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Lactarius subgenus *Russularia* (Basidiomycota, Russulales): novel Asian species, worldwide phylogeny and evolutionary relationships

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ABSTRACT

Lactarius subg. *Russularia* is a large group of milkcaps occurring almost worldwide and dominant in many ecosystems. In this study we focus on new diversity, evolutionary relationships, divergence time, and origin of the subgenus. Six conifer symbionts are described as new to science: *Lactarius atrii*, *L. aurantionitidus*, *L. dombangensis*, *L. flavigalactus*, *L. lachungensis*, and *L. sikkimensis*. Species delimitation is assessed based on the concordance between morphological characteristics and an ITS phylogeny. Infrageneric relationships were studied using a phylogeny constructed from concatenated ITS-*rpb2* data using Maximum Likelihood and Bayesian inference. Results show that species in this subgenus do not cluster together according to their geographic origin. Intercontinental sister relationships between Europe/Asia/North America are common but actual conspecificity is rare. This result suggests that allopatric speciation has played an important role within this subgenus. Only few morphological characteristics tend to be phylogenetically informative, with the most

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important being presence or absence of true cystidia and the pileipellis structure. Two datasets were generated in order to estimate the age of *L.* subg. *Russularia*. The results suggest the origin of *L.* subg. *Russularia* to be in the Mid Miocene period.

Introduction

One of the aims of taxonomy is to delimit and to recognize taxa. The challenge for mycologists is huge since recent hypotheses suggest that the majority of fungal species on the earth is unknown (Hawksworth 1991; Hawksworth & Rossman 1997; O'Brien *et al.* 2005; Kirk *et al.* 2008). Even for the conspicuous macrofungi the biodiversity is often underexplored (Verbeken & Buyck 2002; Mueller *et al.* 2007). More than 400 species have been described in the genus *Lactarius* Pers. (Verbeken 2001; Das *et al.* 2004, 2015; Le *et al.* 2007a, b; Verbeken *et al.* 2014). Many endemic species are recognized by local people, but remain undescribed, particularly in tropical forest ecosystems (Wang *et al.* 2015; Wisitrassameewong *et al.* 2015). Many species are hidden under species names that are described and known from a different continent. The fact that fungal species often only subtly differ in morphology has contributed to our poor understanding of their distribution. The idea that all fungi are everywhere has been abandoned and there is growing evidence that their distribution is shaped by macroecological and community assembly processes, but we still know little about both the general and specific patterns of fungal diversity (Tedersoo *et al.* 2014). Although many major ectomycorrhizal genera like *Russula* Pers, *Inocybe* (Fr.) Fr., *Cortinarius* (Pers.) Gray, *Amanita* Pers. and *Lactarius* Pers. are present on all habitable continents, it is clear that most fungal species do not generally have cosmopolitan distributions. Regional endemism is one of the most consistent results from sequence-based cross-continental-scale studies and climate and isolation seem to determine the patterns of fungal diversity at species level (Koljalg *et al.* 2013; Peay *et al.* 2016). Molecular phylogenetic analyses and detailed morphological studies are needed in *Lactarius* to test intercontinental conspecificity (Nuytinck *et al.* 2007). Species have been overlooked due to subtle morphological differences. We therefore assume that, even though it is one of the best studied genera of macrofungi, there is still an important gap in the knowledge of biodiversity in *Lactarius*.

Within *Lactarius*, three subgenera are currently accepted, with *Lactarius* subg. *Russularia* (Fr.) Kauffman being one (Heilmann-Clausen *et al.* 1998; Buyck *et al.* 2010; Verbeken *et al.* 2014). This subgenus has an almost world-wide distribution but is remarkably absent in tropical Africa (Verbeken & Walleyn 2010). The biodiversity of the subgenus is well-studied in Europe and in North America (Hesler & Smith 1979; Heilmann-Clausen *et al.* 1998; Basso 1999). Recently, a number of described species have been reported from Southeast Asia (Verbeken *et al.* 2001, 2014; Wisitrassameewong *et al.* 2014a, b, 2015; Liu *et al.* 2015). Central and South America, Russia and South Asia are underexplored. For listing generally accepted species of *L.* subg. *Russularia* (see Supplementary Table 1), we followed Hesler & Smith (1979) for North American representatives, and Heilmann-Clausen *et al.* (1998), Basso (1999), and Verbeken & Vesterholt (2008) for the European mycota. These contributions are the most recent and complete monographs for these regions. The subgeneric classification scheme used here has been used before and is partly consistent with the classification system of Heilmann-Clausen *et al.* (1998). Ninety-one species are presently accepted as members of *L.* subg. *Russularia* (see Table 1). Most species are agaricoid, except for three sequestrate species: *Lactarius hispanicus* Calonge & Pegler, *Lactarius borzianus* Cavara and *Lactarius falcatus* Verbeken & Van de Putte (Calonge & Pegler 1998; Nuytinck *et al.* 2003; Vidal 2004; Verbeken *et al.* 2014). The number of accepted species per geographic region are shown in Table 1.

Species traditionally placed in *L.* subg. *Russularia* are characterized by small to medium sized fruiting bodies which have a dry surface and are orange, warm brown to reddish brown. Among the three subgenera of *Lactarius*, latex features of the subgenus *Russularia* appear to be least variable. The latex ranges from transparent and watery white to white and may be unchanging or changing to cream or yellow on exposure. The rather uniform macromorphological features make it particularly difficult to recognize and delimit species and partly explains why many new taxa remain to be described. Microscopic characters have been used for the identification and discrimination of closely related species, but are insufficient to distinguish between all representatives.

Inconsistency in the importance attached to different morphological characters leads to taxonomic and nomenclatural debate. Since a number of species concepts have been established (de Queiroz 1998), the notion of concordance between morphological, ecological and phylogenetic species concepts has emerged (de Queiroz 2007). It has become an effective approach to resolving problems in species delimitation and exploring evolutionary relationships. This approach relies on data of phenotypic variation using the observation of macro- and micro-morphology and genotypic variation using DNA sequence data. Previous studies show that internal transcribed spacer of the nuclear ribosomal DNA (ITS) sequences are adequate to delimit species in *L.* subg. *Russularia*, however, cannot adequately resolve evolutionary relationships of ancestral nodes in the phylogeny (Das *et al.* 2015; Wisitrassameewong *et al.* 2014a, b, 2015). A multi-locus phylogeny can resolve species complexes, higher taxonomic evolutionary relationships, and sub/generic classification. In this study, we selected ITS and the second largest subunit of RNA polymerase II (*rpb2*) sequence data to infer evolutionary relationships of *L.* subg. *Russularia* because these gene markers contain most phylogenetic signal at subgenus and species level, more than the large subunit of the nuclear ribosomal DNA (LSU).

Table 1 – The current number of accepted species of *L.* subg. *Russularia* according to geographic region and references. The total numbers of species per region including morphologically described species but excluding synonyms and species varieties.

Region	Species	References
Europe	19	Basso 1999; Calonge & Pegler 1998; Heilmann-Clausen *et al.* 1998; Vidal 2004; Verbeken & Vesterholt 2008, 2012
North and Central America	33[a]	Hesler & Smith 1979; Kuo *et al.* 2013; Montoya & Bandala 2008; Montoya *et al.* 2014
South America	1	Singer 1962
East Asia	2	Hongo 1957a,b
Southeast Asia	23	Chiu 1945; Liu *et al.* 2015; Verbeken *et al.* 2001, 2014; Wisitrassameewong *et al.* 2014a, b, 2015; Ying 1991
South Asia	4	Das *et al.* 2004, 2015
Oceania	9	Miller & Hilton 1987; Verbeken & Horak 2000

a The sections *Subsquamulosi* and *Triviales* of Hesler & Smith (1979) were excluded.

Within *L.* subg. *Russularia*, several names that were published in European literature turn out to be synonyms or illegimate names, whereas intercontinental conspecificity of many species remains unclear. For example, *Lactarius subsericatus* (Kühner & Romagn.) ex Bon is a synonym of *Lactarius fulvissimus* Romagn. and *Lactarius lapponicus* Harmaja is a synonym of *Lactarius duplicatus* A.H. Sm. (Heilmann-Clausen *et al.* 1998). A number of European names have been applied for Asian specimens without molecular support. Thus, intercontinental conspecificity of *L.* subg. *Russularia*, and in particular European and Asian species, will be discussed in this study.

Molecular clocks can provide the implementation of molecular change into geological times of organisms. All divergences begin when an ancestral species genotypically deviates and eventually develops morphological differences in its progeny. Generally, nucleotide variations and substitution rates vary among organisms. Evolutionary mycologists attempt to estimate divergence times of fungi using sequence data of particular genes and calibration points that are obtained from fungal fossils (Taylor & Berbee 2006; Berbee & Taylor 2010; Floudas *et al.* 2012). In this study we aim to: 1) introduce six new *Russularia* species from India and China, 2) explore the delimitation of the subgenus by including borderline taxa (*Lactarius rufus* (Scop.: Fr.) Fr., *Lactarius helvus* (Fr.: Fr.) Fr., *Lactarius chrysorrheus* Fr. and *Lactarius vinaceorufescens* A.H. Sm.), 3) carry out two locus analyses (ITS–*rpb2*) to test whether we can find concordance between morphology and phylogeny, 4) discuss intercontinental conspecificity and 5) estimate the divergence time of *L.* subg. *Russularia*.

Material and methods

Examined specimens

Indian samples were collected in Sikkim province (Himalaya region) in 2009. Chinese samples were collected in Diqing Tibetan Autonomous Prefecture, Yunnan province from 2012 to 2013. Studied areas, altitude, and plant composition in this study are shown in Table 2. Indian specimens are deposited in the Herbarium Universitatis Gandavensis (GENT), Belgium and Central National Herbarium (CAL), India. Chinese specimens are deposited in Kunming Institute of Botany (KIB), China and GENT. The majority of the European and the remaining Asian specimens used in this study are obtained from GENT and Mae Fah Luang University Herbarium (MFLU), Thailand. All new species are submitted to Faces of Fungi (Jayasiri *et al.* 2015).

DNA extraction, PCR amplification, and sequencing

Fresh tissue stored in 2 × CTAB buffer and dried specimens were used for DNA extraction. Genomic DNA was extracted with the CTAB-based method described by Nuytinck & Verbeken (2003) with a slight modification described in Van de Putte *et al.* (2010). ITS1, 5.8S ribosomal DNA and ITS2 were amplified and sequenced using the ITS1-F and ITS4 primers (White *et al.* 1990; Gardes & Bruns 1993). The fRPB2-5F and fRPB2-7cr primers were used to amplify the 5 to 7 domain of the second largest subunit of RNA polymerase II (*rpb2*) (Liu *et al.* 1999). The protocol for PCR amplification follows Le *et al.* (2007a). DNA sequencing was conducted with an ABI 3730XL or ABI 3700 sequencer by MACROGEN (Amsterdam, the Netherlands). Obtained sequences were assembled and edited with the software Sequencher™ v5.0 (Genecode Corporation, Ann Arbor, Michigan, U.S.A.).

Sequence sampling and alignment

Apart from newly generated sequences, published DNA sequences of *Lactarius herrarae* Montoya, Bandala & Garay (Montoya *et al.* 2014), *Lactarius indochrysorrheus* K. Das & Verbeken (Das *et al.* 2015), *Lactarius politus* Wisitrassameewong & K. D. Hyde and *Lactarius atrobrunneus* Wisitrassameewong & K. D. Hyde (Liu *et al.* 2015) were included in this study, supplemented with some other ITS sequences from the GenBank and UNITE databases. An overview of sequences included in this study is given in Supplementary Table 2. Alignments were constructed using the online version of MAFFT v7 (Katoh & Standley 2013), using the E-INS-I strategy; they were later manually edited and trimmed in Mega 6 (Tamura *et al.* 2013). Poorly aligned positions were excluded using Gblocks v0.91b (Castresana 2000), with settings allowing gaps within selected blocks, smaller blocks (minimum 5 bp)

Table 2 – Studied areas, country, altitude, and plant composition in this study.

Studied area	Country	Altitude (m)	Plant composition
North district, Lachung	India	2800	*Rhododendron*, *Abies densa*, *Tsuga*, and *Picea*
Dombang Valley (5–6 km from Lachung)	India	2940	*Picea*, *Taxus*, *Tsuga*, *Juniperus*, and *Rhododendron*
North district, 2 km South-west of Dombang Valley	India	2840	*Tsuga*, *Abies densa*, *Juniperus*, *Picea*, and *Larix*
North district, 2.5 km South-west of Dombang Valley	India	2840	*Rhododendron*, *Tsuga*, *Abies densa*, *Juniperus*, *Picea*, *Larix*, and *Taxus*
Diqing Tibetan Autonomous Prefecture	China	3212–3341	*Pinus densata*, *Picea likiangensis*, *Rhododendron rubiginosum*, and *Betula platyphylla*

and larger segments with contiguous non-conserved positions (maximum 10 bp). All alignments in this study are available in TreeBASE (S19130).

Phylogenetic reconstruction

Two datasets of DNA sequences of *Lactarius* subg. *Russularia* were generated: (1) ITS dataset, (2) concatenated ITS-*rpb2* dataset. Sequence data were partitioned as follows: (1) ITS was partitioned into the first spacer region (ITS1), the ribosomal gene 5.8S and the second spacer region (ITS2), (2) the second largest subunit of RNA polymerase II (*rpb2*) was partitioned into three different codon positions. *rpb2* comprises two exons from domain 5 to 6 and domain 6 to 7. The intron situated between domain 5 to 6 and domain 6 to 7 of *rpb2* was excluded by Gblocks. Additional *rpb2* sequences (from domain 6 to 7) of European taxa were downloaded from GenBank. Representatives of *Lactarius* subg. *Plinthogalus* were used as the outgroup for both datasets. Maximum likelihood (ML) analyses were performed with program RAxML v7.0.3 (Stamatakis 2006), applying the Rapid Bootstrapping algorithm for 1000 replicates under the GTRCAT option (Stamatakis *et al.* 2008). For the concatenated analysis, we generated 2 alignments containing either ITS or *rpb2* sequences of exactly the same specimens and ran a ML analysis for each marker separately. We investigated the compatibility of both datasets by comparing single locus phylograms. Significant conflict was presumed when two different relationships for any set of taxa were supported by ML bootstrap ≥70 % (Van de Putte *et al.* 2012). MrModeltest v.2.3 (Nylander 2004) was used to determine the model of character evolution of the dataset. MrBayes v3.1.2 (Ronquist & Huelsenbeck 2003) was used to carry out Bayesian inference (BI) analyses. The analyses were performed with four independent runs in which each run had four chains. Each run was performed for 10 million generations. Sampling frequency was set at 100. Convergence, associated likelihood, effective sample sites (ESS) and appropriate burn-in values of each run were determined using Tracer v1.6 (Drummond & Rambaut 2007; Rambaut *et al.* 2014). A majority rule consensus tree was made after discarding the trees from the burn-in. All phylograms were displayed using FigTree v1.3.1 (Rambaut 2009).

Calibration strategies and divergence time estimation

We used Bayesian Evolutionary Analysis Sampling Tree (BEAST) to estimate time of origin and diversification of *Lactarius* subg. *Russularia*. We constructed XML files for BEAST analysis using BEAUTi v.2.0 (Drummond *et al.* 2012; Bouckaert *et al.* 2014) and conducted Bayesian molecular clock analyses using BEAST v.2.3.0. PartitionFinder v1.1.1 (Lanfear *et al.* 2012) was used to define best substitution models for all partitions in both datasets. Two datasets were generated: dataset 1 and 2. An overview of sequences used in both dating phylogenies is given in Supplementary Tables 3 and 4.

Dataset 1 comprises the large subunit of ribosomal RNA (LSU) and *rpb2* sequences of representatives from several orders: Hymenochaetales, Phallales, Polyporales, Agaricales, Boletales, and Russulales. We selected LSU and *rpb2* sequence data to infer phylogenetic relationships of representatives in several orders of Basidiomycota because of their highly informative content at this taxonomic level. The aim of dataset 1 is to estimate the mean ages of genera in Russulaceae. The dataset 1 was analyzed with maximum parsimony (MP), ML, and BI. The substitution model HKY + I + G was used for all gene partitions in dataset 1. For fossil node calibration we used two Agaricomycotina fossils: *Archaeomarasmius leggetti* Hibbett, Grimaldi & Donoghue as the minimum age of the split between *Marasmius* and *Mycena* (Agaricales) (Hibbett *et al.* 1997) (gamma distribution, shape = 1.0, scale = 50.0 and offset = 90.0) and *Quatsinoporites cranhamii* Smith, Currah & Stockey as the minimum age of the Hymenochaetales (gamma distribution, shape = 1.0, scale = 50.0 and offset = 125.0) (Smith *et al.* 2004; Berbee & Taylor 2010). Dataset 2 consists of ITS and *rpb2* sequences of representatives from Russulaceae and *L.* subg. *Russularia*. Secondary calibrations derived from mean estimates of node ages of dataset 1 using the 95 % highest posterior density (HPD) as a normally distributed offset. The substitution model GTR + G, HKY + I, and TRNef + G were used for ITS1, 5.8s rDNA and ITS2 partitions, respectively. For *rpb2*, the SYM + G model was used for the 1st codon position and the TRNef + I + G model was used for 2nd and 3rd codon positions.

All analyses were conducted using three independent Markov chain Monte Carlo (MCMC) runs of 50 000 000 generations. A Yule model and relaxed lognormal clock model were chosen for both datasets. Trees were sampled every 1000 generations for each dataset. As in the BI analyses, we assessed convergence by inspecting the log likelihood distributions and ESS values of individual chains in Tracer v.1.6. An ultrametric maximum clade credibility tree with mean and 95 % HPD node ages was summarized using TreeAnnotator v.2.3.0, with 10 % burn-in.

Morphological studies

Macromorphological characters were observed and described in fresh condition during daylight hours. Micromorphological characters were studied on dried herbarium collections. To describe all morphological characters in this study, we followed the general terminology from Vellinga (1988), Verbeken (1996) and Heilmann-Clausen *et al.* (1998) for the pileipellis structure. Colour coding is according to Kornerup & Wanscher (1978). The observation of latex features was carried out immediately when the latex was exposed to the air, colour reaction is noted after 15–30 min. We also tested latex colour changes by putting a drop of latex on white tissue paper or on a white cotton handkerchief and by mixing a drop of latex with 10 % KOH solution.

Micromorphological characters of Asian and European taxa were observed from the dried herbarium collections obtained from GENT or were derived from the original species descriptions. Microscopic characters of hymenium and pileipellis structure were studied in Congo Red in L4 (Clémençon 1973), using an Olympus CX31 microscope with the aid of a drawing tube, at 1600× magnification. Basidia were measured excluding sterigmata length. Basidiospores were measured in side view, in Melzer's reagent. Basidiospore measurement was conducted on at least 20 spores for each collection, excluding the height of the ornamentation. The measurements are represented as {(MIN) [AVa − 2 × SD] − *AVa* − *AVb* − [AVb + 2 × SD] (MAX)} length × {(MIN) [AVa − 2 × SD] − *AVa* − *AVb* − [AV + 2 × SD] (MAX)} width, in which MIN = the minimum value, MAX = the maximum value, AVa = lowest mean value for the measured collections, AVb = highest mean value for the measured collections and SD = standard deviation. Q corresponds to basidiospore 'length/width ratio' and is given as (MINQa) Qa–Qb (MAXQb), where Qa and Qb are the lowest and the highest mean ratio for the measured collections, respectively. Basidiospore drawings were done using a Zeiss Axioscop 2 microscope and drawing tube at 6000× magnification.

In order to understand concordance between morphology and phylogeny, five major characteristics, traditionally used to delimitate infrageneric taxa, are annotated on the two-locus phylogeny (see Fig 2): 1) latex colour, 2) latex colour change, 3) basidiospore ornamentation, 4) the presence or absence of true cystidia, 5) pileipellis structure. Other potentially delimiting characteristics are also discussed.

Results

ITS and rpb2 *phylogeny*

A set of 114 ITS sequences that comprises 81 putative species of *Lactarius* subg. *Russularia* was used in a global ITS phylogeny. The dataset consists of three partitions: ITS1, 5.8S and ITS 2. All newly proposed species formed strong-supported clades in the ITS phylogeny (Fig 1). The final *rpb2* dataset contains 61 sequences. Due to the low success rate of amplification when using dried collections, several temperate taxa are missing.

The phylogeny generated by the concatenated dataset comprises 51 *L.* subg. *Russularia* species in total (Fig 2). Before combining both markers (ITS & *rpb2*), each marker was analysed separately using ML-based estimation. We compared single locus phylogenies to check for significant conflicts. Minor incongruences in the evolutionary relationships were found between genealogies, but none of those received significant support (>75 % bootstrap support (bs)).

Lactarius subg. Russularia is monophyletic and strongly supported (90 % bs and 1.00 posterior probability (pp)) in the result of the ML and BI analyses of the concatenated dataset (Fig 2). The topology of the ITS-*rpb2* phylogeny is in accordance with the global ITS phylogeny. The subgenus has seven putative Clades: A–G. Clade A is the largest group; it contains 13 known European, ten known Asian, two known North American species and four unknown North American specimens. All newly proposed species form strongly supported clades in Clade A. Clade B contains six species: *Lactarius fuscomaculatus* Wisitrassameewong & Verbeken, *Lactarius rubrobrunneus* H.T. Le & Nuytinck, *Lactarius subumbonatus* Lindgr., *Lactarius crenulatulus* Wisitrassameewong & Verbeken, *Lactarius pasohensis* Wisitrassameewong & Stubbe and *Lactarius atrobrunneus*. This clade has a sister relationship with Clade C where *Lactarius chichuensis* W.F. Chiu, *Lactarius austrorostratus* Wisitrassameewong & Verbeken, *Lactarius rubrocorrugatus* Wisitrassameewong & Nuytinck and *Lactarius camphoratus* (Bulll.: Fr.) Fr. are grouped together. *Lactarius politus*, *Lactarius helvus*, and *Lactarius falcatus* have isolated positions in the phylogeny. Three small-sized species are grouped in Clade D: *Lactarius gracilis* Hongo, *Lactarius glabrigracilis* Wisitrassameewong & Nuytinck and *Lactarius perparvus* Wisitrassameewong & F. Hampe. Clade E consists of two species: *Lactarius atlanticus* Bon and *Lactarius laccarioides* Wisitrassameewong & Verbeken and Clade F consists of two morphologically similar species: *Lactarius chrysorrheus* and *Lactarius vinaceorufescens*. Clade G comprises two species: *Lactarius quietus* (Fr.: Fr.) Fr. and *Lactarius aquosus* H.T. Le & K.D. Hyde.

Divergence time estimation

The chronogram was obtained using two calibration points for estimating the origin of Russulaceae (dataset 1) and is shown in Fig 3. The estimated times for major clades are summarized in Table 3.

The result suggests the estimated divergence time of Russulales to be in the late Jurassic to the Early Cretaceous (157.78 mya, 100.88–226.94 mya; 95 % HPD). The estimated crown age of *Lactarius* is estimated to be in the late Paleogene (28.17 mya, 16.94–41.62 mya; 95 % HPD). The diversification of the genus *Lactarius* might have begun during the Early Miocene (28.17 mya, 16.94–41.62 mya; 95 % HPD) (Fig 3). The analysis of dataset 2 shows that diversification of the major clades within *Lactarius* subg. *Russularia* might have occurred during the Miocene (18.67 mya, 15.85–28.28 mya; 95 % HPD) (Fig 4).

Taxonomy

We extend the diversity knowledge of Asian *L.* subg. *Russularia* by proposing six new species: *L. atrii*, *L. aurantionitidus*, *L. dombangensis*, *L. lachungensis*, *L. flavigalactus,* and *L. sikkimensis*. All new species are associated with mixed coniferous host trees in highland forests in India and China. The description of morphology, diagnostic characters of species and illustration are given as follows.

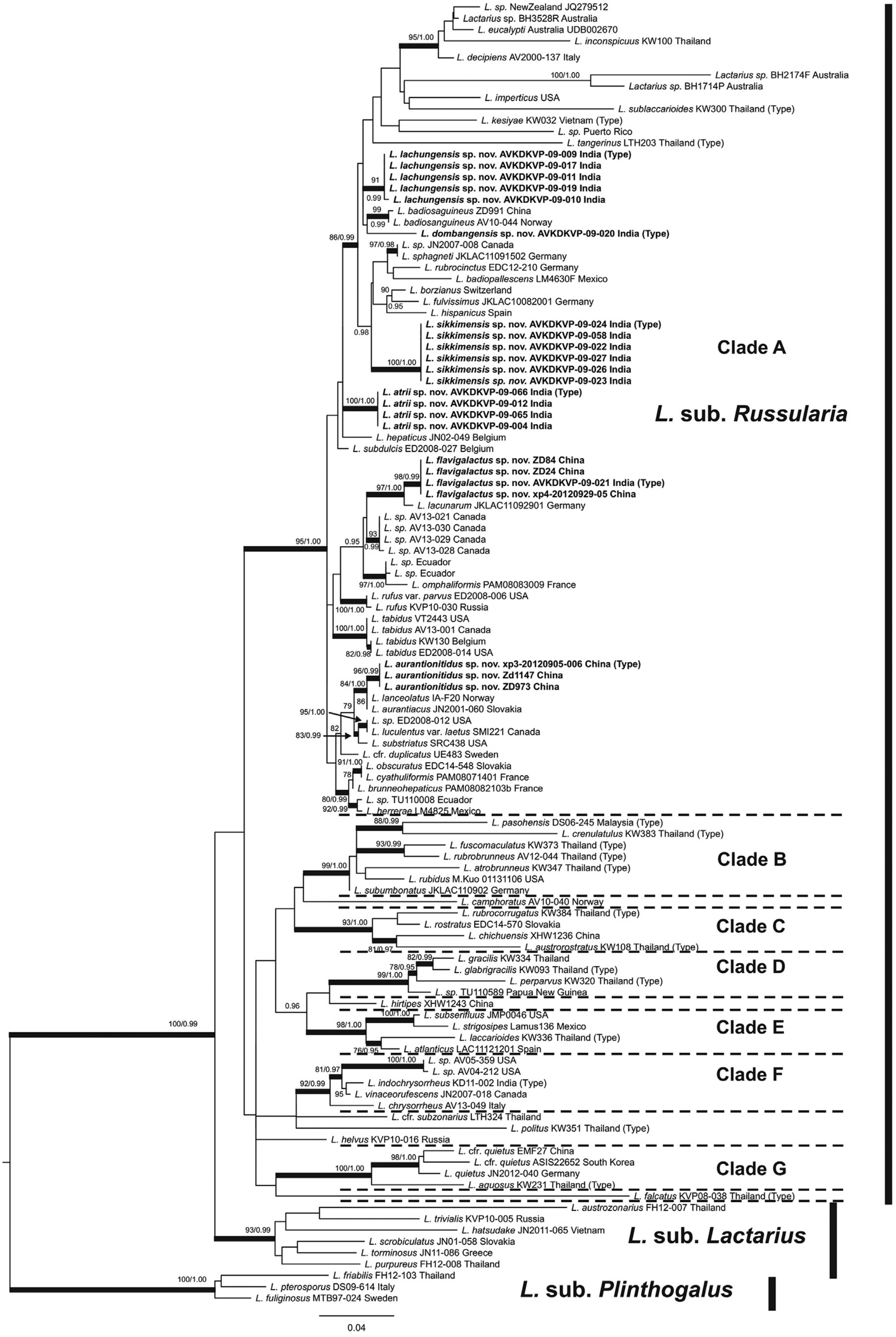

Fig 1 – An ITS tree of *Lactarius* subg. *Russularia*. The thick branches indicate supported clades. Bootstrap support and posterior probabilities are indicated when they exceed 75 % and 0.95 %. New species in this study are in boldface.

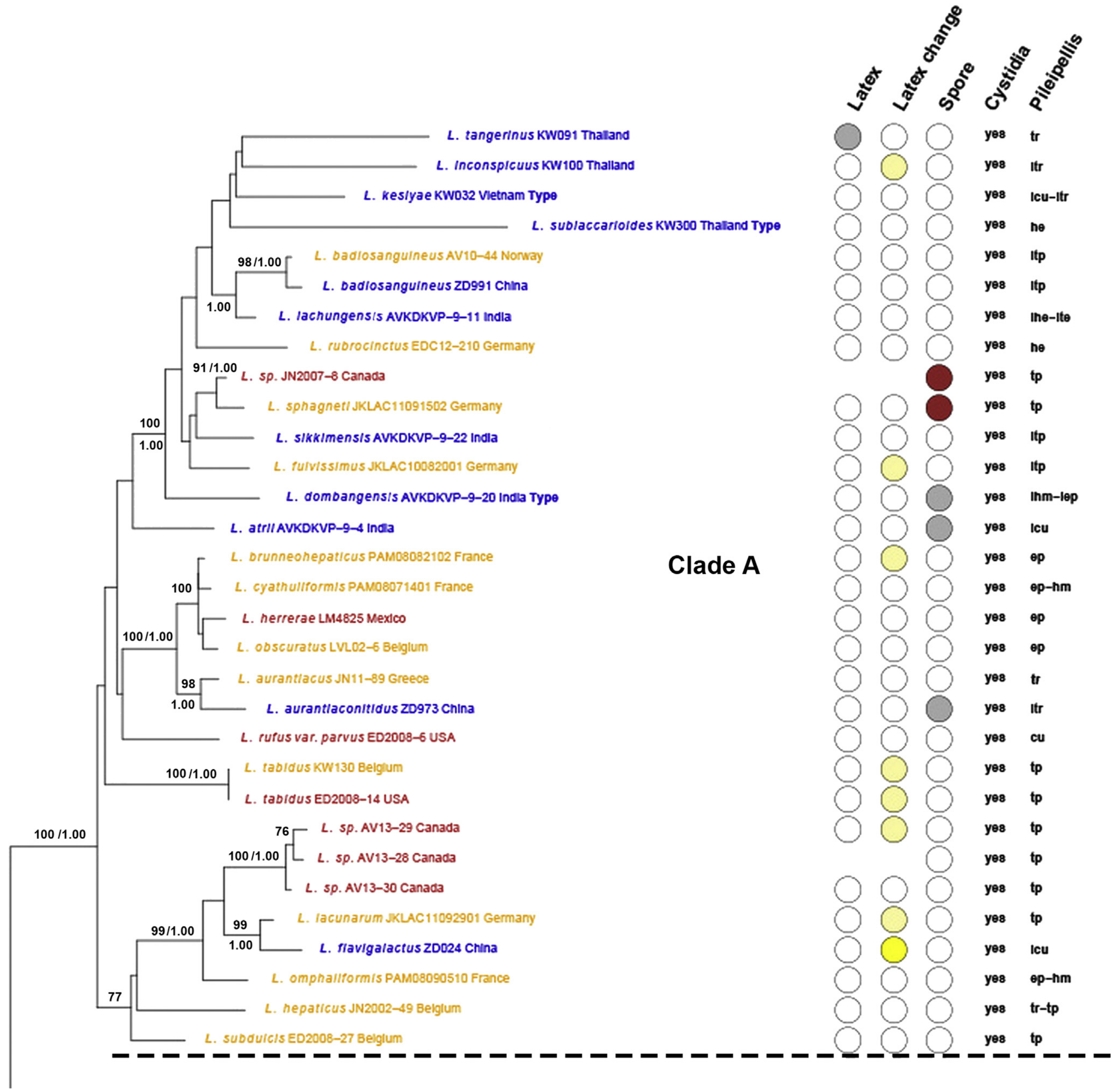

Fig 2 – Tree of the concatenated ITS-*rpb2* dataset of *Lactarius* subg. *Russularia*. Species names are coloured by continent; blue: Asia, orange: Europe, red: North America. Bootstrap values above 75 % and posterior probabilities exceeding 0.95 are indicated. Five morphological characters are plotted right of the tip labels: 1) latex colour: white circle = white latex, grey circle = completely transparent latex, cream circle = pale yellow latex; 2) latex colour change: white circle = unchanging latex, cream circle = pale yellowing reaction, yellow circle = yellowing reaction; 3) basidiospore ornamentation: white circle = incomplete reticulum, brown circle = complete reticulum, grey circle = isolated warts, blue circle = zebroid; 4) cystidia: yes = present, no = absent, rare = rare or infrequently present; 5) pileipellis structure: cu = cutis, icu = ixocutis, tr = trichoderm, itr = ixotrichoderm, tp = trichopalisade, itp = ixotrichopalisade, he = hyphoepithelium, ihe = ixohyphoepithelium, te = trichoepithelium, ite = ixotrichoepithelium, ihm = ixohymenoepithelium, ep = epithelium, iep = ixoepithelium. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

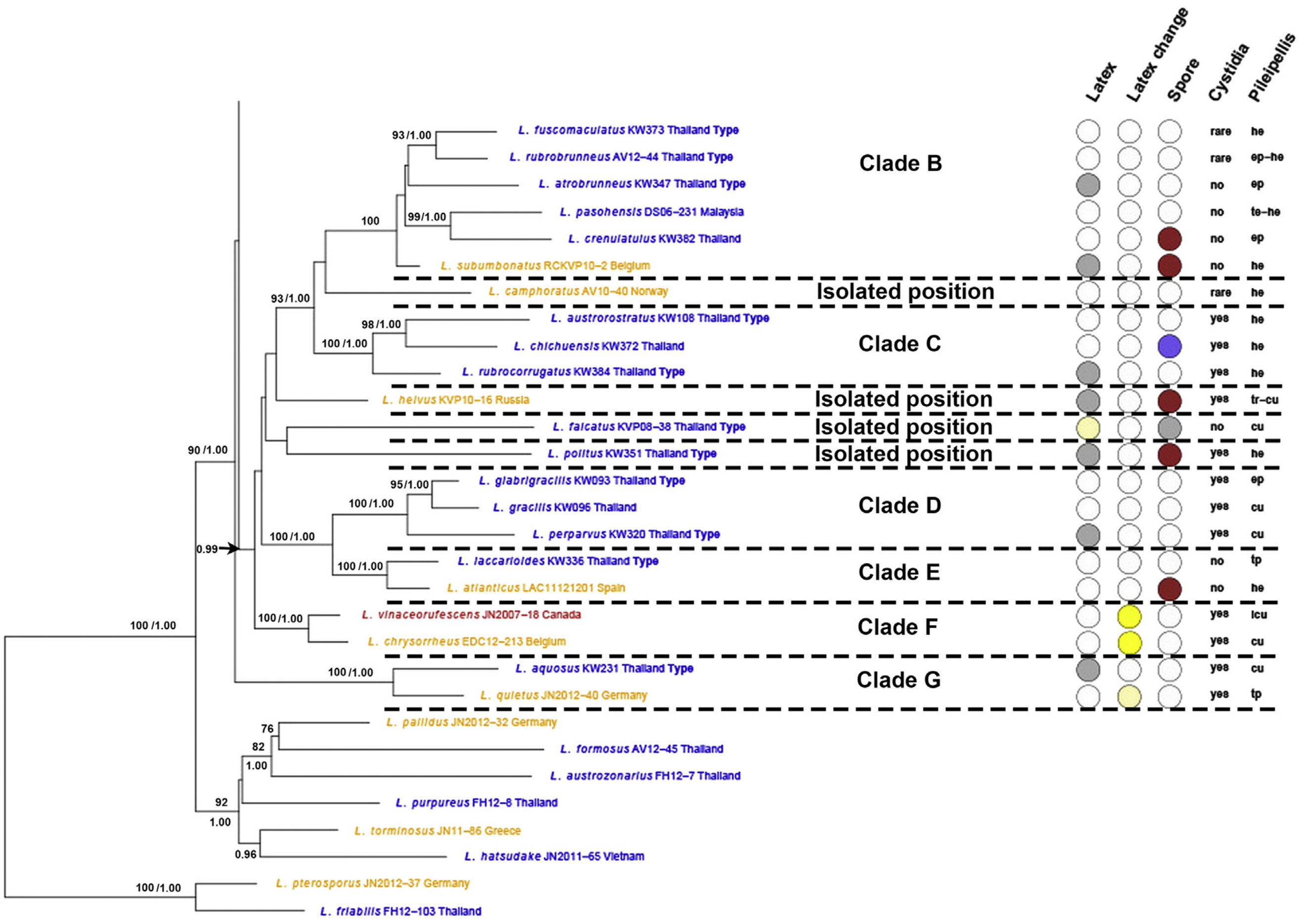

Fig 2 – (*continued*).

Lactarius atrii Van de Putte & K. Das, **sp. nov.** (Fig 5).

MycoBank number: MB812830 **Facesoffungi number:** FoF 00853.

Diagnosis: Medium-sized, light brown basidiocarp with a very strigose base, microscopically recognized by the obtuse, often isolated warts in the basidiospore ornamentation, the large and protruding macrocystidia and an ixocutis as pileipellis structure.

Etymology: 'atrii' commemorating Dr. N.S. Atri for his contribution to Indian *Lactarius*.

Typus: India, Sikkim, North district, Dombang Valley, 5–6 km from Lachung, mixed coniferous forest with *Picea*, *Tsuga*, *Juniperus,* and *Rhododendron* spp., N27°44.27′ E88°46.12′, 2940 m alt., 15/08/2009, AVKDKVP09–066 (Holotypus, GENT!, Isotypus, CAL!).

Pileus 27 mm diam., planoconvex but widely depressed in the centre, with a papilla; surface greasy, sticky, not really smooth, but slightly rugulose, light brown (7D6), paler towards margin, with some darker reddish brown spots which seem to lay under the surface; margin a bit irregularly wavy-crenulate, finely and very slightly striate. *Lamellae* decurrent, sometimes with a long tooth, dense, with abundant lamellulae of different lengths, sometimes forked, rather thick, pinkish, a bit darker when bruised; edge entire, concolourous. *Stipe* 40–45 × 4–5 mm, long and slender, subcylindric, with broadest part in lower half, then tapering downwards; surface smooth, dry, brick-red in lower half, more pinkish in upper half, very strigose at the base. *Context* very thin in pileus, hollow in stipe, pale pinkish to orange reddish-brown; odour sweet, agreeable; taste mild. *Latex* white, not abundant, unchanging on exposure, but staining slightly yellow on white tissue paper.

Basidiospores mostly broadly ellipsoid, sometimes subglobose, sometimes ellipsoid, 7.5 – 8.2 – 8.9 – 10.0 (–10.2) × 6.0 – 6.7 – 6.8 – 7.6 (–7.7), Q = 1.14 – 1.22 – 1.32 – 1.43 (n = 60); ornamentation amyloid, 1–1.7 μm high, composed of distinctly isolated or aligned warts, which are rather obtuse and rarely connected by lower ridges and lines; plage inamyloid to distally amyloid. *Basidia* 55–62 × 16–19 μm, 4–spored, subclavate to clavate, with fine granules and guttate contents. *Pleuromacrocystidia* abundant, 90–119 × 12–15 μm, large and slender, protruding up to 50 μm, subfusiform, irregular, with fine granules, needle-like and guttate contents. *Pleuropseudocystidia* abundant, 3–6 μm diam., cylindrical, straight to tortuous, with an obtuse apex, with fine granules. *Lamellae edge* heterogenous, consisting of basidia, marginal cells and cheilomacrocystidia; marginal cells 13–34 × 7–13 μm, cylindrical to subclavate; cheilomacrocystidia abundant, 40–78 × 7–12 μm,

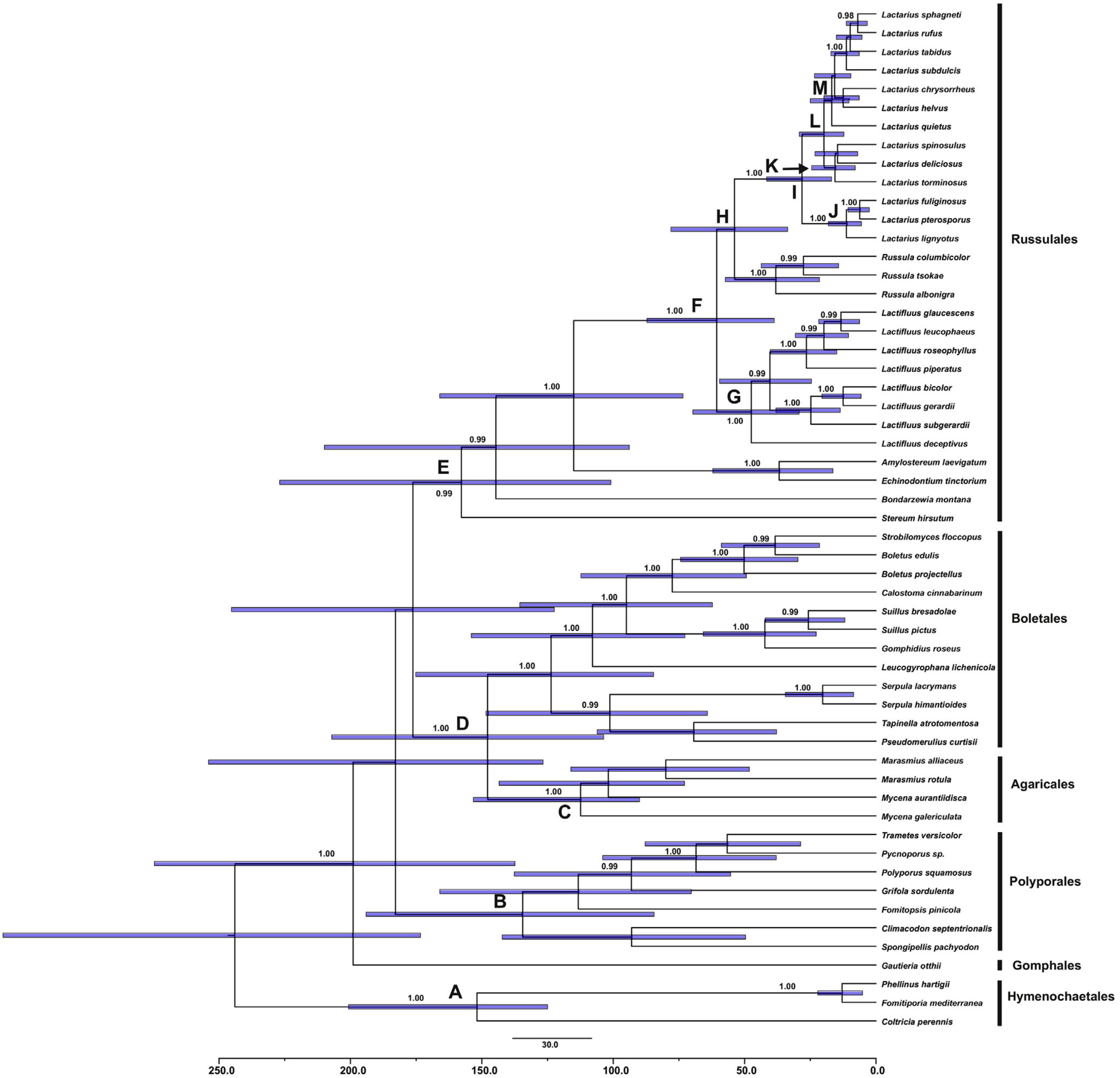

Fig 3 – Fossil-calibrated maximum-clade-credibility tree of the BEAST analysis of dataset 1 (based on LSU and *rpb2*), including representatives in Russulaceae. Two calibration points were used: *Quatsinoporites cranhamii* (125 mya) for the minimum age of Hymenochaetales and *Archaeomarasmius leggetti* (90 mya) for the minimum age of the split between *Mycena* and *Marasmius* (Agaricales). The letters A–M refer to Table 3.

slightly projecting, subcylindrical to narrowly fusiform, with mucronate apex, with fine granulate contents. *Lamellar trama* mixed, consisting of lactifers, hyphae, and sphaerocytes. *Pileipellis* an ixocutis, 60–80 μm thick, covered by a narrow slime layer; terminal hyphae cylindrical, repent to parallel; underlying layer with enlarged and cylindrical hyphae, up to 10 μm diam.

Habitat: gregarious on ground among leaf litter.

Studied material: India, Sikkim, North district, Lachung, mixed coniferous forest with *Abies densa*, *Tsuga, Picea,* and *Rhododendron*, N27°43.51′ E88°44.70′, 2800 m alt., 13/08/2009, AVKDKVP09–004 (GENT!, CAL!);–ibid. 13/08/2009, AVKDKVP09–012 (GENT!, CAL!); Sikkim Prov., Dombang Valley, 5–6 km from Lachung, mixed coniferous forest with *Taxus*, *Tsuga*, *Juniperus*, and few trees of *Rhododendron* sp., N27°44.27′ E88°46.12′, 2940 m alt., 15/08/2009, AVKDKVP09–065 (GENT!, CAL!);–ibid. 15/08/2009, AVKDKVP09–066 (Holotypus, GENT!, Isotypus, CAL!).

Comment: In the field *L. atrii* is characterized by its greasy and sticky surface, pale brown fruiting body with some darker

Table 3 – Estimated mean and 95 % HPD (mya) of each node in the dataset 1 tree. The statistic supports from MP, ML, and BI are included.

Node	MP/ML/PP supports	Mean	95 % HPD
A: Hymenochaetales	100/100/1.00	151.84	125.00–200.73
Fossil calibration: *Quatsinoporites cranhamii* (125 mya)			
B: Polyporales	55/74/–	134.44	84.45–194.02
C: *Marasmius/Mycena*	84/95/1.00	112.42	90.00–153.19
Fossil calibration: *Archaeomarasmius leggetti* (90 mya)			
D: Agaricales/Boletales (Agaricomycetidae)	87/100/1.00	147.82	126.71–253.98
E: Russulales	73/91/0.99	157.78	100.88–226.94
F: *Lactifluus/Russula/Lactarius* (Russulaceae)	100/100/1.00	60.62	38.73–87.15
G: *Lactifluus*	64/97/1.00	47.46	29.26–69.70
H: *Russula/Lactarius*	59/71/0.75	53.86	33.62–78.04
I: *Lactarius*	100/100/1.00	28.17	16.94–41.62
J: *L.* subg. *Plinthogalus*	100/100/1.00	11.27	5.54–18.12
K: *L.* subg. *Lactarius*	15/49/0.70	15.61	7.93–24.46
L: *L.* subg. *Lactarius*/*L.* subg. *Russularia*	46/41/0.85	19.81	12.21–29.19
M: *L.* subg. *Russularia*	13/41/0.72	16.84	10.29–24.98

reddish brown spots and very strigose base. *Lactarius atrii* is reminiscent of few European species such as *L. subdulcis* (Pers. ex Fr.) Gray, *L. fulvissimus,* and *L. tabidus* Fr. Distinctly isolated or aligned warty basidiospores and an ixocutis pileipellis help to separate this new species from *L. subdulcis* which has incompletely reticulate basidiospores and a trichopalisade pileipellis. *Lactarius fulvissimus* has an orange brown pileus, latex that often turns pale yellow, pointed wart on the basidiospores and a trichopalisade pileipellis. *Lactarius tabidus* also has latex that turns yellow on white tissue, but the colour reaction is much brighter; the latex also turns pale yellow when drying and the pileipellis is a hyphoepithelium. *Lactarius atrii* can also be confused with *L. inconspicuus*. The Thai species has similar basidiocarp colours, a dry or glossy surface (in moist condition) and white latex that is staining pale yellow on white tissue paper. *Lactarius atrii* mainly differs from the Thai species by larger basidiospores, ornamented with isolated warts and an ixocutis as pileipellis.

Lactarius aurantionitidus Wisitrassameewong & G. Jiayu **sp. nov.** (Figs 6, 11a and b).

MycoBank number: MB812831 **Facesoffungi number:** FoF 00854.

Diagnosis: Medium sized, yellowish orange fruitbody, with viscid and glossy surface, microscopically characterized by the basidiospore ornamentation consisting of isolated warts and short ridges connected by thin lines, the large macrocystidia and the pileipellis structure being an ixotrichoderm.

Etymology: ‘aurantionitidus’ refers to the glossy and orange pileus.

Typus: China, Yunnan Prov., Diqing Tibetan Autonomous Prefecture, Heping village, mixed forest dominated by *Pinus densata*, *Picea likiangensis*, *Rhododendron rubiginosum*, and *Betula platyphylla*, N27°28.34′, E99°50.92′, 3341 m alt., 05/09/2012, xp3–20120905–06 (Holotypus, KUN!, Isotypus, GENT!).

Pileus 40–48 mm, broadly planoconvex to infundibuliform with depressed centre; surface viscid and glossy, irregular, rather unicolourous, light orange (5A5–A6) to greyish orange (5B4–B6) or sometimes paler, pale cream, sometimes with olive brown tints (4E4–E5); margin shortly striate, crenulate. *Lamellae* decurrent, up to 3 mm broad, crowded, with 4 lamellulae of different lengths, yellowish white (4A2) to pale yellow (4A3), orange brown when bruised. *Stipe* 40 × 5 mm, cylindrical; surface slightly wrinkled, dry to slightly viscid, concolourous, paler on top, with brown tints, often with whitish mycelium at base. *Context* 3 mm thick in pileus, pale cream, partly hollow to hollow in stipe; odour indistinct, taste mild. *Latex* watery white, copious, unchanging on exposure; taste mild.

Basidiospores mostly ellipsoid, rarely subglobose, 7.8 – *8.7* – *8.8* – 9.6 (–10.0) × 6.2 – *6.9* – *7.2* – 8.0 (–8.4) μm, Q = 1.10 – *1.21* – *1.26* – 1.35 (n = 40); ornamentation amyloid, up to 1 μm high, consisting of isolated warts and short and low ridges connected by thin lines, obtuse; plage distally amyloid. *Basidia* 58–65 × 18–20 μm, 4–spored, subclavate to clavate, with fine granules and guttate contents. *Pleuromacrocystidia* abundant, 112–143 × 13–17 μm, projecting up to 70 μm, subfusiform, with mucronate to moniliform apex, with fine granules and guttate contents. *Pleuropseudocystidia* abundant, 3–6 μm diam., cylindrical, straight to tortuous, with round apex, fine granules. *Lamellae edge* heterogeneous, consisting of basidia, marginal cells and cheilocystidia; marginal cells 26–30 × 6–14 μm, subcylindrical to subclavate; cheilocystidia abundant, 33–70 × 8–14 μm, slightly emergent, subfusiform, with mucronate apex, with fine granules. *Lamellar trama* consisting of lactifers, filamentous hyphae and sphaerocytes. *Pileipellis* an ixotrichoderm, covered by a thin to thick slime layer, 100–150 μm thick, consisting of cylindrical hyphae, which are bent to erect and enlarged up to 10 μm diam.

Habitat: solitary or gregarious, growing under *Pinus densata*, *Picea likiangensis*, *Rhododendron rubiginosum*, and *Betula platyphylla*.

Studied material: China, Yunnan Prov., Diqing Tibetan Autonomous Prefecture, Heping village, mixed forest dominated by *Pinus densata*, *Picea likiangensis*, *Rhododendron rubiginosum*, and *Betula platyphylla*, N27°28.34′ E99°50.92′, 3341 m alt., 05/09/2012, xp3–20120905–06 (Holotypus, KUN!, Isotypus, GENT!); Yunnan Prov., Diqing Tibetan Autonomous Prefecture, Heping village, mixed forest dominated by *Pinus densata*, *Picea likiangensis*, *Rhododendron rubiginosum*, and *Betula platyphylla*, N27°28.34′ E99°50.92′, 3291 m alt., 17/08/2013, ZD1147

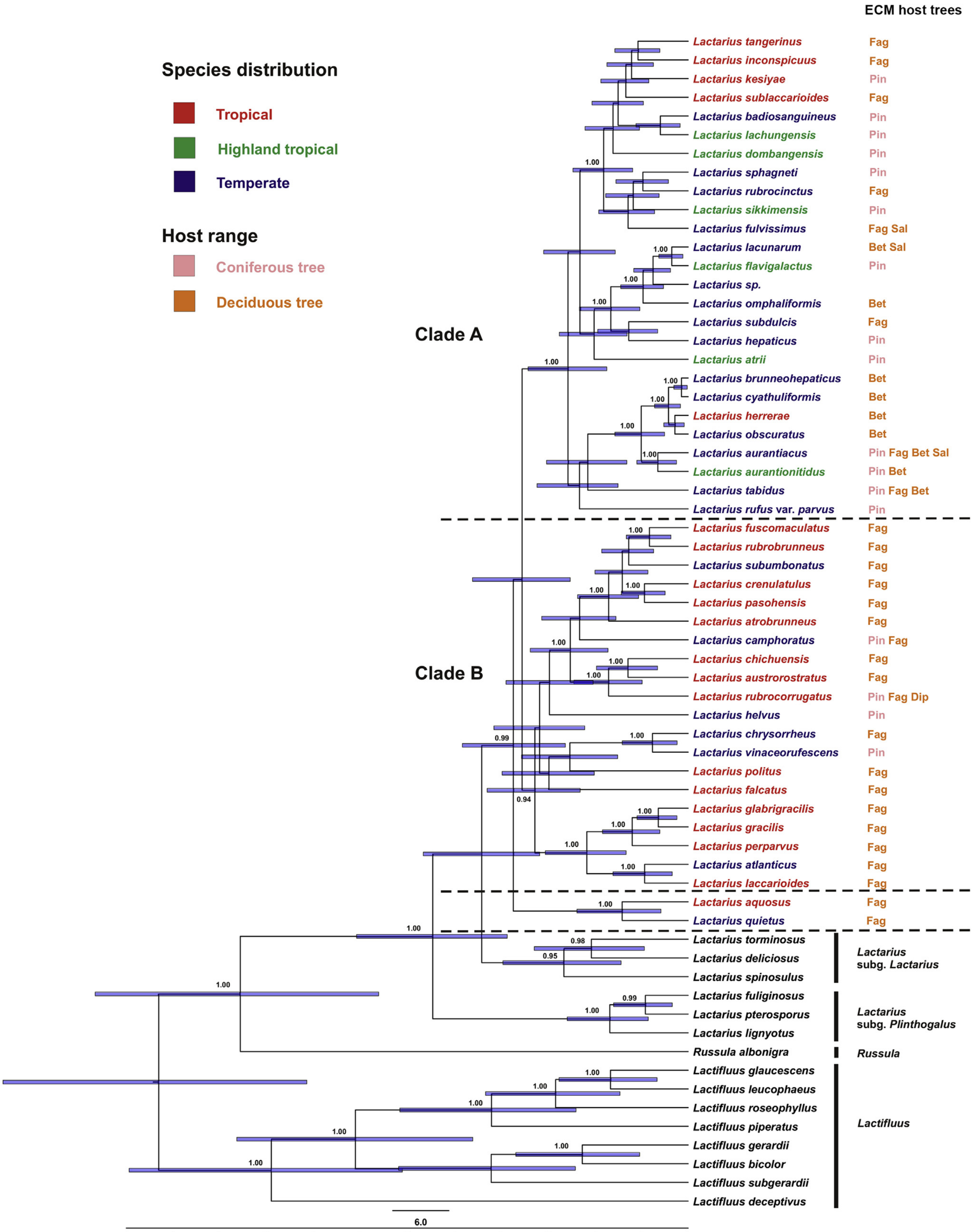

Fig 4 – Fossil-calibrated maximum-clade-credibility tree of the BEAST analysis using dataset 2 (based on ITS and *rpb2*), including representatives of *Lactarius* subg. *Russularia* and Russulaceae. Species distribution are coloured as follows; red = tropical, green = highland tropical, blue = temperate. Families of host trees are annotated and coloured at the right of the tip labels; pink = coniferous tree and orange = deciduous tree. Abbreviations are used for families of trees: Bet = Betulaceae, Dip = Dipterocarpaceae, Fag = Fagaceae, Pin = Pinaceae and Sal = Salicaceae. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

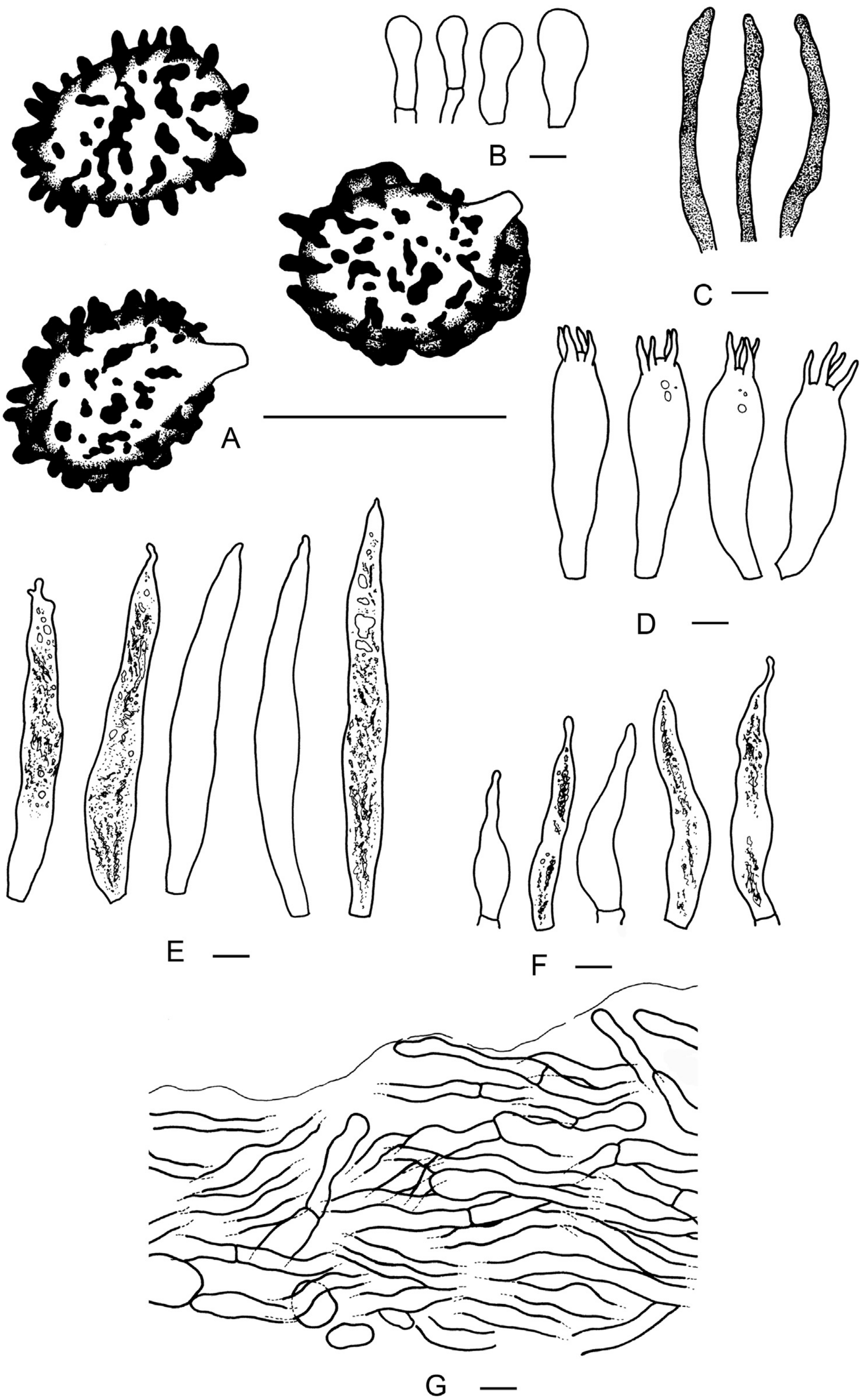

Fig 5 – *Lactarius atrii* (a–g: AVKDKVP09-066, holotype): a. basidiospores, b. marginal cells, c. pseudocystidia, d. basidia, e. pleuromacrocystidia, f. cheilocystidia, g. pileipellis (scale bar = 10 μm).

(KUN!, GENT!); Yunnan Prov., Diqing Tibetan Autonomous Prefecture, Heping village, mixed forest dominated by *Pinus densata*, *Picea likiangensis*, *Rhododendron rubiginosum*, and *Betula platyphylla*, N27°28.34′ E99°50.92′, 3267 m alt., 12/08/2013 ZD973 (KUN!, GENT!).

Comment: *Lactarius aurantionitidus* is a unique species in the subgenus, because of the bright orange pileus and glossy surface. This new species can be distinguished from *L. aurantiacus* (Pers.: Fr.) Gray by the viscid cap, isolated warts on the basidiospores, the large macrocystidia and the ixotrichoderm

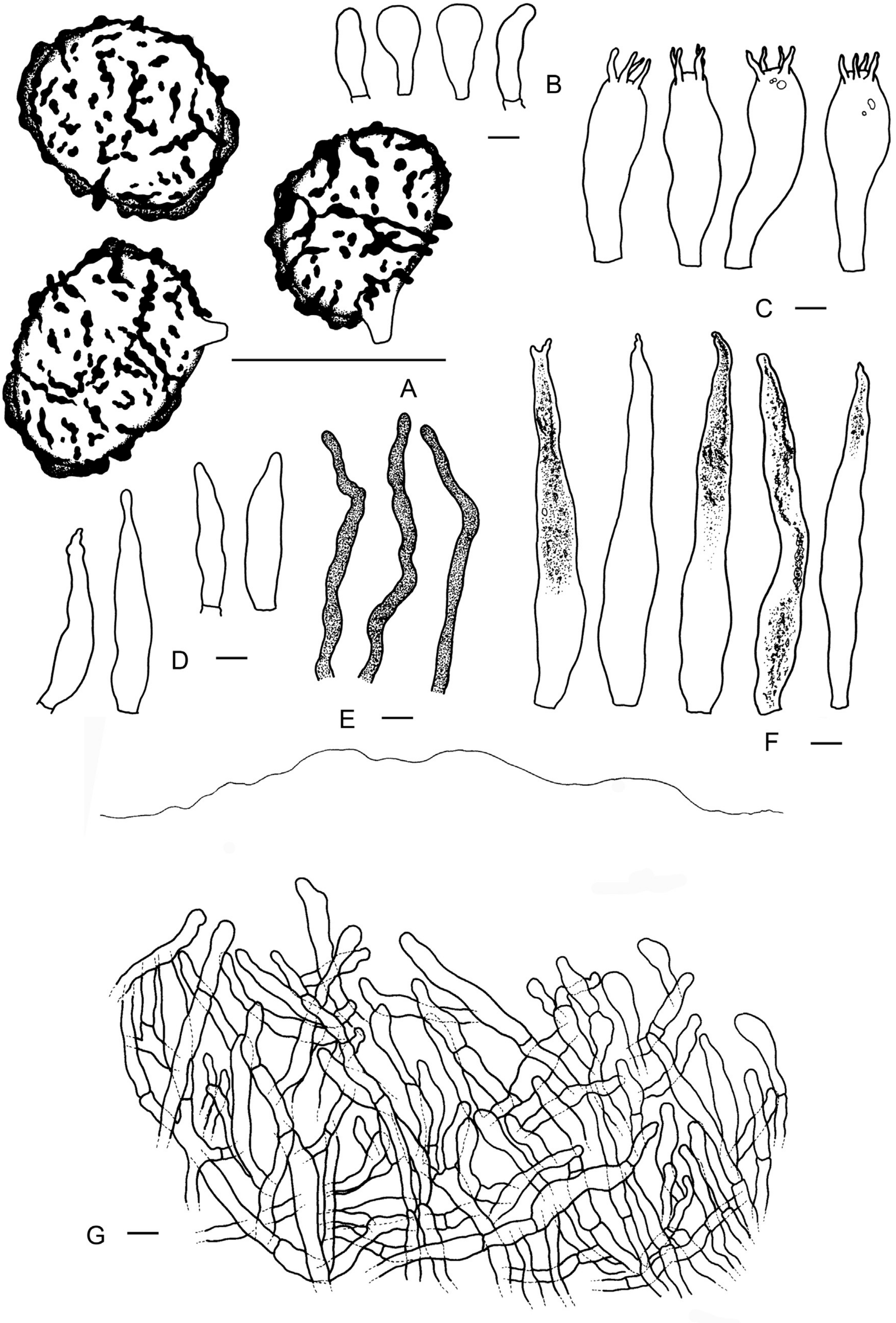

Fig 6 — ***Lactarius aurantionitidus*** **(a–g: xp3-20120905-06, holotype): a. basidiospores, b. marginal cells, c. basidia, d. cheilocystidia, e. pseudocystidia, f. pleuromacrocystidia, g. pileipellis (scale bar = 10 μm).**

pileipellis. *Lactarius lanceolatus* O.K. Mill. Laursen is also similar, but this European species differs from *L. aurantionitidus* by warmer orange brown colours, cracking of the pileus, pinkish buff gills and the ectomycorrhizal association with *Salix*.

Lactarius kesiyae is a species with a glossy and viscid surface that occasionally has a bright orange pileus, though it is usually much paler. Wisitrassameewong *et al.* (2015) stated that the latex of *L. kesiyae* turns strongly yellow on white handkerchief. Microscopically, the basidiospore ornamentation is a good distinguishing character between these species. The basidiospores of *Lactarius aurantionitidus* have isolated warts or short ridges and are infrequently forming long ridges and a subreticulum, whereas *L. kesiyae* has incompletely reticulate basidiospores. In addition, *L. kesiyae* is found in pure *P. kesiyae* forest in Northern Thailand.

Lactarius dombangensis Verbeken & Van de Putte **sp. nov.** (Fig 7).

MycoBank number: MB812832 **Facesoffungi number:** FoF 00855.

Diagnosis: A remarkably small species, with warm reddish orange basidiocarp and crenulate margin, microscopically characterized by the basidiospore ornamentation with obtuse warts connected by lower ridges, forming an incomplete reticulum and an ixohymenoepithelium to ixoepithelium as a pileipellis structure.

Etymology: 'dombangensis' refers to Dombang valley of North Sikkim, the type locality.

Typus: India, Sikkim, North district, 2.5 km South-west of Dombang Valley, mixed coniferous forest with *Rhododendron*, *Tsuga*, *Abies densa*, *Juniperus,* and *Taxus*, N27°43.96′ E88°45.70′, 2840 m alt., 14/08/2009, AVKDKVP09–020 (Holotypus, GENT!, Isotypus, CAL!).

Pileus 2–5 mm diam. in the very young specimens, later expanding up to 15 mm diam.; surface smooth, brownish orange (6C5) to light brown (7D6) but paler towards margin; margin sometimes irregularly wavy, rather regularly crenulate in oldest specimen, striate up to sulcate in older specimens, with striations almost up to the centre. *Lamellae* adnate, up to 3 mm broad, moderately distant, up to 10 L + l/half a pileus, with abundant lamellulae of different lengths, sometimes but not always with a regular pattern, orange white (6A2) to pale orange (6A3), sometimes irregular and anastomosing; edge entire, concolourous. *Stipe* 20–25 × 3–4 mm, smooth, concolourous with pileus, a bit darker, sometimes rather brick-coloured, hollow. *Context* extremely thin-fleshed, flesh-coloured to reddish, hollow in stipe; taste mild; odour indistinct. *Latex* white, sparse.

Basidiospores mostly broadly ellipsoid, sometimes subglobose, 7.6 – 8.4 – 9.2 (–9.3) × (6.3–) 6.4 – 7.0 – 7.7 μm, Q = 1.10 – *1.19* – 1.26 (n = 20); ornamentation amyloid, composed of ridges up to 1.8 μm high; high isolate warts up to 1 (1.5) μm present; warts obtuse to subacute, with connexions by lower ridges, forming an incomplete reticulum; plage inamyloid. *Basidia* 59–74 × 14–20 μm, 4–spored, subclavate to clavate, with fine granules and guttate contents. *Pleuromacrocystidia* not abundant to fairly abundant, slightly emergent up to 20 μm, 42–70 × 8–14 μm, subfusiform, with moniliform to mucronate apex, with fine granules and guttate contents. *Pleuropseudocystidia* abundant, 3–5 μm diam., cylindrical, straight to tortuous, with an obtuse apex, with finely granulate contents. *Lamellae edge* heterogenous, consisting of basidia, marginal cells, and cheilomacrocystidia; marginal cells abundant, 18–36 × 11–14 μm, cylindrical to subclavate; cheilomacrocystidia abundant, 49–57 × 11–14 μm, subfusiform with mucronate apex, with fine granules and guttate contents. *Lamellar trama* consisting of hyphae, lactifers, and sphaerocytes. *Pileipellis* an ixohymenoepithelium to ixoepithelium, 50–80 μm thick, covered by a narrow slime layer; terminal hyphae broad and short, subclavate; underlying layer compact layer of globose cells, up to 30 μm diam.

Habitat: growing in mixed coniferous forest with *Rhododendron*, *Tsuga*, *Abies densa*, *Juniperus*, *Taxus*, some on a big log of dead wood, some directly on the wood, some among the mosses covering the wood.

Studied material: India, Sikkim, North district, 2.5 km Southwest of Dombang Valley, mixed coniferous forest with *Rhododendron*, *Tsuga*, *Abies densa*, *Juniperus,* and *Taxus*, N27°43.96′ E88°45.70′, 2840 m alt., 14/08/2009, AVKDKVP09–020 (Holotypus, GENT!, Isotypus, CAL!).

Comment: The specimens are remarkably small-sized, reminding of the group around *Lactarius obscuratus* (Lasch: Fr.) Fr. in Europe: *L. obscuratus*, *L. cyathuliformis* Bon and *L. omphaliformis* Romagn. These three European species are associated with *Alnus* whereas *L. dombangensis* is found in mixed coniferous forest in high altitude. A microscopic examination revealed that the three European species lack a mucus layer on the pileus cuticle. We observe a narrow mucus layer in this Indian species. In addition, this new species has smaller pleuromacrocystidia (42–70 × 8–14 μm) and basidiospores with isolated warts connected by low ridges.

Lactarius crenulatulus, *L. perparvus,* and *L. glabrigracilis* have similar dimensions but they grow in tropical evergreen forests dominated by members of Fagaceae. *Lactarius crenulatulus* differs from this new species by having a paler fruitbody colour, by the completely reticulate and dense basidiospore ornamentation and the absence of pleuromacrocystidia. *Lactarius perparvus* differs in the pileipellis structure which is a cutis. *Lactarius glabrigracilis* has smaller basidiospores and an epithelium as pileipellis. Another small species with striate margin is *L. liliputianus* Verbeken & E. Horak. This Papuan species differs from *L. dombangensis* by having a spore ornamentation completely composed of high, isolated warts and a trichoderm as pileipellis. In addition, the Papuan species grows under *Castanopsis acuminatissima*.

Although we described this species based on a single collection, the combination of characters, e.g. basidiospore ornamentation and pileipellis structure, habitat and molecular evidence, are convincing that *L. dombangensis* is a new species. The very small basidiocarps may have affected the frequency of finding *L. dombangensis* in this study.

Lactarius flavigalactus Verbeken & K. Das **sp. nov.** (Figs 8 and 11c).

MycoBank number: MB812833 **Facesoffungi number**: FoF 00856.

Diagnosis: Medium-sized, shiny, and glossy cap with or without knobby centre, reddish brown, remarkably yellowing milk, microscopically characterized by the incompletely reticulate basidiospore ornamentation, the presence of macrocystidia and an ixocutis as pileipellis structure.

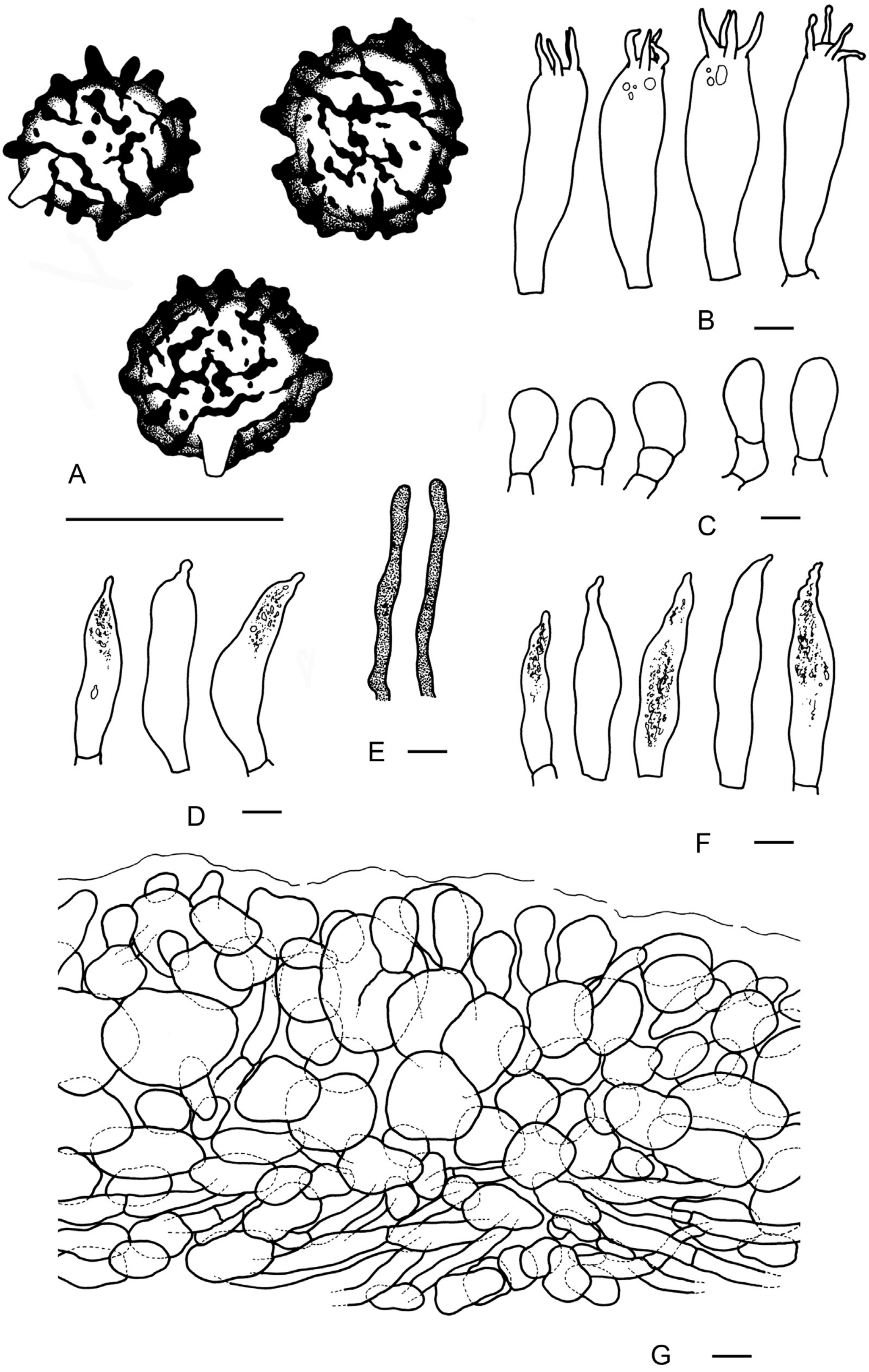

Fig 7 – *Lactarius dombangensis* (a–g: AVKDKVP09-020, holotype): a. basidiospores, b. basidia, c. marginal cells, d. cheilocystidia, e. pseudocystidia, f. pleuromacrocystidia, g. pileipellis (scale bar = 10 μm).

Etymology: 'flavigalactus' refers to the yellowing milk.

Typus: India, Sikkim, North district, 2 km South-west of Dombang Valley, mixed coniferous forest with *Tsuga, Abies, Juniperus, Picea,* and *Larix*, N27°44.13′ E88°45.96′, 2840 m alt., 14/08/2009, AVKDKVP09–021 (Holotypus, GENT!, Isotypus, CAL!).

Pileus 20–35 mm diam., planoconvex to widely and undeeply infundibuliform, usually without blunt papilla in the centre; surface greasy, often shiny-glossy, hardly wrinkled but slightly knobby in some specimens, rarely cracking in some older specimens, with some rare scrobicules present, with some knobs forming concentric circles; reddish brown, warm rusty, darker reddish brown in some circles and in the centre brown (7DE5–6); margin distinctly and regularly crenulate, not striate, but sometimes a bit shortly sulcate because of the crenulate margin. *Lamellae* broadly adnate to slightly decurrent, dense, 10 L + l cm^{-1}, with lamellulae of different lengths, but with the very short ones especially abundant, often forked, especially near the margin, brittle and papery thin, pale orange (5A3) to light orange (5A4) to greyish orange (6B3) or a bit paler; edge entire, concolourous. *Stipe* 18–30 × 5–8 mm, cylindric to subcylindric, sometimes tapering downwards; surface smooth, dry, light brown (7D5–6), paler on top to almost pinkish (in some specimens). *Context* rather thick, especially in the centre of the pileus, hollow in stipe, at least in the under half, whitish cream to flesh-coloured, slightly and locally yellowing because of the milk; odour not remarkable; taste first mild, then soon bitter and acrid, very disagreeable. *Latex* white, rather abundant, changing chrome-yellow on the fruiting body in a few minutes, also changing yellow when isolated, and golden yellow with KOH, drying yellow, staining yellow on white tissue paper.

Basidiospores mostly broadly ellipsoid, sometimes subglobose, sometimes ellipsoid, 6.6 – *7.3* – 7.5 – 8.1 × 5.3 – 5.9 – 6.2 – 6.8 μm, Q = 1.10 – *1.21* – 1.23 – 1.38 (n = 40); ornamentation amyloid, composed of ridges up to 1 μm high, which are irregular and forming an incomplete reticulum; isolate warts present; plage inamyloid. *Basidia* 54–69 × 14–16 μm, 4–spored, subclavate to clavate, with fine granules and guttate contents. *Pleuromacrocystidia* abundant, not emergent to slightly emergent, 62–103 × 7–11 μm, very narrowly fusiform to subcylindrical, rather slender, often with moniliform to mucronate or even tortuous apex, with fine granules and guttate contents. *Pleuropseudocystidia* abundant, 3–4 μm diam., cylindrical, straight to tortuous, with an obtuse apex, with finely granulate contents. *Lamellae edge* heterogeneous, consisting of basidia, marginal cells, and cheilomacrocystidia; marginal cells 19–30 × 5–10 μm, cylindrical to subclavate; cheilomacrocystidia abundant, 60–70 × 8–12 μm, slender, subfusiform with mucronate apex, fine granules, and guttate contents. *Lamellar trama* consisting of hyphae, lactifers, and sphaerocytes. *Pileipellis* an ixocutis, 130–150 μm thick, covered by a thin slime layer; terminal hyphae cylindrical, repent to parallel; underlying layer a dense layer of cylindrical hyphae with some enlarged hyphae and small globose cells, up to 10 μm diam.

Habitat: solitary or gregarious, exposed, in open parts between rocks, mosses, and plants, with *Juniperus* and *Picea* in the neighbourhood.

Studied material: India, Sikkim, North district, 2 km South-west of Dombang Valley, mixed coniferous forest with *Tsuga*, *Abies densa*, *Juniperus*, *Picea,* and *Larix*, N27°44.13′ E88°45.96′, 2840 m alt., 14/08/2009, AVKDKVP09–021 (Holotypus, GENT!, Isotypus, CAL!); China, Yunnan Prov., Diqing Tibetan Autonomous Prefecture, Heping village, grassland nearby forest dominated by *Betula platyphylla*, *Pinus densata*, and *Picea likiangensis*, N27°28.45′ E99°51.63′, 3212 m alt., 04/07/2013, ZD024, (KUN!, GENT!); Yunnan Prov., Diqing Tibetan Autonomous Prefecture, Heping village, grassland 1 km from forest dominated by *Betula platyphylla*, *Pinus densata*, and *Picea likiangensis*, N27°28.60′ E99°51.84′, 3215 m alt., 08/07/2013, ZD084 (KUN!, GENT!).

Comment: We found *L. flavigalactus* in mixed coniferous forest in India and China. In the field this new species is reminiscent of the European *L. badiosanguineus* Kühner & Romagn. in its brick red fruiting body, cinnamon gills and similar habitat. The Indian species is easily distinguished from *L. badiosanguineus* by the yellow discolouration of the milk. *Lactarius sphagneti* (Fr.) Neuhoff is also similar, but this species has a paler pileus margin, unchanging milk and completely reticulate basidiospores. *Lactarius hepaticus* Plowr. has similar colours, but the cap surface is dry and not glossy which is microscopically reflected in the absence of a thin glutinous layer in the pileipellis structure. In addition, the almost complete reticulum connected by fine ridges in the basidiospore ornamentation also allows to distinguish this European species from *L. flavigalactus*.

Lactarius lachungensis Verbeken & Van de Putte **sp. nov.** (Figs 9 and 11d).

MycoBank number: MB812834 **Facesoffungi number:** FoF 00857.

Diagnosis: Medium-sized, warm orange brown basidiocarps, without any reddish or brick tinge, incompletely reticulate basidiospore, protruding macrocystidia and a transition between an ixohyphoepithelium and an ixotrichoepithelium pileipellis.

Etymology: 'lachungensis' refers to Lachung of North Sikkim, the type locality.

Typus: India, Sikkim, North district, Lachung, *Rhododendron*, *Abies densa*, *Tsuga*, *Picea*,, N27°43.49′ E88°44.83′, 2800 m alt., 13/08/2009, AVKDKVP09–009 (Holotypus, GENT!, Isotypus, CAL!).

Pileus 25–50 mm diam., applanate with a central depression; surface greasy, viscid and sticky, smooth to slightly but distinctly rugulose to wrinkled near the margin, brownish orange (6C4–6), light brown (6D6–7), orange (6C8), brown (6D6–D8), without any reddish or brick tinge, a bit darker in the centre, paler to pinkish orange at margin; margin striate up to 1 cm. *Lamellae* broadly adnate to narrowly decurrent or slightly decurrent, moderately distant, 12 L + l cm^{-1}, with lamellulae of different lengths, without regular pattern, pink, orange white (6A2); edge entire, concolourous. *Stipe* 35–40 × 5–6 mm, subcylindric, sometimes tapering downwards, sometimes slightly swollen at the base, reddish brown on top, brick-coloured in the under half or near the base, brownish orange (6C5), light brown (6D5) (or paler). *Context* very thin and fragile, hollow in the stipe, pale orange to flesh-coloured; taste mild; odour sweet. *Latex* extremely scarce, white, unchanging on exposure, unchanging on white tissue paper.

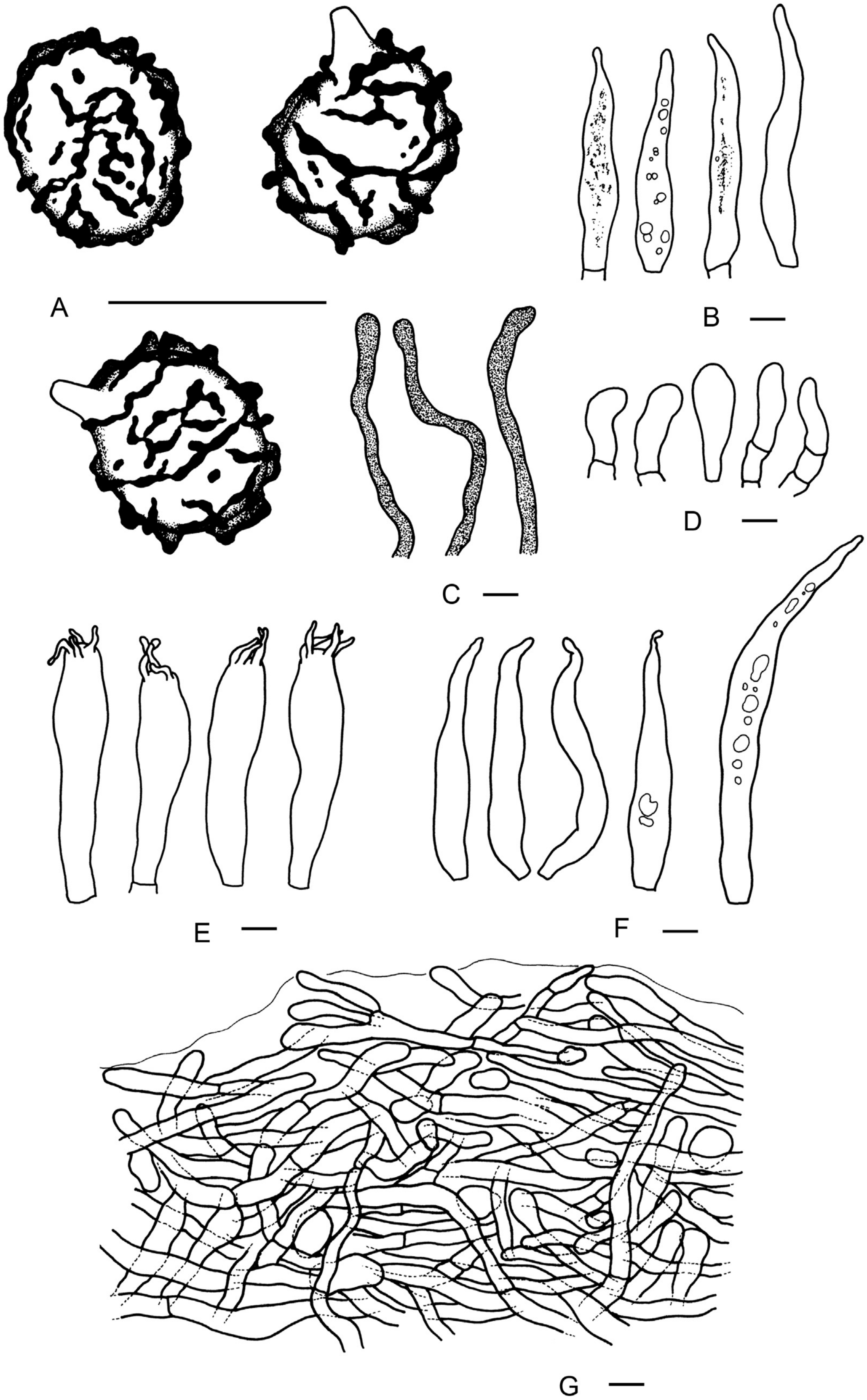

Fig 8 – ***Lactarius flavigalactus*** **(a–g: AVKDKVP09-021, holotype): a. basidiospores, b. cheilocystidia, c. pseudocystidia, d. marginal cells, e. basidia, f. pleuromacrocystidia, g. pileipellis (scale bar = 10 μm).**

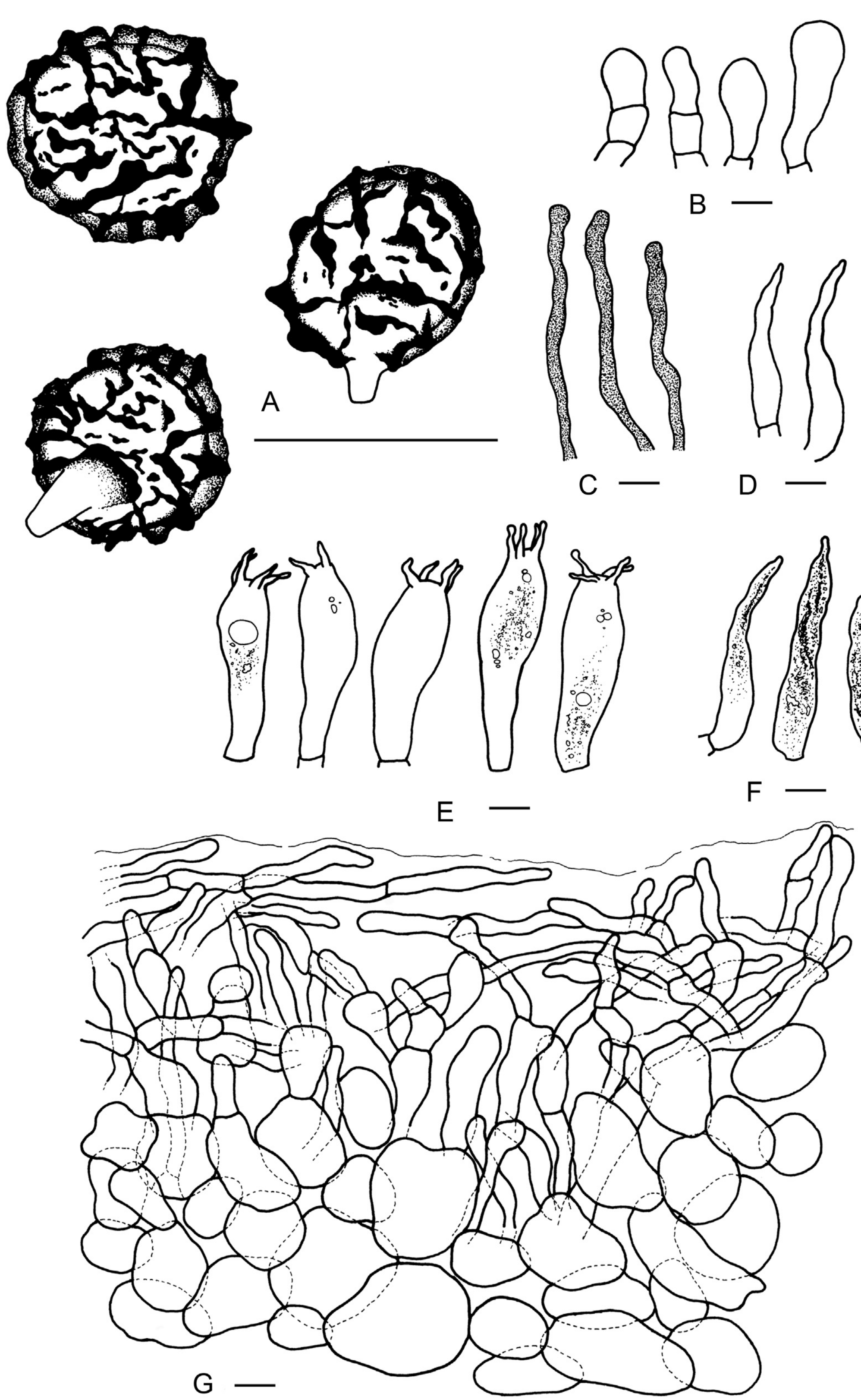

Fig 9 – ***Lactarius lachungensis*** **(a–f: AVKDKVP09-009, holotype, g: AVKDKVP09-010): a. basidiospores, b. marginal cells, c. pseudocystidia, d. cheilocystidia, e. basidia, f. pleuromacrocystidia, g. pileipellis (scale bar = 10 μm).**

Basidiospores subglobose to ellipsoid, 7.0–7.7–8.0–8.7 × 5.9–6.6–7.1.–7.7 μm, Q = 1.08 – *1.13* – *1.20* – 1.30 (n = 60); ornamentation amyloid, composed of ridges up to 1.2–1.4 μm high which are irregular, obtuse to subacute and sometimes crenulate, forming an incomplete reticulum to subreticulum, connected by thin ridges; isolated warts present; plage inamyloid to distally amyloid. *Basidia* 50–61 × 13–21 μm, 4–spored, sometimes 2–spored, subclavate to clavate; with fine granules and guttate contents. *Pleuromacrocystidia* abundant, 57–114 × 9–13 μm, large and slender, protruding up to 70 μm, subcylindrical to subfusiform, straight to bent, with a mucronate to moniliform apex, with fine granules and guttate contents. *Pleuropseudocystidia* abundant, 3–5 μm diam., cylindrical, straight to tortuous, with rounded apex, with fine granules. *Lamellae edge* heterogenous, consisting of basidia, marginal cells, and cheilomacrocystidia; marginal cells 12–40 × 7–15 μm, subcylindrical to subclavate; cheilomacrocystidia abundant, 40–78 × 7–12 μm, slightly projecting up to 10 μm, subfusiform, with mucronate to moniliform apex, with fine granules. *Lamellar trama* consisting of hyphae, lactifers, and sphaerocytes. *Pileipellis* a transition between an ixohyphoepithelium and an ixotrichoepithelium, covered by a narrow slime layer of 20 μm thick; terminal hyphae cylindrical, repent to erect; underlying layer 40–60 μm thick, composed of enlarged hyphae and globose cells which are up 30 μm diam.

Habitat: gregarious on ground among leaf litter.

Studied material: India, Sikkim, North district, Lachung, *Rhododendron*, *Abies densa*, *Tsuga*, *Picea*, N27°43.49′ E88°44.83′, 2800 m alt., 13/08/2009, AVKDKVP09–009 (Holotypus, GENT!, Isotypus, CAL!):–ibid. 13/08/2009, AVKDKVP09–010 (GENT!);–ibid. 13/08/2009, AVKDKVP09–011 (GENT!, CAL!); ibid., North district, Lachung, *Rhododendron*, *Abies densa*, *Tsuga*, *Picea*,, N27°43.51′ E88°44.70′, 2800 m alt., 13/08/2009, AVKDKVP09–017 (GENT!, CAL!); ibid., North district, 2.5 km South-west of Dombang Valley, mixed coniferous forest with *Tsuga*, *Abies densa*, *Juniperus*, *Picea* and *Larix*, N27°43.96′ E88°45.70′, 2840 m alt., 13/08/2009, AVKDKVP09–019 (GENT!).

Comment: *Lactarius lachungensis* is recognized by its unicoloured orange brown basidiocarps in the field. The species is reminiscent of the European *Lactarius tabidus* but *L. lachungensis* has a warmer orange brown colour and unchanging milk. Under the microscope, a thin glutinous layer in the pileipellis distinguishes this new species from *L. tabidus*. Due to the presence in similar habitat and similar basidiocarp colour, *L. lachungensis* can be confused with *L. flavigalactus* and *L. sikkimensis*. *Lactarius flavigalactus* occasionally has uni-coloured orange pileus but this species is characterized by its yellowing milk. For the differences between *L. lachungensis* and *L. sikkimensis*, see under the comment of *L. sikkimensis*.

Lactarius sikkimensis Verbeken & K. Das **sp. nov.** (Figs 10 and 11e).

MycoBank number: MB812835 **Facesoffungi number**: FoF00858.

Diagnosis: Medium-sized, dark liver brown species with a pale and crenulate margin, whey like latex, microscopically characterized by the incomplete to almost complete reticulate basidiospores, the presence of macrocystidia and an ixotrichopalisade for pileipellis structure.

Etymology: 'sikkimensis' refers to Sikkim province, the type locality.

Typus: India, Sikkim Prov., 2 km South-west of Dombang Valley, mixed coniferous forest with *Tsuga*, *Abies densa*, *Juniperus*, *Picea*, and *Larix*, N27°44.13′ E88°45.96′, 2840 m alt., 14/08/2009, AVKDKVP09–024 (Holotypus, GENT!, Isotypus, CAL!).

Pileus 25–45 mm diam., applanate and slightly depressed to concave or widely infundibuliform, sometimes with blunt papilla in the centre, with margin remarkably straight; surface soft, smooth to slightly knotty, chamois-leather like, not shiny at all, more greasy in young ones; young specimens reddish brown (9E6–E7) to dark brown (9F4 to 9F6–F7), strikingly dark liver brown with an abruptly very narrow pale pinkish (9A2) margin; colour soon fading in mature specimens, with the margin still paler and pale pinkish but never that abruptly; margin faintly and shortly striate, already in young specimens. *Lamellae* broadly adnate to slightly decurrent, moderately distant, 11 L + l cm^{-1}, with lamellulae of different lengths, without regular pattern, pink, orange white (6A2) to pale orange (6A3); edge entire, concolourous. *Stipe* 40–75 × 5–8 mm, subcylindric, sometimes tapering downwards, usually swollen halfway, reddish brown, brick-coloured, brownish orange (6C5) to light brown (6D5). *Context* very thin and fragile, hollow in the stipe, pale orange to flesh-coloured; taste mild; odour sweet. *Latex* very scarce, whey to watery whitish, unchanging on exposure.

Basidiospores broadly ellipsoid to ellipsoid, rarely subglobose, (–7.4)7.5 – 8.2 – 8.5 – 9.2 (–9.3) × 5.9 – 6.4 – 6.6 – 7.1 (–7.3) μm, Q = 1.14 – *1.27* – *1.30* – 1.42 (n = 80); ornamentation amyloid, composed of ridges up to 1.2 μm high which are irregular and forming an incomplete to almost complete reticulum, connected by thinner ridges; isolate warts present; plage mostly inamyloid, sometimes distally amyloid. *Basidia* 50–60 × 14–17 μm, 4–spored, subclavate to clavate, with fine granules and guttate contents. *Pleuromacrocystidia* abundant, 57–89 × 12–15 μm, emergent up to 25 μm, subfusiform, mucronate to moniliform apex, with fine granules and guttate contents. *Pleuropseudocystidia* abundant, 3–6 μm diam., cylindrical, straight to tortuous, with round apex, fine granules. *Lamellae edge* heterogenous, consisting of basidia, marginal cells, and cheilomacrocystidia; marginal cells 20–33 × 9–14 μm, subcylindrical to subclavate; cheilomacrocystidia abundant, 34–50 × 6–10 μm, slightly projecting up to 10 μm, subfusiform, with mucronate to moniliform apex, with finely granulate contents. *Lamellar trama* consisting of lactifers, hyphae, and sphaerocytes. *Pileipellis* an ixotrichopalisade, 50–70 μm thick, covered by a thin slime layer, consisting of cylindrical hyphae which are bent to erect; underlying layer with enlarged hyphae and globose cells, up 20 μm diam.

Habitat: solitary or gregarious, growing in mixed coniferous forest with *Tsuga*, *Abies densa*, *Juniperus*, *Picea,* and *Larix*, some growing with *Rhododendron* sp.

Studied material: India, Sikkim, North district, 2 km South-west of Dombang Valley, mixed coniferous forest with *Tsuga*, *Abies densa*, *Juniperus*, *Picea,* and *Larix*, N27°44.13′ E88°45.96′, 2840 m alt., 14/08/2009, AVKDKVP09–022 (GENT!);–ibid. 14/08/2009, AVKDKVP09–023 (GENT!, CAL!);–ibid. 14/08/2009, AVKDKVP09–024 (Holotypus, GENT!, Isotypus, CAL!); –ibid. 14/08/2009, AVKDKVP09–027 (GENT!, CAL!); Sikkim, North

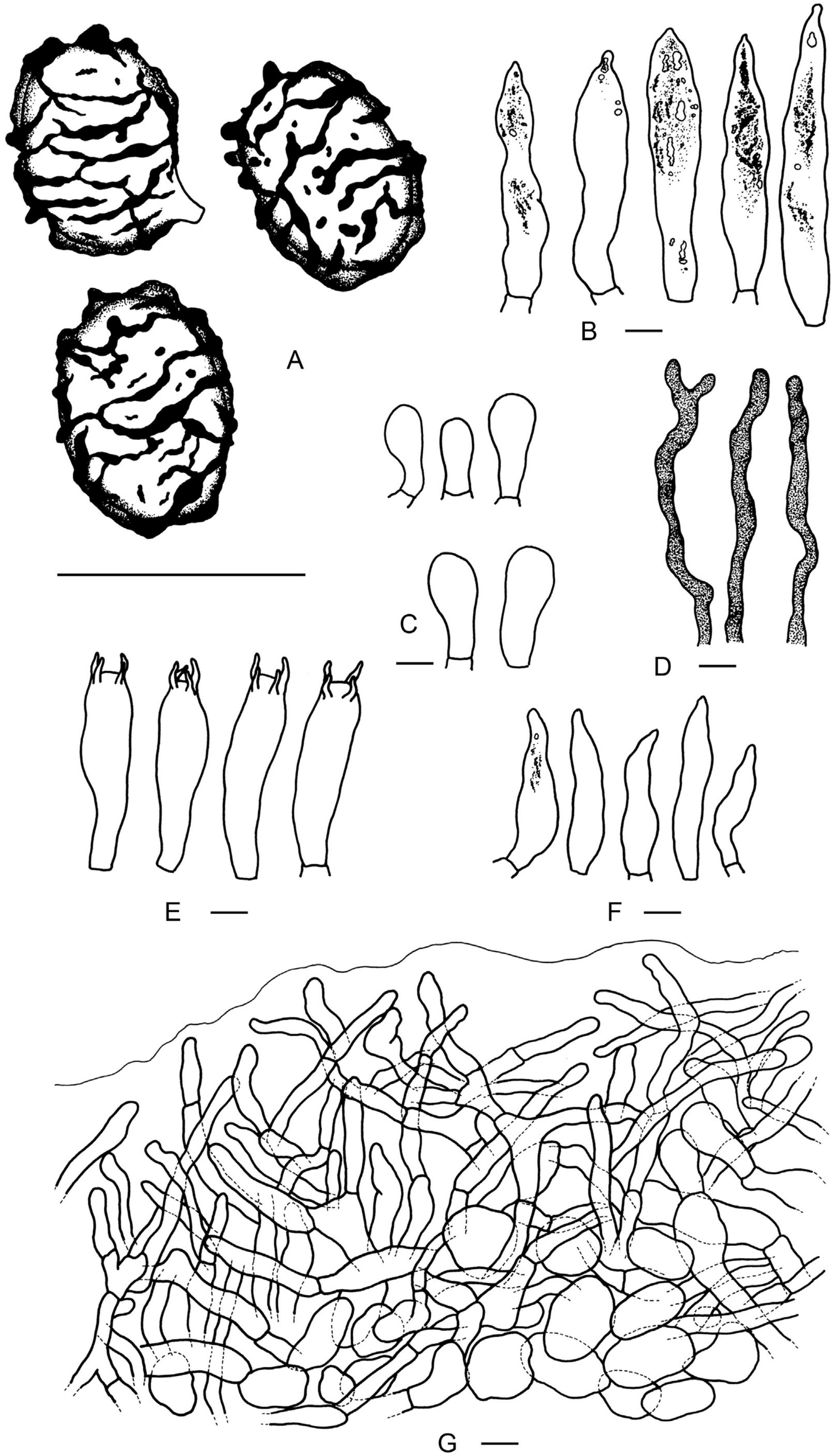

Fig 10 – *Lactarius sikkimensis* (a–f: AVKDKVP09-024, holotype, g: AVKDKVP09-058): a. basidiospores, b. pleuromacrocystidia, c. marginal cells, d. pseudocystidia, e. basidia, f. cheilocystidia, g. pileipellis (scale bar = 10 μm).

district, 2.5 km South-west of Dombang Valley, mixed coniferous forest with *Tsuga*, *Abies densa*, *Juniperus*, *Picea,* and *Larix*, N27°43.96′ E88°45.70′, 2840 m alt., 14/08/2009, AVKDKVP09–025 (GENT!);–ibid.14/08/2009, AVKDKVP09–026 (GENT!); Sikkim Prov., Dombang Valley, 5–6 km from Lachung, mixed coniferous forest with *Picea*, *Tsuga*, *Juniperus*, *Rhododendron* spp., N27°44.51′ E88°46.32′, 2940 m alt., 15/08/2009, AVKDKVP09–058 (GENT!, CAL!).

Comment: In the field *Lactarius sikkimensis* is easily recognized by its greasy and dark liver brown cap with an abrupt and very narrow pale pinkish margin and a crenulate margin in mature specimens. The species is similar to *L. lachungensis*, but it differs by the brick-red tinges and pale pileus margin which are absent in *L. lachungensis*. The other significant difference is that the basidiospores of *L. sikkimensis* are larger.

Lactarius sikkimensis is easily confused with a few European species. *Lactarius sphagneti* also has a two-coloured cap, crenulate pileus margin and similar pileipellis structure. The rather complete reticulate ornamentation in *L. sphagneti* basidiospores and molecular evidence allow to distinguish the European species from *L. sikkimensis*. The general features of *L. sikkimensis* are also similar to *L. camphoratus*. However, *L. camphoratus* has dry pileus, basidiospores ornamented by acutely pointed warts and a hyphoepithelium pileipellis.

In Asia, *L. mukteswaricus* described by Das *et al.* (2004) has similar morphological features, pileus size 30–50 mm, leathery like surface, reddish brown to brown pileus becoming paler towards the margin. A hyphoepithelium pileipellis allows us to distinguish *L. mukteswaricus* from this new species. *Lactarius tangerinus* has an orange brown to reddish brown pileus with pale yellowish margin. However, we frequently observed a glossy surface in *L. sikkimensis* specimens, which reflects the presence of a glutinous layer in pileipellis (ixotrichopalisade). In addition, the Thai species has completely transparent latex, smaller basidiospores, a pileipellis without a glutinous layer (trichoderm) and it is found in deciduous forest dominated by Fagaceae.

Discussion

Taxonomy

Pileus zonation is a striking field character that is considered typical for many members of *Lactarius* subg. *Lactarius* (Heilmann-Clausen *et al.* 1998). Molecular results from this study show that strongly zonated caps can also occur in *Lactarius* subg. *Russularia*. Two species traditionally treated as members of *L.* subg. *Lactarius*, *Lactarius chrysorrheus,* and *Lactarius vinaceorufescens*, are firmly placed in *L.* subg. *Russularia* and are thus classified in this subgenus in our study. *Lactarius chrysorrheus* is described from Europe, but is also known in North American literature, whereas *L. vinaceorufescens* is described from North America. The zonation and the strong and quickly yellowing latex are features that argue for a classification in *L.* subg. *Lactarius*, as done by Hesler & Smith (1979) and Heilmann-Clausen *et al.* (1998). Other characteristics of both species, like the dry basidiocarp and hairless pileus, do fit with the morphological concept of *L.* subg. *Russularia*. Recent work (Das *et al.* 2015) has also shown that these two species form a distinct clade in *L.* subg. *Russularia*, together with *Lactarius indochrysorrheus* and an undescribed, related species from North America (collections AV04-212 and AV05-359, GENT).

The classification of *Lactarius helvus* and *Lactarius rufus* are contradictory in different monographs. Both species are treated as members of *L.* subg. *Russularia* by Hesler & Smith (1979) and Basso (1999). They have been classified by many authors in a separate subgenus *Lactarius* subg. *Colorati* and Heilmann-Clausen *et al.* (1998) transferred these species to *L.* subg. *Lactarius* section *Colorati*, a section characterized by dry, felty or squamulose pilei, whitish or watery latex with no colour reaction and a cutis or trichoderm pileipellis. *Lactarius rufus* has a dry, brick to dark brick or orange brown cap, a weak *Lactarius quietus* odour, white, unchanging latex and a cutis pileipellis. *Lactarius helvus* has a dry, velutinous, pinkish buff cap, transparent latex, an odour of acidic with a curry component, like lovage (*Levisticum officinale*) and a transition between cutis to trichoderm as pileipellis structure. The molecular results suggest that these species should be placed in *L.* subg. *Russularia*.

Evaluation of morphology and phylogeny

Comparative studies of multiple gene genealogies and the concordance between molecular and morphological data have been explored in some Asian milkcaps, using *Lactifluus*[1] (Pers.) Roussel subg. *Lactifluus* as the studied model (Van de Putte *et al.* 2010, 2012). Pleurolamprocystidia, pileipellis hairs and pileus colour are important diagnostic characteristics for the Thai taxa (Van de Putte *et al.* 2010), whereas lamellar density, pileus colour, and pileipellis hairs provided information to distinguish Indian taxa (Van de Putte *et al.* 2012). Stubbe *et al.* (2010) split *Lf.* subg. *Gerardii* (A.H. Sm. & Hesler) Stubbe from *Lactarius* subg. *Plinthogalus* (Berk.) Hesler & A.H. Sm., using the concordance between morphology and multi-locus molecular phylogeny. The authors also establish a clear-cut morphological definition between these two subgenera; spore print colour, basidiospore ornamentation, the presence or absence of true cystidia and the presence or absence of well-developed cellular layer in the pileipellis were proposed as discriminative characters.

We evaluated five characteristics that are considered important for species delimitation and classification in *Lactarius* subgenus *Russularia* by adding the respective information to the outcome of the analysis of the concatenated dataset: latex colour, latex colour change, basidiospore ornamentation, the presence or absence of true cystidia and pileipellis structure (Fig 2). The result suggests that the combination of two morphological characters, i.e. pileipellis structure and the presence or absence of true cystidia, is phylogenetically informative in most clades in this subgenus. True cystidia are absent in multiple unrelated lineages or species in the evolutionary history of the subgenus *Russularia*.

Existing classification systems are entirely based on either European or North American taxa. Since we have included mainly European and Asian taxa, the classification schemes of the subgenus in the most recent European literature, by Basso (1999) and Heilmann-Clausen *et al.* (1998) are

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[1] *Lactifluus* is abbreviated as *Lf.* in this paper.

considered. Our result differs from the classification of Basso (1999) because here *Lactarius* subg. *Rhysocybella* Bon species sensu Basso (1999) are included within *L.* subgenus *Russularia*. Pileipellis structure and true cystidia were mentioned as important characters in many studies (Bon 1983; Heilmann-Clausen *et al.* 1998; Basso 1999). In Clade A (see Fig 2), all species have true cystidia. The majority of species in this clade (22 out of 31 species) have a pileipellis with typical hyphae e.g. ixocutis (icu), (ixo−)trichoderm (tp and itp), (ixo−)trichopalisade (tp and itp). All European taxa in Clade A have been classified in the sections *Russularia* and *Tabidi* in the European monograph of Heilmann-Clausen *et al.* (1998). However, it would be wrong to conclude that this character is phylogenetically informative for this clade due to the occurrence of species with completely isodiametric cells in the pileipellis, e.g. with epithelium (ep) or hymenoepithelium (hm). Within this clade such pileipellis structures are restricted to the Indian *Lactarius dombangensis* and the *Alnus*-associated taxa: *Lactarius cyathuliformis*, *Lactarius obscuratus*, *Lactarius brunneohepaticus* M.M. Moser, *Lactarius herrerae,* and *Lactarius omphaliformis*. A pileipellis structure with isodiametric cells, e.g. hyphoepithelium (he) and epithelium (ep), is a shared character for species in Clade B and C. The major difference between representatives in Clade B and C is the presence or absence of macrocystidia. Similarly, the distinction between species in Clade D and E is the presence and absence of macrocystidia. Clade G contains two species: *Lactarius quietus* and *Lactarius aquosus*. Both species have an inconspicuous zonation and true cystidia.

The colour of the latex and colour change of the latex when exposed to the air are important characters for defining infrageneric groups of milkcaps in general and especially in *Lactarius* subg. *Lactarius* (Hesler & Smith 1979; Heilmann-Clausen *et al.* 1998). *Lactarius* subg. *Russularia* displays only a very narrow variability of this feature. Many species possess white, unchanging latex and in most cases the only change that can be observed is a yellowing change (pale to bright yellow or sulphur). This study shows that strongly yellowing milk is at least informative for the group of *Lactarius chrysorrheus* (in Clade G, Fig 2). Latex turning (paler) yellow appears to have multiple origins, and thus contains less or no phylogenetic information. In addition, water-like, transparent latex does not seem to give taxonomic information on section level in the subgenus (Fig 2).

Spore morphology is usually very important for species delimitation, but this characteristic gives little information on evolutionary relationships within this subgenus. In some cases, it is difficult to quantify the degree of ornamentation, particularly to distinguish between a subreticulum and a complete reticulum. There is always a certain degree of intraspecific variation in the ornamentation type. The basidiospore ornamentation of most species is composed of ridges up to 1.5 μm high, forming a very incomplete reticulum or subreticulum, with some or many isolate warts or spines present. An ornamentation with a high and complete reticulum or zebroid pattern is restricted to some species that are not closely related, e.g. *Lactarius chichuensis* (zebroid), *Lactarius crenulatulus* (complete reticulum), *Lactarius politus* (ridges up to 2.5 μm forming a complete reticulum), *Lactarius helvus* (complete reticulum) (Fig 2). The same is true for an ornamentation completely composed of isolated warts (occurring in *Lactarius falcatus*, *Lactarius atrii,* and *Lactarius aurantionitidus*).

Concerning the general habits, most representatives of *L.* subg. *Russularia* are agaricoid. So far, three angiocarpic species have been described and pleurotoid species have not been discovered in this subgenus. The angiocarpic habit evolved more than once. *Lactarius hispanicus* Calonge & Pegler and *Lactarius borzianus* Cavara might have evolved from the same recent common ancestor but *L. falcatus* has a different origin. Fruiting body size has little or no phylogenetic information. There are, however, two clades dominated by very small species: the *Lactarius gracilis*-group and the *L. obscuratus*-group.

The lamellar density is a reliable character for species delimitation but it is not phylogenetically informative within the subgenus. Most species have crowded to subdistant lamellae. Four very distantly gilled species (*Lactarius sublaccarioides*, *Lactarius pasohensis*, *Lactarius atrobrunneus* Wisitrassameewong & K.D. Hyde and *Lactarius laccaroides* Wisitrassameewong & Verbeken) were included in this study. However, they all seem to have evolved independently.

Other characteristics that could be studied in more detail are the chemical composition of latex which is related to the taste (pungency), the colour and the eventual colour change. The pungency is correlated with a few fatty acid esters of sesquiterpenoids. After bruising the fruiting body, these esters are converted to pungent, unsaturated sesquiterpene dialdehydes (Gry & Andersson 2014). However, the degree of pungency is hard to quantify by tasting the latex. Imprecision and subjectivity is also an issue for the characterization of odours.

Intercontinental specificity

The discussion of distribution patterns is necessarily limited by the sampling of mainly European and Asian representatives. Representatives from the New World are especially lacking in this analysis to fully explore distribution patterns. Phylogenetic studies often focus on linking clades to geographical distribution (e.g. are species grouped according to continents or northern versus southern hemisphere). Our results show that many lineages have spread across continents. The species do not at all cluster together according to their geographic origin. On the other hand species diversification is also observed in adjacent regions of the same continent. This suggests that both allopatric and sympatric speciation might have occurred.

None of the European taxa are conspecific with the tropical Southeast Asian taxa. Consequently temperate names should not be applied to tropical Asian species. We did find conspecificity of species between Europe/Asia and North America/Europe. The European and Chinese specimens identified as *Lactarius badiosanguineus* group together in a clade with high support (98 % bs and 1.00 pp, in Fig 2) and the sequences of this clade show low sequence variability, as can be expected at infraspecific level. Morphological characters are concordant with the molecular result. The combination of the reddish brown cap, incompletely reticulate basidiospores with isolated warts, the presence of true cystidia and an ixotrichoderm pileipellis has also been observed in the Chinese material (ZD991). In Europe, *L. badiosanguineus* can be associated with *Picea* and *Abies*; the Chinese material is recorded with *Picea likiangensis*. The Chinese specimen was collected in a high elevation coniferous forest (3302 m alt.) in Northwest Yunnan. Conspecificity also occurs between North America and Europe in the case of *Lactarius tabidus* (Fig 2). The Belgian sample of *L. tabidus* (KW130) grouped with the American *L. tabidus* (ED2008-14) in a strongly supported group (100 % bs/1.00 pp). Specimens identified as *Lactarius quietus*, have recently been reported from Dujiangyaan area in Sichuan Province, China (JF273529) and South Korea (KM052571). Both sequences group in a clade (with low bootstrap support; 63 %) that is sister to the European *L. quietus* (Fig 1). Hence we prefer to name them as *L.* cfr. *quietus* in this study. More markers and additional Asian collections need to be included; coupled with a thorough microscopic comparison of the European and Asian specimens this will address the true relationships of these collections.

According to the online sequence database, UNITE, DNA sequence data from mycorrhiza or soil samples shows that European taxa occur in different continents. *Lactarius rufus*, *Lactarius helvus,* and *Lactarius camphoratus* are known to occur in North America (Kõljalg *et al.* 2013). *Lactarius subdulcis* has been recorded in the United States and Iran (Kõljalget *al.* 2013). The event of temperate species crossing continents is possible, but is generally a rare occasion (Nuytinck *et al.* 2007). More molecular and morphological data are necessary to resolve the question of conspecificity for some of the other *Lactarius* subg. *Russularia* species.

Divergence time and historical host relationships

A reliable and well-supported phylogeny is a prerequisite for a dating analysis. Before estimating the divergence times of major clades in dataset 1 (containing LSU and *rpb2* sequences of representatives from Russulales, Hymenochaetales, Phallales, Polyporales, Agaricales, and Boletales), we tested different phylogenetic algorithms to infer the phylogeny: MP, ML and BI. The estimates for the calibrated nodes, the ancestor of Hymenochaetales and the *Mycena/Marasmius* split were 151.84 and 112.42 mya, respectively (Fig 3 and Table 3). The node values for major clades H, K, L and M (*Russula/Lactarius*, *Lactarius* subg. *Lactarius*, *L.* subg. *Lactarius/Lactarius* subg. *Russularia,* and *L.* subg. *Russularia*, respectively) are poorly supported because of two possible factors: (1) there is a low support in the ML and BI trees for most of these splits, e.g. the *L.* subg. *Lactarius/L.* subg. *Russularia* is not well-supported because there is a problem with the delimitation of *L.* subg. *Lactarius*. These clades might be resolved using additional sampling and more markers, but resolving clades on this level is beyond the purpose of this BEAST analysis; and (2) we value the support value of subgenus *Russularia* from the ML and BI trees generated by the concatenated dataset comprising 51 *L.* subg. *Russularia* species in total (Fig 2) more than that of the BEAST analysis. The tree topology and divergence dates of dataset 1 are also consistent with other studies (Hibbett & Matheny 2009; Sánchez-Ramirez *et al.* 2014; Chen *et al.* 2015). A recent study (Looney *et al.* 2016) used secondary calibrations from a dated genome-based phylogeny to infer a chronogram of Russulaceae and found similar but older crown ages for Russulales (~169 mya) and *Lactarius* (~35 mya) and a younger crown age for Russulaceae (~55 mya).

Hibbett & Matheny (2009) suggest that Boletales and Russulales might be young enough to have been plesiomorphically associated with either rosids or Pinaceae. Our results support this, with the estimated crown age of Russulales inferred as 157.78 mya (100.88–226.94 mya; 95 % HPD), which is younger than the estimated crown age of the family Pinaceae (~189 mya, Lin *et al.* 2010) and rosids (~175 mya, Bell *et al.* 2010). The age of ancestral species of *L.* subg. *Russularia* is estimated to be 18.67 mya (13.11–24.10 mya; 95 % HPD) which is in the middle Miocene (dataset 2, see Fig 4). From the ancestor two main evolutionary lineages seem to have diverged: Clade A (~12.83 mya, 8.68–17.09 mya; 95 %HPD) and B (~16.36 mya, 11.53–21.46 mya; 95 %HPD). Whereas, the divergence of the *Lactarius aquosus/Lactarius quietus* group remains uncertain due to its low support (19 % bs/0.48 PP). This clade appears to be sister with Clade A and B. Generally, evolutionary processes of ECM fungi are thought to relate with distribution or migration of their host trees (den Bakker *et al.* 2004). ECM fungi are likely to migrate with their symbiotic partners (Vellinga *et al.* 2009). Hence, the range expansion and isolation of ancestral *L.* subg. *Russularia* species might be related to the distribution and migration of their symbiotic partners. The migration of plant species can be influenced by environmental factors (e.g. climatic change), geologic changes (e.g. separation of continents, uplift of mountains) or even human-caused introductions.

Thus, we hypothesize that the ectomycorrhizal host association might have played an important role in species evolution within *L.* subg. *Russularia*. One of the most specialized cases of ectomycorrhizal (ECM) symbiosis is that between *Alnus* trees and their symbionts (Rochet *et al.* 2011; Põlme *et al.* 2013). Our study shows that most *Alnus*-associated species of *L.* subg. *Russularia* form a supported clade (Fig 2). This is in accordance with Rochet *et al.* (2011) and confirms that *Lactarius brunneohepaticus*, *Lactarius obscuratus*, *Lactarius cyathuliformis,* and *Lactarius herrerae* appear to have evolved from the same common ancestor, while *Lactarius omphaliformis* has an independent origin. Most of these species are associated with hosts of *Alnus* section *Alnus*, except for *L. brunneohepaticus* which is found in association with *Alnus alnobetula* (*A.* section *Alnobetula*). *Lactarius obscuratus* and *L. cyathuliformis* are associated with *Alnus incana*, *Alnus glutinosa,* and *Alnus cordata* (*A.* section *Alnus*). Montoya *et al.* (2015) described the ectomycorrhizal association of the Mexican *L. herrerae* with *Alnus acuminata* (*A.* section *Alnus*). *Lactarius omphaliformis* has been reported with *A. glutinosa* and *A. acuminata* (*A.* section *Alnus*). Rochet *et al.* (2011) and Põlme *et al.* (2013) suggest that the observed evolutionary patterns of *Alnus* associated ectomycorrhizal fungi can be explained possibly by coevolution and the association is relatively specific at the level of plant subgenus and section. *Alnus* and associated fungal species might have a co-migration pattern across a wide range of geographic locations (Kennedy *et al.* 2011). Põlme *et al.* (2013) hypothesized that the migration of ancestral *Alnus* species might have occurred after the last glacial maximum and the migration pattern might be from Eurasia to North America and from Southern Europe to Northern Europe.

The ectomycorrhizal association of many other *L.* subg. *Russularia* species remains uncertain in mixed vegetations, e.g. tropical forests dominated by Fagaceae, mixed deciduous and coniferous forests and coniferous forest with *Pinus*, *Picea,* and *Abies*. Also, some species have a broad host range, e.g. *Lactarius aurantiacus* and *Lactarius fulvissimus*. Digging up soil and collecting ectomycorrhizal rootlets underneath the collected fruitbodies as well as DNA sequencing of plant host and fungal partner from ectomycorrhizal rootlets are needed to answer questions concerning the ecology and symbiotic interaction of ECM species.

In this study, we annotated possible host ranges in the dated phylogeny of *L.* subg. *Russularia* in Fig 4. A multitude of host preferences can be observed in the species in Clade A. Some species in this clade are generalists, e.g. *L. aurantiacus*, *L. tabidus,* and *Lactarius fulvissimus* which suggest that host switches or host expansions might have occurred in the evolutionary history of these species. *Alnus*-associated *L.* subg. *Russularia* species, e.g. *L. brunneohepaticus*, *L. cyathuliformis*, *L. herrerae*, *L. obscuratus*, *L. omphaliformis*, are specialists. Ancestral species of *Alnus* might have originated in the Eocene epoch (Rochet *et al.* 2011) and thus the origin of the genus *Alnus* predates the divergence of the *Alnus* associated *Russularia* species. Many species associate with Pinaceae, e.g. *Lactarius hepaticus*, *Lactarius badiosanguineus,* and *Lactarius rufus* as well as the newly proposed species in this study. All newly proposed species and some tropical lowland species, e.g. *Lactarius sublaccarioides*, *Lactarius inconspicuus* H.T. Le & F. Hampe and *Lactarius tangerinus* H.T. Le & De Crop, seem to have diverged from temperate species and not from other tropical lowland species. Recent phylogeographic studies report on the origin and possible paleodispersal routes of Pinaceae trees. Pinaceae might have evolved during Early Jurassic with subfamilies and genera diversifying during the Mid Jurassic and lower Cretaceous (Lin *et al.* 2010). Ran *et al.* (2006) proposed a biogeography and phylogeny of *Picea* based on chloroplast and mitochondrial DNA. Their results suggested a North American origin of *Picea* which is consistent with the earliest pollen fossil of *Picea* in USA in Paleogene (Wilson & Webster 1946). Moreover, many cone fossils of *Picea* have also been recorded from the Eocene sediments of North America (Axelrod 1998; LePage 2001). A North American origin is also proposed for *Abies* (Xiang *et al.* 2015). The origin and early diversification of *Abies* is likely to have occurred around high latitude areas in Western North America during the Eocene epoch and possibly migration to eastern Asia occurred through BLB during the Middle Miocene.

In Clade B, the majority of taxa associate with tropical Fagaceae. The high concentration of tropical lowland species and the prominent species diversification of Southeast Asian species in Clade B might suggest an Asian origin of the subgenus. Fagaceae genera are most abundant in tropical Southeast Asia. A high diversity of Fagaceae species is presented in the

Fig 11 – Basidiocarps, a–b. *L. aurantionitidus*, (a: xp3-20120905-06, b: ZD973, photo: Guo Jiayu), c. *L. flavigalactus* (ZD84, photo: Guo Jiayu), d. *L. lachungensis* (AVKDKVP09-017, photo: Kobeke Van de Putte), e. *L. sikkimensis* (AVKDKVP09-024, photo: Kobeke Van de Putte).

subtropical and evergreen tropical forests of Central America, southern continental Asia and the Malayan Archipelago (Manos *et al.* 2001). The high concentration of Fagaceae species in these regions suggests that these plants might have a tropical origin (Qiang 1996). Ancestral area reconstruction analyses suggested an Asian origin for the genus *Fagus* whereas the origin of the genus *Quercus* remains ambiguous (Manos & Standford 2001). White oaks (section *Quercus* s.s.) might have a New World origin and might have dispersed in the Northern hemisphere (Manos *et al.* 1999). The migration events possibly occurred in the late Eocene (~40 mya) and the Middle Miocene (~15 mya) (Tiffney 1985). Other dominant mycorrhizal trees in the vegetation of Southeast Asia belong to the Dipterocarpaceae. Molecular dating analyses suggest that the origin of ancestral Dipterocarpaceae was in Africa in the early Cretaceous and that later migration to the Deccan plate and Southeast Asia occurred. Fossil records show the occurrence of Dipterocarpaceae in Southeast Asia in the late Oligocene and the earliest Miocene (30–25 mya) (Gunasekara 2004).

The detailed historical biogeography and origin of *L.* subg. *Russularia* remain unclear because insufficient data are available at the moment. The crown node of *L.* subg. *Russularia* (~18 mya) is younger than the estimated ages of the families of host trees. Further studies on biogeography and ancestral area reconstruction analyses, including more representatives from different continents will address the distribution pattern of *L.* subg. *Russularia* (See Fig 11).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.funbio.2016.08.004.

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