frequently septate, thin- to slightly thick-walled hyphae; terminal cells cylindrical, narrowly to broadly clavate or bulbous. **Clamp connections** absent.

**Comments:** The orange to brown colored pileus and stipe, unchanging latex and hymeniderm to trichopalisade structure of the pileipellis fit well for *L. subg.* *Russularia*. *Lactarius aquosus* H.T. Le & K.D. Hyde, reported from Thailand and sharing the exudation of transparent latex, looks similar to *L. indoaquosus* in the field but the former differs in a smaller (21-37 mm diam.) zonate pileus, presence of pleuromacrocytidia and a cutis as pileipellis structure (hymeniderm to trichopalisade in the present species) (Wisitrassameewong et al. 2015).

*Lactarius helvus*, a European species with transparent latex appears to be closest (showing only 94% identity under 100% query coverage) to the present undescribed species in our phylogenetic analysis but is morphologically very different. *Lactarius helvus* has distinctly larger basidiomata (pileus 25-130 mm diam., stipe 15-100 × 6-22 mm) with faintly zonate, pinkish buff to yellowish brown, very dry pileus, basidiospores with low (0.5-1.0 μm) ornamentations, presence of pleuro- and cheilomacrocytidia and a trichoderm to cutis as a pileipellis structure (Heilmann-Clausen et al. 1998). Furthermore, *L. helvus* is recognized by the strong odour of fenugreek, celery or maggi while our new species has a mild spicy odour.

Two Asian species possessing reddish-brown pileus and zebroid ornamentation of basidiospores, e.g. *Lactarius verbekenae* K. Das, J.R. Sharma & Montoya and *L. chichuensis* W.F. Chiu may be confused with *L. indoaquosus*. But, unlike the latter one both *L. verbekenae* (originally reported from India) and *L. chichuensis* (originally reported from China) have colored (never transparant) latex (“yellowish-white” in *L. verbekenae* and “milk white” in *L. chichuensis*), smaller basidiospores with distinctly lower ornamentations [“6-7.8 × 5.8-7 μm” with ornamentations “up to 1.5 μm” high in *L. verbekenae* and “(5.7-)6.2-7.5(-8.5) μm” high in *L. chichuensis*].
Two new Lactarius species from India

× (5.2-)5.7-6.6(-8.0) μm” with ornamentations “up to 1 μm” high in L. chichuensis] and presence of pleuromacrocytidia in the hymenium layer (Wang & Liu 2002; Das et al. 2004).

Two other Asian species with a zebroid spore ornamentation but with a more brownish and less reddish cap are L. corrugatus Verbeken & E. Horak and L. castanopsis Hongo. Lactarius corrugatus (originally reported from Papua New Guinea) has basidiospores with remarkably abundant short ridges and warts between the wings, which is not the case in L. indoaquosus; it furthermore differs by the completely cellular pileipellis and sterile lamellae edge. In the field, L. corrugatus is recognized by the strong L. quietus-like smell. L. castanopsis has larger spores and remarkably large and fusoid to mucronate cheilocystidia.

Several Indian Lactarius, namely L. sikkimensis Verbeken & K. Das, L. atrii Van de Putte & K. Das, L. lachungensis Verbeken & Van de Putte and L. flavigalactus Verbeken & K. Das, also appear to be genetically close to our species but these four species have white to whitish latex. Moreover, L. sikkimensis has an ixotrichopalisade, L. atrii and L. flavigalactus have an ixocutis and L. lachungensis has an ixohyphoepithelium to ixotrichoepithelium as pileipellis structure (Wisitrassameewong et al. 2016).

Acknowledgements. The authors are thankful to the Director, Botanical Survey of India, Kolkata and the Director, Agharkar Research Institute, Pune for providing facilities, to the Scientist-in-Charge, BSI, SHRC, Gangtok and the entire Forest Department of Sikkim for issuing permit to KD and DC during the present studies. Help rendered by Subhash Pradhan (BSI, SHRC) is duly acknowledged.

REFERENCES


