Diversity and phylogeny of *Lactarius* subgenus *Russularia* in Southeast Asia

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Rationale of the study

Fungi are a large and diverse group of organisms, which are estimated to be the second largest group of organisms after the group of insects. However, the diversity of fungi remains understudied. The real number of fungal species on earth might be higher than previously hypothesized (O'Brien et al. 2005). This actual number has been estimated to be up to 1.5 million species (Hawksworth 1991, Hawksworth & Rossmann 1997) or even 5.1 million species (Blackwell 2011), whereas about 100,000 species are described so far (Kirk et al. 2008). Numerous new species are still waiting to be explored. Species delimitation of fungi mainly relies on the morphological species concept (MSC) up to the middle of the 20th century. This species concept is based on the similarity and difference of morphological characteristics. However, fungal taxonomists encounter difficulty in delimiting fungal species because many groups of fungi are morphologically rather simple. Furthermore groups with a lot of morphological variation or morphological differences may not have evolved yet or underwent convergent evolution. In addition, the nomenclature and classification system is often unstable and relationships between fungi are poorly understood because of the limited sampling that has been done by only a few experienced mycologists.

Fungi play an important role in many ecosystems, as decomposers or pathogens or symbionts (i.e. lichens and mycorrhiza). The ectomycorrhizal (ECM) plant-fungal mutualism is diverse and widespread in soil communities. It has long been hypothesized that the ECM mutualism might have a paleotropic origin (Pirozynski 1983, Thoen 1993, Buyck et al. 1996, Alexander 2006, Matheny et al. 2009) during the Cretaceous period when both major groups of ECM plant, Pinaceae and rosids, have diversified (Brundrett 2002, Hibbett &

Matheny 2009). However, ECM fungi and their symbiotic interaction are understudied in the tropics due to the lack of trained mycologists, contemporary collections and molecular characterization. The genus *Lactarius* Pers. is an important group of macrofungi. It is ecologically important as one of the major groups of ECM fungi in many ecosystems worldwide (Heilmann-Clausen et al. 1998). In this PhD study I focus on the diversity of one of the three subgenera in *Lactarius,* i. e. *L.* subg. *Russularia* (Fr. ex Burl.) Kauffman. It is the least documented subgenus out of the three known subgenera. Many areas remain understudied. The delimitation of the subgenus remains problematic. Southeast Asia was chosen as the study area because previous reports revealed the richness of the *Lactarius* mycoflora in this region (Le et al. 2007a, b, Stubbe 2007, 2008, Van de Putte et al. 2010). Northern Thailand was suggested as one of the hotspots in this subcontinent (Le 2007).

1.2 Milkcaps in a new generic landscape

Lactarius Pers., Tent. Disp. Meth. Fung.: 63. 14 Oct – 31 Dec 1797 (‘*Lactaria*’), nom. et orth. cons. Typus: *L. torminosus* (Schaeff.: Fr.) Pers. (*Agaricus torminosus* Schaeff.), typ. cons., see Buyck et al., 2010.

Lactarius was raised by Persoon (1797) and little discussion existed about its delimitation. *Lactarius* or milkcaps are easily recognized among the agaricoid mushrooms since they exude latex (milk) when bruised, a rare feature within the Basidiomycota. Besides milkcaps, latex exudation is also occuring in certain *Mycena* species, where it is only visible in the stipe, and in some boletes (*Suillus* spp.) or some rare *Amanita* species. After 1800, many taxonomists placed milkcaps in different sections of *Agaricus* (Persoon 1801, de Candolle 1815, Fries 1818 and 1821). In 1806, Roussel raised milkcaps to the genus *Lactifluus* (Pers.) Roussel. Afterwards, Gray (1821) was the first authority to establish the name *Lactarius*. *Galorrheus* (Fr.: Fr.) Fr., *Lactariella* Schröter, *Lactariopsis* Henn., *Gloeocybe* Earle and *Pleurogala* Redhead & Norvell are now widely accepted as synonyms of *Lactarius* (Redeuilh et al. 2001). Together with the even larger genus *Russula* Pers., *Lactarius* forms the core of the family Russulaceae. Until recently, more than 400 species were accepted under the name *Lactarius* (Verbeken 2001). In 2008, a multi-gene based phylogeny of the Russulaceae revealed that the structure of the family actually comprised four major groups (Buyck et al.

2008, Figure 1.1). A small group of *Russula*, namely *R.* subsect. *Ochricompacta* Bills & Miller, was shown to be separate from the major monophyletic clade of *Russula* and classified together with *L. furcatus* Coker. This resulted in the establishment of the genus *Multifurca* Buyck & V. Hofstetter. The remaining milkcaps were divided in two clades. One major clade containing about 75% of all the representatives remains *Lactarius*. It contains members in three subgenera: *L.* subg. *Lactarius*, *L.* subg. *Plinthogalus* (Burl.) Hesler & A.H. Sm. and *L.* subg. *Russularia*. In addition, sequestrate genera *Arcangeliella* Cavara, *Zelleromyces* Singer & A.H. Sm. and *Gastrolactarius* J.M. Vidal also nest in the *Lactarius* clade with angiocarpic representatives present in the three subgenera.

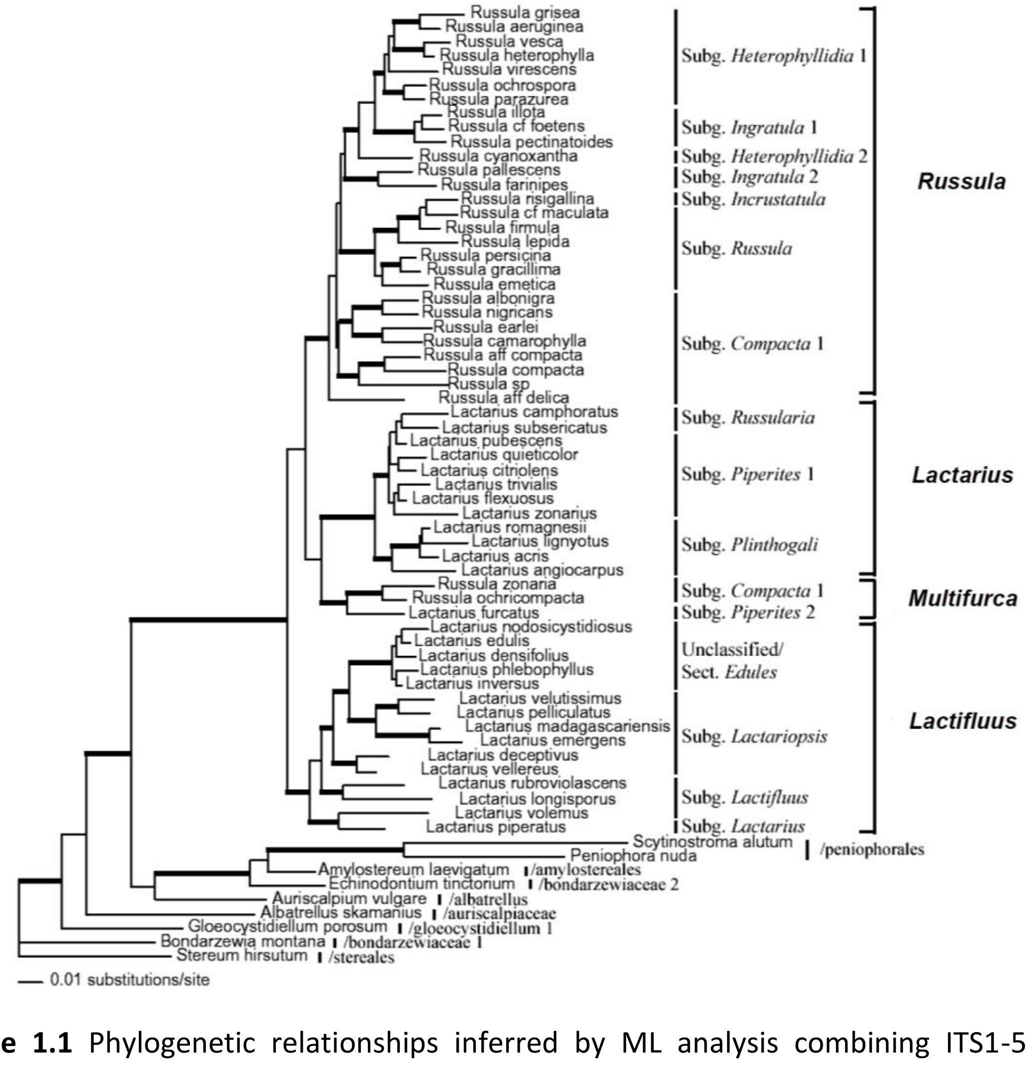


Figure 1.1 Phylogenetic relationships inferred by ML analysis combining ITS1-5.8S-ITS2, nucLSU and *rpb2* sequence data for 67 taxa. Thick branches received ML bootstrap ≥70% and Bayesian posterior probability ≥95%. This picture is reproduced from Buyck et al. 2008.

The second milkcap clade contains the species that belonged to former *Lactarius* subg. *Gerardii* Stubbe, *L.* subg. *Lactifluus* (Burl.) Hesler & A.H. Sm., *L.* subg. *Lactariopsis* (Henn.) R. Heim, *L.* subg. *Russulopsis* Verbeken, *L.* sect. *Edules* Verbeken and *L.* sect. *Panuoidei* Singer (Buyck et al. 2008, 2010). All species in this clade are now treated in the genus *Lactifluus* (Pers.) Roussel (Verbeken et al. 2011, 2012, Stubbe et al. 2012).

1.3 Morphology of *Lactarius* and *Lactifluus*

Despite the clear molecular distinction between the two milkcaps genera *Lactarius* and *Lactifluus*, it remains hard to distinguish these two groups using morphology. The latex colour and colour changes are taxonomically not informative because most character states are present in both genera. There is no clear-cut character that helps both genera. A combination of characters is necessary to distinguish them in the field (Verbeken 2015).

Glutinous and slimy caps, hairy or bearded pileus margins only occur in *Lactarius* and the same goes for pileus zonation. So far these characters are not represented in *Lactifluus*. On the other hand strongly velutinous pileus surfaces and an annulus or velar remnants are only present in *Lactifluus*. Angiocarpic forms (a sporocarp morphology in which basidiospores are retained in the closed fruiting body) are only known in *Lactarius*, whereas pleurotoid forms are only found in *Lactifluus*. Microscopically, thick-walled elements (as in a lamprotrichoderm) in the pileipellis and thick-walled cystidia (lamprocystidia) in the hymenium are often present in *Lactifluus* species. These elements are uncommon in *Lactarius*. Cystidia in *Lactarius* are thin-walled and are typical macrocystidia, as occurring often in *Russula* spp. Gills with a completely cellular trama (composed of sphaerocytes) are common in *Lactifluus*, while rarely present in *Lactarius*.

1.3.1 Macromorphology of *Lactarius*

Most representatives in *Lactarius* are agaricoid (with pileus and stipe, bearing the hymenium on vertically arranged gills underneath the pileus). Angiocarpic representatives, the so-called false truffles, have been derived from the agaricoid forms (Miller et al. 2001). *Lactarius* is a genus which is rich in pigment diversity and in variation in pileipellis structures (ranging from pure filamentous hyphae to both inflated filamentous hyphae and globose cells, over pure globose cells). Although the majority of the species have white latex, we see extraordinary colours like sulphur-yellow, orange, red, blue, brown and pink in some species or species groups. The oxidation of latex colour on exposure is also diverse, sometimes leading to spectacular and fast colour changes, sometimes requiring a careful observation of subtle changes. The abundance of latex can vary from species to species and of course also depends on the age of the fruiting body. Generally, latex is best observed and most abundant in immature to mature specimens. In old specimens or in dry condition, latex can be scarce or even almost absent.

Most *Lactarius* species have a typical convex to planoconvex pileus, which later becomes more expanded, often with a central depression. The pileus gradually lifts up and eventually becomes infundibuliform. The range of pileus size is rather extreme. The pileus diameter can be up to 300 mm in *L. controversus* Pers.: Fr., while the smallest one is probably *L. perconicus* Verbeken & E. Horak, with a pileus of maximum 8 mm.

Pileus colours vary from white, pale cream, pale pink brown, vivid orange, vinaceous red, olive green, indigo blue, buff, brown to even strong dark brown. Pileus colours and features related to the pileipellis structure (such as strong zonation, bearded margin or velutinous aspect) are very important when it comes to identification of species, or recognition of the infrageneric groups. The pileus features are more diverse in *Lactarius* than in *Russula* or *Lactifluus*. The pileus can be dry or velvety to sticky or even slimy, and smooth, rugulose, (strongly) veined. Often the sticky or slimy surfaces become inconspicuous in dry condition. The best way to determine the nature of a glutinous layer on the pileus is the kissing test, by gently touching the cap surface with your moistened upper-lip (Heilmann-Clausen et al. 1998). The macroscopic cap texture is reflected in the microscopic pileipellis structure (see further down). Lamellae features include distance between the lamellae, colour of the gills and gill edge. In most cases the stipe is smooth to slightly wrinkle. Many species in *L.* subg. *Lactarius* have a sticky stipe surface with scrobicules. A faintly velvety stipe surface can be found in the species with a velvety cap. Some species, particularly in *L.* subg. *Russularia*, have a strigose stipe base.

As in *Russula* and *Lactifluus*, the taste is an important field character. Most *Lactarius* species are mild, some are extremely acrid, or first mild and then turning acrid. Bitter tasting species also occur. The taste of the context is usually linked to the latex taste, although not in all cases. It is necessary to taste the latex separately from the flesh. There is also an important and amazing variation in odours, which certainly contributes to the attractiveness of the genus. Some smell like apples (*L. acerrimus* Britzelm.), some smell like coconut (*L. glyciosmus* (Fr.: Fr.) Fr.). The strong smell of maggi or *Levisticum offinale* occurs in many representatives of *L.* subg. *Russularia* and remains very strongly in dried herbarium specimens.

In some cases, the spore print can be useful as an identification tool. The spore print colour allows to distinguish *L.* subg. *Plinthogalus* from *Lactifluus* subg. *Gerardii.* Cream-coloured or ochraceous buff colours are often found in *L.* subg. *Plinthogalus* and white spore prints are well-represented in *Lf.* subg. *Gerardii* (Stubbe et al. 2011). However, one has to keep in mind that the colour of spore prints in a single species can be perceived as variable due to the density of the print (Hesler & Smith 1979).

1.3.2 Micromorphology

Microscopic characters are as diverse as macroscopic characters in the genus. The observation of microscopic characters is best studied using dried material, because in fresh condition the presence of latex will often hinder the microscopic observations. Clamp connections are absent in all Russulaceae. A very characteristic basidiospore feature of most Russulales is the amyloid spore ornamentation. The ornamentation stains bluish black when treated with iodine containing reagents (e.g. Melzer’s reagent and Lugol’s solution) (Figure 1.2c). This ornamentation is variable among different species but quite consistent within a single species. Thus the pattern of basidiospore ornamentation is like an identity card for individual species and is considered as a very useful characteristic for species recognition and delimitation. It ranges from a low ornamentation with isolated warts over an incomplete or complete reticulum to a high and winged ornamentation, sometimes forming a zebroid pattern. The only species recorded so far that has a distinct ornamentation with a remarkably weak amyloid reaction is *L. inamyloideus* Verbeken & E. Horak (Verbeken & Horak 2000).

Elements of the hymenium in lamellae are observed by means of manual cross sections through the lamellae (Figure 1.2b). The basidia (spore producing structures situated on the lamellae) are mostly subcylindric to subclavate, thin-walled, and have a guttate to granular content (Figure 1.2a). They usually have four sterigmata but variation in the number of sterigmata is occasionally found in most species; some rare two-spored basidia occur. *Lactarius acerrimus* is characterized by its exclusively two-spored basidia.

The central part of the lamellae (called the lamellar trama or hymenophoral trama) is sterile. One of the synapomorphies of Russulales is the presence of a gloeoplerous systems. The gloeoplerous system consists of vascular hypha containing fluid that typically stains black in sulfoaldehyde compounds. One type of gloeoplerous hyphae is lactiferous hyphae or lactifer (Figure 1.2e). Lactiferous hyphae are non-septate hyphae which contain latex. Latex becomes macro- and microscopically visible when the tissue is bruised and the lactiferous hyphae are damaged. These hyphae function as a storage depot for secondary metabolites. These hyphae are distributed in the whole context and their extremities ascend in the hymenium as pseudocystidia. Pseudocystidia are present in Lactarius and in Lactifluus, not in Russula. Their features are generally not considered as an important character in infrageneric taxonomy (though they can be deviating in some *Lactifluus* groups and in some species of *Lactarius* subg. *Plinthogalus*).

Besides gloeoplerous systems, most members in Russulaceae possess specialized cells, called sphaerocytes. A sphaerocyte is a globose cell that commonly occurs in clusters (so-called rosettes) in the pileus, lamellar and stipe trama. The presence of these globose cells in tissues is related to the brittle consistency of *Lactarius, Russula* and *Lactifluus*. The rosettes of sphaerocytes are abundant in the trama of *Russula* and *Lactifluus*. In *L.* subg. *Russularia*, the lamellar trama is heteromerous, consisting of sphaerocytes and lactiferous hyphae.

True cystidia are relatively large cells typically found on the hymenium. The presence or absence, shape, size and abundance of true cystidia are useful in taxonomy. Macrocystidia are the most common type of true cystidia present in *Lactarius*. Unlike in many *Lactifluus* species, the cystidia found in *Lactarius* are thin-walled. They are subcylindrical to fusiform structures arising from deep in the hymenium and usually situated on the face of gills, called pleuromacrocystidia, or at the edge of gills, called cheilomacrocystidia. Generally the shape of macrocystidia depends on the age of the fruiting body. The cystidia typically contain fine granular or guttate or needle-like contents. The smaller cylindrical macrocystidia with a rounded apex can be found in immature specimens and are often confused with paracystidia. In a more mature stage, they become larger and subcylindrical or fusiform macrocystidia with a moniliform, mucronate or very pointed apex. On the lamellar edge, cheilocystidia, if present, are intermingled with paracystidia. The edge of the lamellae can be heterogenous (with some basidia present between cheilocystidia and paracystidia) or sterile (without basidia and consisting of paracystidia and cheilocystidia).

One of the most useful microscopic characters for the identification of *Lactarius* at different taxonomic levels is the pileipellis structure (Figure 1.3). The pileipellis is the upper layer of the pileus, microscopically completely differently structured from the underlying trama. Observation of the pileipellis structure can be performed by means of manual radial sections through the pileipellis. The structure of the pileipellis depends on the age of the fruitbody. The terminal hyphae are often compact and more ascending in immature specimens, later they can become more enlarged when the pileus expands. To ascertain the presence of a mucus layer in the pileipellis structure, the section is prepared in congo-red solution. When present, the mucus layer is usually seen as a congophobic and amorph mass under the microscope (Figure 1.2d). This character can be more inconspicuous in older specimens. The presence of brown pigments in the pileipellis hyphae is one of the important features for representatives of *L.* subg. *Plinthogalus*. These pigments can easily be observed by viewing sections in a 10% aqueous potassium hydroxide solution. A palisade-like structure is the common type of pileipellis present in *L.* subgenus *Plinthogalus* whereas the presence of a mucus layer is common in *L.* subg. *Lactarius*. Many different stages of pileipellis structure occur in *L.* subg. *Russularia* (see more details in the next section).

In order to define pileipellis types in *Lactarius*, we follow the terminology from Verbeken (1996) and Heilmann-Clausen et al. (1998). There are three major types of pileipellis structure as shown below (Figure 1.3):

1 Pileipellis composed entirely of filamentous hyphae, and where isodiametric cells are completely absent:

1.1 Cutis: consisting of repent or parallel hyphae.

1.2 Ixocutis: like a cutis but containing a thin mucus layer covering the terminal hyphae.

1.3 Trichoderm: consisting of hyphae that rise from the basal layer, like a palisade structure. In some specimens the terminal layer can also be the intermediate between ascending and oblique hyphae.

1.4 Ixotrichoderm: like a trichoderm but terminal hyphae covered by a thin mucus layer.

2 Pileipellis composed of a distinct layer with isodiametric cells:

2.1 Epithelium: consisting of subglobose to globose cells, without terminal filamentous hyphae.

2.2 Ixoepithelium: like an epithelium but with the presence of a thin mucus layer.

2.3 Hyphoepithelium: consisting of an upper layer of repent, parallel and elongated hyphae above a distinct layer of isodiametric cells. This type is called a palisade by Verbeken (1996, 1998).

2.4 Ixohyphoepithelium: like a hyphoepithelium but with the presence of a thin mucus layer.

2.5 Trichoepithelium: consisting of ascending hyphae in the terminal layer above a distinct layer of isodiametric cells.

2.6 Ixotrichoepithelium: like a trichoepithelium, but with the presence of a thin mucus layer.

2.7 Hymenoepithelium: like a trichoepithelium but ascending hyphae are shorter and broader. This type is also called a palisade by Verbeken (1996, 1998).

2.8 Ixohymenoepithelium: like a hymenoepithelium but with the presence of a mucus layer.

3 Pileipellis heterogenous, composed of both filamentous hyphae and enlarged or globose cells, but these are not forming a distinct layer:

3.1 Trichopalisade: intermediate between a trichoderm and a trichoepithelium in which filamentous hyphae are more inflated.

3.2 Ixotrichopalisade: like a trichopalisade but with the presence of a mucus layer.

When the terminal elements have thickened walls I call the subsequent pileipellis structures a lamprotrichoderm, lampropalisade, lamprotrichopalisade (Verbeken 1996, 1998).

Three subgenera are currently recognized in *Lactarius*. *Lactarius* subg. *Lactarius* is the largest group and is characterized by firm basidiocarps, sticky to slimy caps, often with concentric zones or a hairy pileus margin, often with scrobicules and a mucus layer. *Lactarius* subg. *Plinthogalus* can be recognized by dry and velvety caps, often with dark or dull colours, often with extraordinary latex colour and colour change, a lack of true cystidia

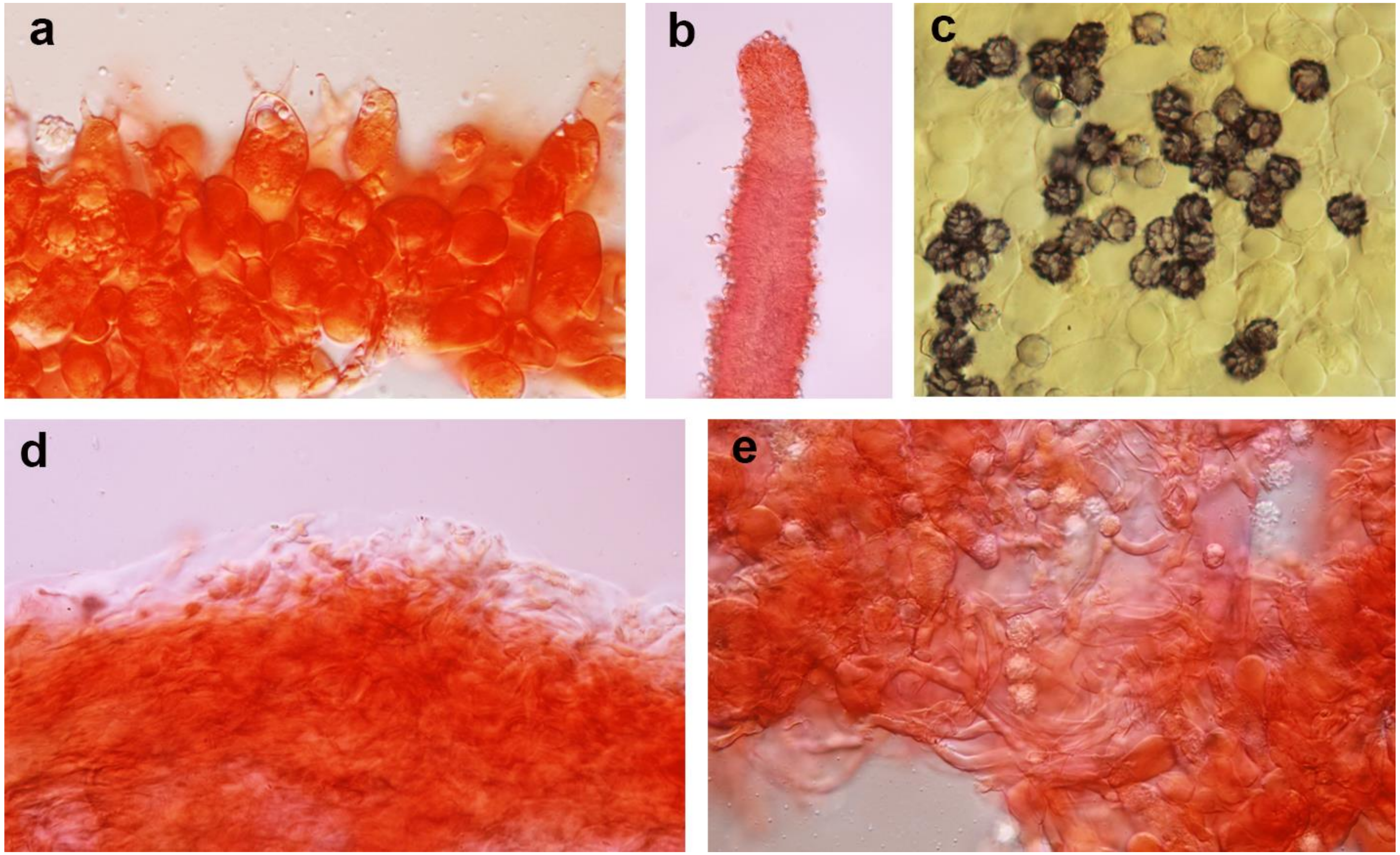
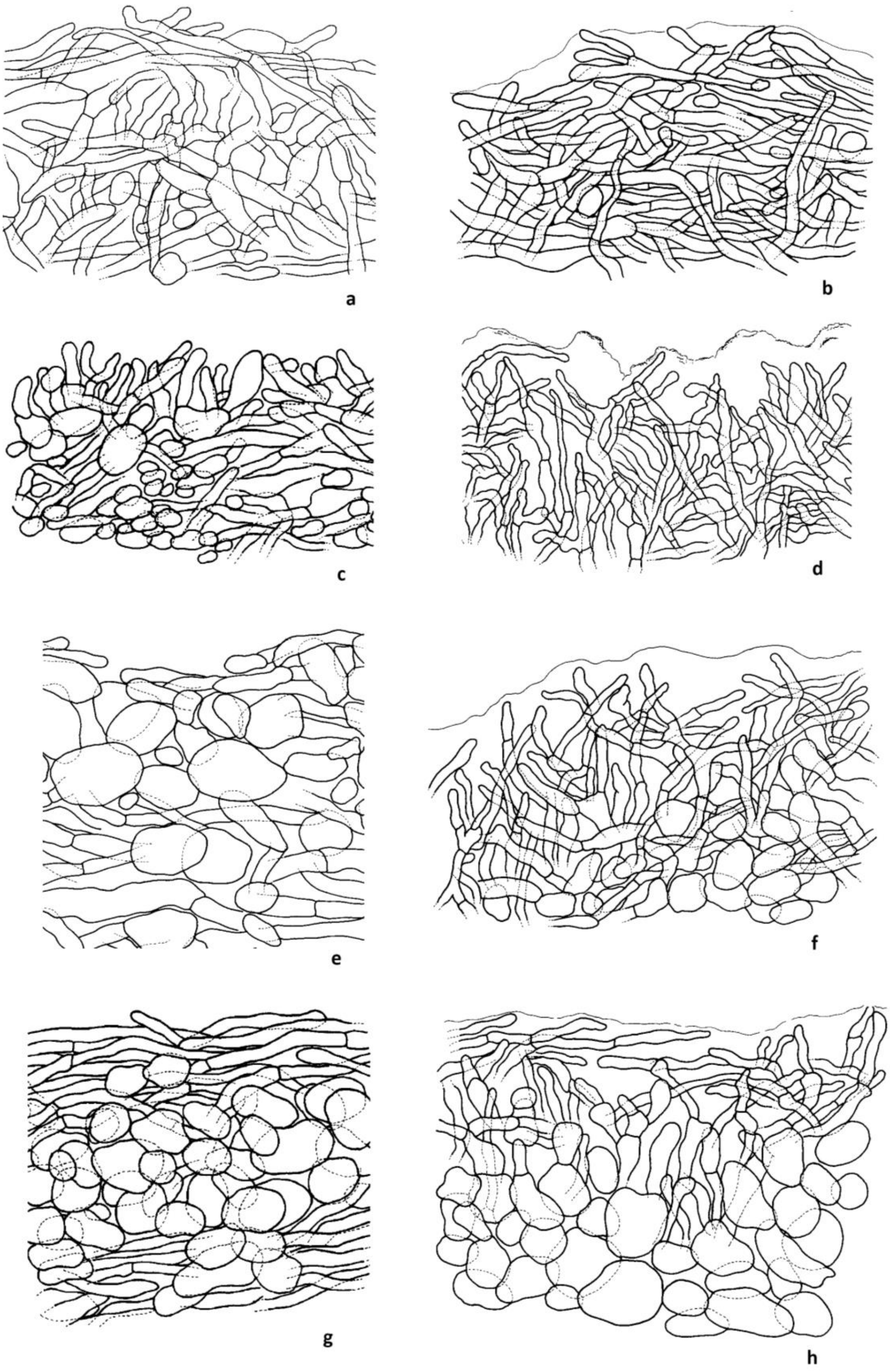


FIGURE 1.2. Microscopic characters of *Lactarius*: a. basidia on a hymenium of *L. politus*, b. cross section of lamella of *L. kesiyae*, c. basidiospore of *L. politus*, d. ixocutis pileipellis with a mucus layer of *L. kesiyae*, e. lactiferous hyphae of *L. politus* (a-b and d-e observed in Congo red solution, c observed in Melzer's reagent).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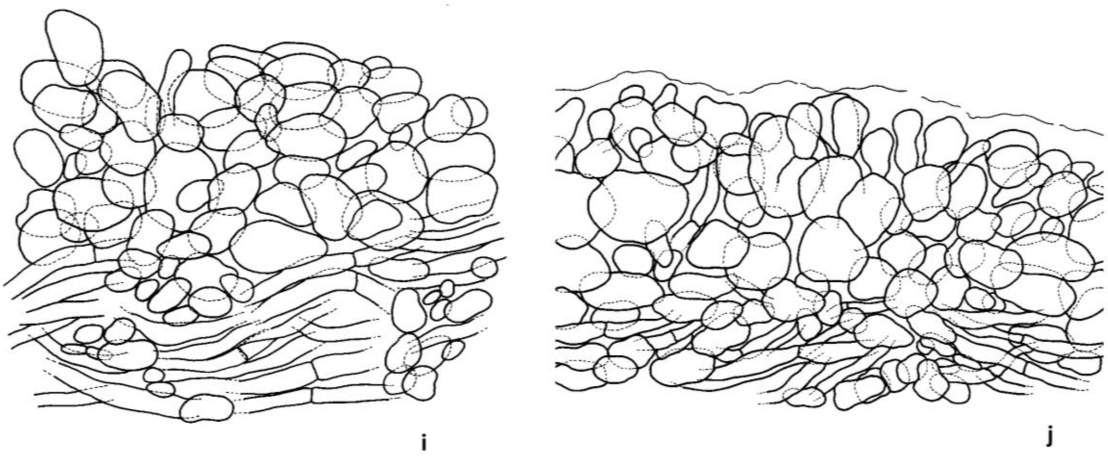

FIGURE 1.3. Pileipellis structures found in *Lactarius*; A: cutis (*L. aquosus*), B: ixocutis (*L. flavigalactus*), C: trichoderm (*L. tangerinus*), D: ixotrichoderm (*L. inconspicuus*), E: trichopalisade (*L. laccarioides*), F: ixotrichopalisade (*L. sikkimensis*), G: hyphoepithelium (*L. chichuensis*), H: ixohyphoepithelium/ixohyphotrichopalisade (*L. lachungensis*), I: epithelium (*L. atrobrunneus*, Liu et al. 2015), J: ixohymenoepithelium (*L. dombangensis*).

in the hymenium, highly reticulate or winged basidiospores and a trichoepithelium or palisade-like pileipellis which often contains a brown intracellular pigmentation. A dry cap surface, fragile habit and orange brown or brown or reddish brown colours are general features in most species in *L.* subg. *Russularia*.

1.4 *Lactarius* subgenus *Russularia*: a short taxonomical and nomenclatural overview

***Lactarius* subg. *Russularia* (Fr. ex Burl.) Kauffman.**

Agaricaceae of Michigan 105. 1918. Type: *L. subdulcis* (Pers.: Fr.) Gray (Figure 1.4).

Agaricus (tribus) *Galorrheus* Fr., Syst. Mycol. 1: 61. 1821.

Lactarius (tribus) *Russulares* (Fr.: Fr.) Epicr. Syst. Mycol.: 341. 1838. Nomen invalid, rang incorrect, type: *L. subdulcis*.

Lactarius (tribus) *Russularia* Fries, Hymen, Europa 431. 1874. Nomen invalid, rang incorrect, type: *L. subdulcis*.

Lactarius stirps *subdulcis* Bon. Doc. Mycol. 10(40): 57. 1980. Nomen invalid, no latin diagnosis, type: *L. subdulcis*.

Lactarius sect. *Russulares* Fr., Syst. Mycol. 1: 68. 1821, type: *Agaricus subdulcis*.

Lactarius sect. *Russularia* Fr. ex Burl. Mem. Torrey bot. Club 14(1): 14. 1908, *L. subdulcis*.

FIGURE 1.4. *Lactarius subdulcis* (Pers.: Fr.) Gray showing latex exudation (voucher collection: KW482, collected in Cerina, Slovakia).

Lactarius subg. *Russularia* is one of the three currently accepted subgenera in *Lactarius* and forms a large group of milkcaps that is especially well-represented in the Northern hemisphere. In the Southern hemisphere, records are known from South America and Australasia, but the subgenus is remarkably absent in tropical Africa. Morphologically, the subgenus is characterized by small to medium-sized basidiocarps, with typically dry, sometimes sticky caps and with colours mostly ranging from orange to orange brown to reddish brown to dark brown. Many species are fragile and small-sized with more or less uniform colours of cap and stipe. *Lactarius perconicus* Verbeken & E. Horak is probably the smallest *Russularia* species known before this study (maximum 8 mm pileus diam.). Large and firm basidiocarps are rather exceptional but occur e.g. in *L. javanicus* Verbeken & E. Horak with a pileus diam. up to 190 mm. Pileus features are rather uniform among species within this group. None of the species have a slimy surface. Pileus zonation and hairy pileus margins are rather rare; they are restricted to species like *L. gracilis* Hongo (hairs at the pileus margin) and *L. subzonarius* Hongo (zonations). The odour of Pentatomidae bugs is prominently present in several species; sweetish or fruity smells are exceptional but do occur. Most species have a bitter or mild taste. Among the three subgenera of *Lactarius*, this subgenus shows the least spectacular latex features and therefore this character is less taxonomically informative than in the other groups. In most cases, the latex is watery white or white. Some species have completely transparent and colourless latex like water. Colour changes are not very common, but weak oxidation of latex occurs in several species, and is typically pale yellow to yellow. *Lactarius duplicatus* A.H. Sm. has white latex which is soon turning sulphur-yellow. Chrome-yellow latex is present in *L. sulphurescens* Verbeken & E. Horak.

The pileipellis structure shows a large variation in this group: from pileipellis structures completely composed of hyphae (i.e. cutis, trichoderm) over a more differentiated layer (i.e. hyphoepithelium, trichopalisade) to a parenchymatous-like pileipellis (i.e. epithelium, hymenoepithelium). A thin mucus layer covering the pileipellis can be found in several species. Basidiospore ornamentation is most often an incomplete to almost complete reticulum, but completely isolated warts do occur in some species as does a complete reticulum.

Due to the lack of spectacular latex colours and the rather uniform pigments in cap and stipe make species of this subgenus among the most difficult to delimit and to recognize especially in the field. Hence, the knowledge of *L.* subg. *Russularia* is rather restricted in several geographical areas. The major problems in assessing the biodiversity of this group are the subtle morphological differences, both macro- and microscopically. Furthermore, European and North American names have been used in other continents without checking or confirming true conspecificity. Even in well studied areas, such as Europe, there is no consensus about the status of some species. Some of the most frequently discussed species or species complexes in the genus *Lactarius* are assigned to this subgenus: e.g. *L. serifluus* (DC: Fr.) Fr./*L. subumbonatus* Lindgr., *L. mitissimus* (Fr.: Fr.) Fr./*L. aurantiacus* (Pers.: Fr) Gray, *L. theiogalus* sensu Neuhoff/*L.tabidus* Fr. A microscopic study of related species in *L.* section *Tabidi* Fr. (see the most recent infrasubgeneric classification systems in the appendix 2) revealed that *L. theiogalus* is a synonym of *L. tabidus* (Idzerda 1997). *Lactarius* *aurantiacus* and *L. mitissimus* were recognized as separate species by Bon (1980) but later *L. mitissimus* was included under the name *L. aurantiacus* (Heilmann-Clausen et al. 1998). The delimitation between *L. serifluus* and *L. subumbonatus* remains unclear so far.

Taxonomic issues have also occurred outside of Europe. Especially the intercontinental conspecificity of species has been debated. European names were often mentioned in North American and Asian literature. The names *L. tabidus*, *L. camphoratus* (Bull.: Fr.) Fr., *L. quietus* (Fr.: Fr.) Fr. were mentioned in the American monograph of Hesler and Smith (1979). In Asia, documentation of species description is often incomplete and identification of several species are imprecise. *Lactarius squamulosus* Z.S. Bi & T.H. Li is in fact *L. gracilis* (Wang 2007). European names were mentioned in the literature of Chinese and Indian mycoflora. Some Chinese collections were labelled *L. camphoratus* but the pileipellis structure is contradictory to the concept of the European *L. camphoratus*. The names *L. serifluus* and *L. subdulcis* were mentioned in the checklist of the family Russulaceae in India (Joshi et al. 2012).

Apart from debates on nomenclature and conspecificity, we also see the incongruency in classification schemes among different authors. Singer (1962) did not accept the group at subgeneric level, but as a section with five subsections: *Colorati*, *Olentini*, *Rufini*, *Obscuratini* and *Subdulcini*. In the monograph on North American milkcaps, Hesler & Smith (1979), recognized *L.* subg. *Russularia* with five sections: *Triviales* Hesler & A.H. Sm., *Pseudo-Aurantiaci* Hesler & A.H. Sm., *Thejogali* Hesler & A.H. Sm., *Russularia* Hesler & A.H. Sm. and *Subsquamulosi* Hesler & A.H. Sm. These sections are mainly based on pileus and latex features. Bon (1980, 1983) distinguished between filamentous cuticles and subcellular pileus cuticles and also emphasized that pileipellis structure plays a crucial role in the infrageneric classification of *Lactarius*. This author classified the subgenus into two sections: *Russularia* (Fr.) Kauffman and *Pseudoaurantiaca* A.H. Sm. and also established an extra subgenus, *Rhysocybella* Bon.

In the European literature, modern infrageneric treatments on the genus *Lactarius* are Heilmann-Clausen et al. (1998) and Basso (1999). Both authors, however, treat the subgenus *Russularia* rather differently. Heilmann-Clausen et al. (1998) largely follow Hesler and Smith's classification concept for *L.* subg. *Russularia* but exclude the two sections, *L.* sect. *Subsquamulosi* and *L.* sect. *Triviales*. They consider the pileipellis structure to be the most important character for infrageneric classification. Three sections *L.* sect. *Russularia*, *L.* sect. *Olentes* Bat. and *L.* sect. *Tabidi* were accepted in the subgenus. The classification of Basso (1999) accepts the following three sections in this subgenus: *L.* sect. *Colorati* (Bat.) Hesler & A.H. Sm., *L.* sect. *Russulares* (Fr.) Fr. and *L.* sect. *Mitissimi* Neuh. Ex. M. Bon, whereas *L.* sect. *Olentes* and many species formerly placed in *L.* subg. *Russularia* in the work of Heilmann-Clausen et al. (1998) are transferred in a separate subgenus, *L.* subg. *Rhysocybella*, as proposed by Bon (1980, 1983).

From differences in these and previous classification systems, I conclude that the delimitation of *L.* subg. *Russularia* is not very clear and some species, such as *L. chrysorrheus* are sometimes placed in *L.* subg. *Lactarius*, sometimes in *L.* subg. *Russularia*. The status of *L.* subg. *Colorati* is also uncertain, with the most recent classifications (i.e. Hesler & Smith 1979, Heilmann-Clausen et al. 1998, Basso 1999) placing some of its species in *L.* subg. *Lactarius*, others in *L.* subg. *Russularia*.

1.5 Species delimitation and previous phylogenetic study

Species are the fundamental units of biology and important for communication in different fields of biological research ranging from taxonomy, systematics, phylogeny, ecology, and biochemistry to applied sciences. The issue of species delimitation has long been confused with species concept and boundary (de Queiroz 1998, 2007). This is also true for the species concept of fungi. A number of species concepts have been proposed and used (Petersen & Hughes 1999, Taylor et al. 2000, Cai et al. 2011). Incompatibility among different criteria has existed ever since. This has led to taxonomic debates on species boundaries and resulted vagueness in number of described species. Morphological species concept (MSC) delimitates species based on gaps in morphology (Darwin 1859). Biological species concept (BSC) recognizes species boundary based on the ability of interbreeding (Mayr 1970, 1972). A population or group of the same species has the potential to interbreed and reproduce viable and fertile offspring in nature. Ecological species concept (ESC) delimitates species based on occupancy of an ecological niche (van Valen 1976). The emergence of molecular techniques, genetic information of organisms has been generated. This has resulted in a new species concept called Phylogenetic Species Concept (PSC) (Hennig 1966). This species concept delimitates species when species evolve in a supported monophyletic clade. The Genealogical Concordance Phylogenetic Species Recognition (GCPSR) uses the phylogenetic concordance to indicate "species/species clade (O'Donnell et al. 1998, Taylor et al. 2000, Dettman et al. 2003a, b, de Queiroz 2007).

In accordance with most traditional systematic classifications, the fungal species concept is mainly based on the MSC and the BSC. However, many fungi have simple morphology, hence the MSC often lead to misidentification. Whereas the BSC cannot apply for several groups of fungi because some fungi reproduce only asexually, some fungi are unable to coax into breeding in cultivation.

Ideally, not only morphological and molecular information but also data of different genes are combined in order to draw conclusions in species delimitation and phylogeny. Generally ribosomal markers have fewer problems with PCR aplification and sequencing than protein-coding markers. The nuclear ribosomal DNA of internal transcribed spacer (ITS) is most widely used in fungi and is selected as the universal barcode because it has a clearly defined barcode gap (Schoch et al. 2012). The ITS gene consists of two non-coding regions, called spacer, situated between the small subunit and the large subunit of the rDNA gene. It can be a powerful marker when dealing with phylogenetically more distant species (Kiss 2012), but often does not allow to discriminate phylogenetically closely related species and does not resolve nodes at a higher phylogenetic level because of the frequency of indels. Apart from ITS regions, the 18S nuclear ribosomal small subunit rRNA gene (SSU), large subunit rRNA gene (LSU) and protein-coding genes, e.g. the largest subunit of RNA polymerase II (*rpb1*), the second largest subunit of RNA polymerase II (*rpb2*) etc., are commonly used in fungal taxonomy. However, amplification failure and sequencing success are obstacles often encountered when amplifying protein-coding genes. Concordance between multiple gene genealogies has been applied for species delimitation in many groups of ectomycorrhizal macrofungi, for example, *Paxillus* Fr. (Hedh et al. 2008), *Lactifluus* (Van de Putte et al. 2010, 2012, Stubbe et al. 2011), *Hebeloma* (Fr.) P. Kumm. (Eberhardt et al. 2013). Moreover, a phylogram calculated from concatenated data can ameliorate statistically phylogenetic supports at stem nodes in a phylogram.

Before this study, molecular research in *L.* subg. *Russularia* was restricted to analysis of ITS sequences for species delimitation and identification (Montoya et al. 2010, 2015, Lamus et al. 2012, Triantafyllou et al. 2015), but was not used to resolve questions on evolutionary relationships between different species. Montoya et al. (2010) used Maximum Parsimony (MP) to provide evidence of the ectomycorrhizal symbiosis of *L. badiopallescens* Hesler & A.H. Sm. with *Fagus*. Moreau et al. (2011) used ITS for investigating the evolutionary history of host association of *Lactarius* spp., including some members of *L.* subg. *Russularia*. The comparison of rDNA sequences based on ITS regions of basidiome and ectomycorrhizal root tips of *L. areolatus* Hesler & A.H. Sm. and *L. strigosipes* Montoya & Bandala were shown by Lamus et al. (2012). Two Mexican species, *L. herrerae* Montoya, Bandala & Garay and *L. cuspidoaurantiacus* Montoya, Bandala & Garay, were confirmed to be strictly associated with *Alnus* based on comparison of ITS sequences from basidiomes and ectomycorrhizal roots (Montoya et al. 2015).

ITS data have also been used to investigate the position of some angiocarpic representatives, formerly included in the genera *Arcangeliella*, *Zelleromyces* etc. The first officially recombined species *Lactarius borzianus* (Cavara) Verbeken & Nuytinck has been included in *L.* subg. *Russularia* (Nuytinck et al. 2003). The only, more general, conclusions on phylogeny based on a multiple-locus analysis can be retrieved from Buyck et al. 2008 (see figure 1.1). Eberhardt (2000) used ITS and LSU data to investigate the infrageneric relationship of a by current standards small number of members of the genus *Lactarius*, including some members of *L.* subg. *Lactarius*. Eberhardt & Verbeken (2004) used ITS and LSU to describe the first Afrian sequestrate *Lactarius*. Verbeken et al. (2014) used ITS, *LSU* and *rpb2* sequences to show that an angiocarpous species, *L. falcatus* Verbeken & Van de Putte, described from Thailand, also belonged to subg. *Russularia.* Therefore, the total number of angiocarpic species known in this subgenus is now three: *L. borzianus*, *L. hispanicus* Calonge & Pegler and *L. falcatus*.

This study is the first one to tackle questions on evolutionary relationships and phylogenetic correlation of morphological characters of species in *L.* subg. *Russularia* using a phylogram based on two-locus (ITS-*rpb2*) dataset. All described species in this study are distinguished based on a polyphasic approach, combining morphological, ecological and phylogenetic species concepts, thence adapting the the Consolidated Species Concept (CSC) as recently defined by Quaedvlieg et al. (2014).

1.6 Ecology and biogeography

Lactarius subg. *Russularia* is one of the dominant ectomycorrhizal genera present in many ecosystems almost all over the world (but remarkably absent in tropical Africa). The number of basidiocarps covering the forest floor is usually very high, and much higher than in other groups of milkcaps or *Russula* species. In Europe, it is not exceptional to find 50 to 100 basidiocarps of *L. lacunarum* or *L. hepaticus*. Representatives of *L.* subg. *Russularia* form ectomycorrhizal associations with several families of host trees, both deciduous and coniferous taxa. Fagaceae (e.g. *Castanopsis*, *Lithocarpus*, *Quercus* and *Fagus*) and Dipterocarpaceae (*Dipterocarpus* and *Shorea*) are the dominant groups of host trees widespread in tropical rainforests. Fagaceae is also distributed in boreal and temperate regions. Pinaceae (i.g. *Pinus*, *Picea*, *Abies*) is an important ECM group that is widely distributed in temperate regions as well as in highlands in subtropical areas. Several *L.* subg. *Russularia* species are associated with Betulaceae (*Alnus* and *Betula*) and Salicaceae (*Populus* and *Salix*) trees.

Many *L.* subg. *Russularia* species have a strict association with a certain genus (or even subgenus or species) of host tree, for example, *L. quietus* is only associated with *Quercus*, *L. duplicatus* only with *Betula*, and *L. lanceolatus* O.K. Mill. & Laursen only with dwarf *Salix*. *Lactarius obscuratus* (Lasch: Fr.) Fr. and *L. cyathuliformis* Bon associate with *Alnus glutinosa*, *A. incana* and *A. cordata* (subg. *Alnus*), while *L. brunneohepaticus* M.M. Moser associates with *A. betula* (subg. *Alnobetula*) (Rochet et al. 2011). Some species of *L.* subg. *Russularia* have been reported having a wide range of ectomycorrhizal partners. *Lactarius fulvissimus* Romagn. forms ectomycorrhiza with *Fagus*, *Quercus*, *Tilia*, *Populus*, *Corylus*, as well as conifers. *Lactarius tabidus* is associated with *Betula*, *Quercus* and *Picea*. *Lactarius areolatus* has been reported with *Quercus, Carpinus, Betula*, *Fagus* and *Picea.* In North America as well as in Europe, many species are strictly found in coniferous forest (e.g. *Lactarius subflammeus* Hesler & A.H. Sm. in USA, *L. hepaticus* Plowr. in Europe) (Hesler & Smith 1979, Heilmann-Clausen et al. 1998).

Soil conditions (e.g. humidity and nutrient) are important for *Lactarius* growth and fructification. *Lactarius tabidus* Fr. occurs in humid condition. *Lactarius sphagneti* (Fr.) Neuhoff is present in very acid conditions. Some species, such as *L. rubrocinctus* Fr., *L. subsericatus* Kühner & Romagn. ex Bon and *L. duplicatus* A.H. Sm. typically grow on more alkaline soil (Heilmann-Clausen et al. 1998). A rare European species, *L. rostratus* Heilman-Clausen has been found in acidophilus beech forest (Běták 2013).

The subgenus is well-represented in the Northern hemisphere. The total number of described species is unclear due to the use of synonyms and varieties in the most recent monographs, Hesler & Smith (1979), Heilmann-Clausen et al. 1998 and Basso (1999). For counting the total number of species, I included species in *L.* subg. *Rhysocybella* in Basso (1999) and excluded species in the sections *Subsquamolosi* and *Triviales* of Hesler & Smith (1979) and varieties of species. Synonyms of species that have been used in Hesler & Smith (1979) and Basso 1999 are not counted as accepted species. Before this PhD study, 74 species have been accepted as members of *L.* subg. *Russularia* (Table 1.1). The total number of accepted species before and after this PhD study is shown in the appendix 6.

Europe is the best-explored area. For counting the number of currently accepted species in Europe, the number of described species excludes the symnonyms in Europe. I follow the species delimitation of Heilmann-Clausen (1998), Basso (1999) and Verbeken & Vesterholt (2012) and therefore recognize 19 species. A number of varieties and synonyms were indicated in Basso (1999). Outside of Europe, the *Lactarius* mycoflora in North America is diverse and well-documented. Thirty-three species are accepted in the subgenus (Hesler & Smith 1979, Montoya & Bandala 2008, Montoya et al. 2014). However, the lack of molecular data supporting taxon limits for most of these taxa present serious obstacles for giving a good estimate of the true number of described species. The subgenus is poorly represented in South America; only *L. alni* Singer has been reported (Singer 1962). According to the monograph regarding the milkcaps in tropical African areas, it has been noted that indigenous species appeared to be absent (Verbeken 1996, Verbeken & Walleyn 2010). In South Africa only one species has been reported: *L. hepaticus*. This European species has been introduced along with *Pinus* (not included in the table). The reported species from Northern Africa are similar to Mediterranean European representatives (Verbeken 2001). *Lactarius* subg. *Russularia* in Oceania is assumed to be endemic; nine species are reported from Australia and Papua New Guinea (Miller & Hilton 1987, Verbeken & Horak 2000). In Asia, the diversity is assumed to be rich but few regions are well-studied. Only twelve species have been described. Two species were described from Japan, *L. gracilis* and *L. subzonarius* Hongo (Hongo 1945a, b). *Lactarius gracilis* is an easily recognizable species which is also reported from Southern China and Northern Thailand. The distribution area of *L. subzonarius* ranges from Japan to China. The additional studies on diversity of *L.* subg. *Russularia* in Asia were carried out in Indonesia (Verbeken et al. 2001), India (Das et al. 2004, 2015) and Thailand (Verbeken et al. 2014); eight species were described from these countries. Apart from the angiocarpic species *L. falcatus*, the delimitation of all Asian taxa is based on morphological characters only and have never been documented by molecular data. But the same is actually true for most European and almost all American taxa.

TABLE 1.1. The number of known species according to geographic region before this PhD. study.

Region	Number	References
Europe	19	Vidal 2004, Calonge & Pegler 1998, Heilmann-Clausen et al. 1998, Basso 1999, Verbeken & Vesterholt 2012.
North and Central America	33 [a]	Hesler & Smith 1979, Montoya et al. 2008, 2014, Kuo et al. 2013
South America	1	Singer 1962
Asia	12	Chiu 1945, Hongo 1957a, 1957b, Ying 1991, Verbeken et al. 2001, 2014, Das et al. 2004, Das et al. 2015
Oceania	9	Miller & Hilton 1987, Verbeken & Horak 2000

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

1.7 Uses

1.7.1 Edibility

None of *Lactarius* species is strongly poisonous and they are hardly confused with deadly poisonous mushrooms because of the latex. Most species are assumed to be edible and some are really delicious. In culinary point of view, members of *L.* sect. *deliciosi* have the best reputation, e.g. *L. deliciosus* (L.: Fr.) Gray and *L. sanguifluus* (Paulet) Fr. in Europe; *L. hatsudake* Tanaka and *L. akahatsu* Tanaka in Asia and *L. indigo* (Schwein.) Fr. in North America.

Members of *L.* subg. *Russularia* are not often consumed, possibly due to their inferior taste, their fragile texture and small size. The odour is rather unpleasant in some species (bug-like). Although in Finland and Eastern Europe, people consume the very acrid *L.*

rufus. In Southeast Asia, *L.* subg. *Russularia* is totally unappreciated by local people and is thought to be pungent and bitter. *Lactarius helvus* (Fr.: Fr.) Fr., a representative of *L.* subg. *Russularia*, has been reported to cause nausea, vomiting, vertigo and abdominal pain (Bresinsky & Besl 1990, Benjamin 1995).

1.7.2 Secondary metabolites

Apart from culinary purposes, macrofungi can be very useful for secondary metabolites. In milkcaps, the latex has received attention. Latex is a complex mixture of a number of secondary metabolites. In some species sesquiterpenoids, being responsible for the pungency and bitterness of the milk, as well as for the colour changes once exposed to air, have been isolated from the latex. Possibly, they have antimicrobial (Luo et al. 2005) and antifeedant properties (Dowd & Miller 1990, Kopczacki et al. 2001). Most *Lactarius* sesquiterpenes are lactaranes, secolactaranes, marasmanes, isolactaranes, norlactaranes, or caryophyllanes (Luo et al. 2006). The sesquiterpene derivatives are supposed to derive from fatty acid precursors which differ in different groups of *Lactarius*. Guanine sesquiterpenes are found in species with bright latex colour, e.g. *L.* sect. *Deliciosi* (Fr.: Fr.) Redeuilh, Verbeken & Walleyn. Chromene derivatives are presented in *L.* subg. *Plinthogalus*, while lactarane sesquiterpenoids were often reported from *L. subg. Russularia*; i.e. *L. aurantiacus* and *L. subdulcis* (Gilardoni et al. 2014). Luo et al. (2006) have isolated five humulane sesquiterpenoids from *L. aurantiacus*.

1.8 Objectives

Although the group is the dominant *Lactarius* component in many ecosystems, *L.* subg. *Russularia* is the least studied subgenus in *Lactarius*. The value of certain characters (macroscopic as well as microscopic) and their degree of variation are still poorly understood, and hence also the taxonomy and relationships between species.

This study aims to evaluate species boundaries and evolutionary relationships in the subgenus, in order to provide a tool for biodiversity assessment. For Southeast Asia, a potential hotspot and poorly explored area, this study will provide detailed morphological descriptions and illustrations of the species, as well as molecular characterization. The more specific questions to addressed are:

1.8.1 Biodiversity assessment in Southeast Asia

For *L.* subg. *Russularia*, significant taxonomic treatments are restricted to Europe (Heilmann-Clausen et al. 1998, Basso 1999, Verbeken & Vesterholt 2012) and North America (Hesler & Smith 1979), but in Asia, the knowledge on biodiversity remains substandard. Because of suitable climate conditions and presence of ectomycorrhizal host trees, Southeast Asia is predicted to be a hotspot for this group. Therefore we aim to add a significant portion of new species and to contribute to a more comprehensive monograph of *L.* subg. *Russularia* in Southeast Asia (see chapter 2-5).

1.8.2 Delimitation of the subgenus

From previous classification systems and preliminary molecular analyses (ITS) it is not clear how to delimitate and characterize the subgenus and whether it forms a well-supported monophyletic group. A two locus based phylogeny with a broader sampling from different continents will provide an overview of the subgenus.

1.8.3 Delimitating species and evaluating morphological characters

Of the three subgenera in *Lactarius*, *L.* subg. *Russularia* shows the most uniformous latex features and basidiome colours. What are assumed to be differentiating characters between species are often very subtle differences in character states. By combining morphological and molecular multiple gene analysis, I will evaluate the phylogenetic signal of several traditionally used morphological characters.

Cryptic species occur when morphology does not differentiate between species. Two or more cryptic species can be hidden under one species name and can only be differentiated by means of molecular data. According to Van de Putte et al. 2010, 2012 on cryptic diversity of milkcaps, this event has been reported many times in *Lactifluus*. For *Lactarius*, we hypothesize that *Lactarius* has higher morphological variation than molecular variation and hence we expect less cryptic speciation.

In addition, I will also investigate whether morphological characters are phylogenetically informative. Several morphological characters have been considered important characters for infrageneric classification without molecular support. Latex features are assumed not to be taxonomically informative at this level. Differences in structure of pileipellisis is considered as one of the most single frequently used characters for infrasubgeneric classification in recent literature, e.g. Heilmann-Clausen et al. (1998) and Basso (1999). Hence I assume that pileipellis structure might be related with evolutionary process of individual species. Apart from pileipellis structure, true cystidia might also be an important indicator for evolutionary process due to the absence of this character occurred in several species. Basidiospore ornamentation is either an incomplete or complete reticulum while a minority has isolated warts or a zebroid ornamentation.

To tackle these questions, I show a phylogram combined with potentially significant morphological characters (e.g. pileipellis structure, presence or absence of true cystidia, spore ornamentation, latex feature, etc.) and evaluate the phylogenetic signal of the different characters (see chapter 6).

1.8.4 Intercontinental conspecificity

European *Russularia* names were often applied in North American and Asian literature, which can mean two things: (1) representatives are indeed conspecific between continents and have a large distribution area; (2) representatives in fact represent different species, sharing comparable morphology but each occurring in one of the continents. To find out what the actual distribution is, I compare morphological characteristics and molecular phylogeny using ITS and *rpb2* data. This study tests the intercontinental conspecificity between extant species from Europe and Asia (see chapter 6).

1.8.5 Infrasubgeneric classifications

Infrasubgeneric classification systems of *L.* subg. *Russularia* have been proposed by several mycologists (Hesler and Smith 1979, Heilmann-Clausen et al. 1998 and Basso 1999). These three recent classifications of the subgenus (see the appendix 2) are based on morphological characters but have never been tested with molecular tools so far. In addition, these classification systems are all based on sampling in only one continent. Therefore, it is plausible that incompatibility exists, both in the used key characters and the resulting classification schemes. In this PhD study, a phylogram based on two genes and a wider sampling area should reveal whether subclades can be distinguished and are concordant with morphological and geographical data or not. We will use our results to evaluate the existing classification systems.

1.8.6 Divergence time estimation

In this study, Bayesian Evolutionary Analysis Sampling Trees (BEAST) allows the exploration of evolutionary relationships and the diversification of *L.* subg. *Russularia* in time using concatenated sequence data of representatives from Europe and Asia. This will allow to show a reasonable timeframe of the emergence of ancestral species of *L.* subg. *Russularia* and preliminary assumption on their distribution between these continents.

1.9 Outline of this thesis

Species is a universal system used for naming orgamisms. Accurate species delimitation and identification are crucial as a fundamental step in biological research. Up to present fungi are considerec as one of the most understudied groups due to the large portion of its diversity remaining undiscovered and undescribed. The knowledge of many groups of fungi is poor and fragmentary and the genus *Lactarius* is no exception. Like many other groups of fungi, the species boundaries and current classification systems of *Lactarius* are still based on traditional morphological characters. In the last decade, after the invention of molecular methods, DNA sequence data have been widely used. This resulted in a more reliable species delimitation and concept. The establishment of the genera *Multifurca* and *Lactifluus* resulted in a smaller genus *Lactarius* with only three subgenera currently recognized (Buyck et al. 2008, 2010). Among the three subgenera of *Lactarius*, *L.* subg. *Russularia* is a large group but many species in this subgenus are difficult to identify because of its uniform morphology. This PhD study aims to fill the gap in knowledge of the diversity and phylogeny of this subgenus mainly by exploring species from Southeast Asia and adjacent areas. Most chapters in this PhD thesis deal with the diversity and species delimitation using the concordance of morphological and molecular supports.

Almost every chapter (2, 3, 4 and 5) in this PhD thesis corresponds with published articles, except for chapter 6. Hence the reader will notice overlapping contents in introduction and material and method sections in most chapters. In the chapter 6, information on GenBank and numbers are lacking for the moment. This information will be available as soon as this paper is published.

Chapter 2 and **Chapter 3** describe two morphologically distinct groups in *L.* subg. *Russularia*. The species delimitation in these groups is based on a combination of morphological and molecular data, the latter using ITS phylogeny. Four species with strikingly distant gills are described from Thailand and Malaysia in **Chapter 2**. A discussion on the morphological differences of these look-alike species is given.

In **Chapter 3**, four very small-sized species are reported from Thailand, which are three new and one known species. A thoroughly morphological study, illustrations and discussion on morphological differences of described species are also given in this chapter.

In **Chapter 4**, eight new species are described from montane rainforest in Thailand and Vietnam. *Lactarius chichuensis* W.F. Chiu is reported for the first time from Thailand. A summary of identification keys for all described Southeast Asian species is given.

In **Chapter 5**, *Lactarius politus* Wisitrassameewong & K.D. Hyde and *L. atrobrunneus* Wisitrassameewong & K.D. Hyde are described as part of a larger paper which wants to illustrate the fact that Northern Thailand is a hotspot for fungal diversity. Morphological and molecular data confirmed that both new *Lactarius* species are members of the subgenus *Russularia*. This chapter is an adapted and shortened version of the complete paper from Liu et al. (2015).

In **Chapter 6** the author hypothesizes the estimated age and origin of the subgenus using BEAST analysis. A discussion about recent boundary of the subgenus and informative characters relies on the genealogical approach based on ITS and *rpb2* DNA sequences data. Additionally, the overview of described species is given and confirmed by a global ITS phylogeny and six new species from Sikkim Himalaya, India and regions around Shangri-la in Yunnan, China are described in this study.

Chapter 7 offers a general discussion, future perspectives and conclusion of this thesis.

Chapter 8 provides a summary in English and Dutch.

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CHAPTER 2

SPECIES WITH VERY DISTANT GILLS

Abstract

This article introduces four new species of *Lactarius* subgenus *Russularia* from Southeast Asia with descriptions and illustrations of macromorphological and microscopic characters. *Lactarius laccarioides* and *L. sublaccarioides* were discovered in Northern Thailand, and *L. pasohensis* and *L. stubbei* in Malaysia. These four species share some striking macroscopic features that are unique in the subgenus: distant gills and a very thin-fleshed and deeply striate to sulcate pileus, which gives them the aspect of *Laccaria* species in the field. Molecular analysis of the ITS gene region shows that these four species are not as closely related within *Lactarius* subgenus *Russularia* as their similar appearance in the field would suggest.

Modified from: Wisitrassameewong, K., Nuytinck, J., Hyde, K.D. & Verbeken, A. (2014a) − *Lactarius* subgenus *Russularia* (Russulaceae) in South-East Asia: Species with very distant gills. *Phytotaxa* 158(1): 23−42.

Introduction

Since the splitting of the milkcaps among *Lactarius* Pers., *Lactifluus* (Pers.) Roussel and *Multifurca* Buyck & V. Hofst. (Buyck et al. 2008, 2010), *Lactarius* sensu novo now comprises three large subgenera: *L.* subg. *Plinthogalus* (Burl.) Hesler & A.H. Sm., *L.* subg. *Lactarius* (Fr. ex J. Kickx f.) Kauffman and *L.* subg. *Russularia* (Fr. ex Burl.) Kauffman. *Lactarius* species are one of the most important ectomycorrhizal taxa in many ecosystems worldwide. *Lactarius*, in contrast to *Lactifluus*, seems to have its main distribution in the Northern hemisphere, particularly in temperate regions, but is also well represented in the Southern hemisphere. In Southeast Asia and Australasia, the biodiversity of the genus is best explored in Malaysia, Indonesia, Thailand and Papua New Guinea. In contrast to the other subgenera, *Lactarius* subg. *Plinthogalus* seems evenly distributed in both temperate and tropical regions (Stubbe et al. 2007). Extensive surveys in tropical Asian regions resulted in 24 new taxa described from Indonesia (Verbeken et al. 2001), Papua New Guinea (Verbeken & Horak 2000, Verbeken et al. 2002), Malaysia (Stubbe et al. 2007, 2008) and Northern Thailand (Le et al. 2007b). *Lactarius* subg. *Lactarius* has numerous representatives in temperate areas, both in Europe and in North America, but is very poorly represented in tropical Africa (Verbeken & Walleyn 2010), and about 20 species have been described from tropical Asia (Verbeken & Horak 1999, 2000, Verbeken et al. 2001, Nuytinck et al. 2006, Le et al. 2007a). The third subgenus, *L.* subg. *Russularia*, also seems to be dominant in temperate zones. It is poorly represented in South America, and apparently completely absent in tropical Africa (except for an introduced species with pine plantations (*L. hepaticus*) in South Africa (Verbeken 1996, Verbeken & Walleyn 2010). A thorough overview of the diversity in this subgenus is lacking, mainly due to undersampling in many areas, but also due to the lack of local identification tools which has resulted in the uncritical use of European and North American names (Wang & Liu 2002, Wang 2007). Research in Southeast Asia has shown that representatives of this subgenus are ectomycorrhizal with trees in Fagaceae (*Quercus* spp., *Castanopsis* spp. and *Lithocarpus* spp.), Dipterocapaceae (*Dipterocarpus* spp. and *Shorea* spp.) and Pinaceae (*Pinus* spp.) (Yuwa-Amornpitak et al. 2006, Stubbe et al. 2007, Le et al. 2007a, Phosri et al. 2012).

Species of *Lactarius* subg. *Russularia* are morphologically well-characterized by a small to medium-sized cap which is dry, rarely sticky and has a dominant reddish-brown or orange colour. The latex is white or transparent, and shows very little colour and pigment variation as compared to the other two subgenera in the genus *Lactarius*. In most species it is unchanging, in some it turns sulphur-yellow or yellow (sometimes very slowly and sometimes only on white tissue). Some of the species have remarkable smells (pentatomid bugs, maggi-like, sweetish). Microscopically, the spores are globose, subglobose or ellipsoid, ornamented with an incomplete to complete reticulum, and have an inamyloid or distally amyloid plage. The pileipellis structure is an epithelium, hyphoepithelium, trichopalisade or cutis, and in some species is gelatinised (Heilmann-Clausen et al. 1998, Das & Sharma 2005).

Previous studies have clearly shown that *L.* subg. *Plinthogalus* is a monophyletic group (Eberhardt & Verbeken 2004, Shimono et al. 2004, Buyck et al. 2008). The delimitation of *L.* subg. *Russularia* and *L.* subg. *Lactarius* are more problematic. Molecular phylogenetic analyses based on the ITS region show that *L.* subg. *Russularia* clusters with *L.* subg. *Lactarius* and forms a large and complex group. The ITS gene clearly does not resolve relationships at subgenus level (Eberhardt & Verbeken 2004, Buyck et al. 2008).

Lactarius subg. *Russularia* is considered a complex and difficult group due to the similarity among species concerning habit, pileus colour and latex colour. Differences are often subtle. Mycologists, especially in Europe, have debated on nomenclatural and taxonomic confusion of some closely related species or complex groups. However, some look-alike species have also been recorded and discussed in Asia, e.g. *L. chichuensis* W.F. Chiu and *L. hirtipes* J.Z. Ying (Wang & Liu 2002). In addition, *Lactarius* species described from China have been re-examined and discussed by Wang (2007). From Indonesia and Papua New Guinea, 11 species have so far been described (Verbeken & Horak 2000, Verbeken et al. 2001). None of the known species in Asia is conspecific with any taxon from other continents.

This paper is the first of a series, resulting from a study to assess the biodiversity of *L.* subg. *Russularia* in Southeast Asia based on both morphological characteristics, ecological and molecular data. Here we propose four new species which are extraordinary in the subgenus and easily recognisable in the field because they have strikingly distant gills. In addition, we performed a molecular phylogenetic analysis. The new species are compared with closely related species and morphological observations are discussed in view of the phylogenetic results.

Material and Methods

Sampling

Collections were made during extensive fieldwork in May to October 2011 and 2012 in Northern Thailand (mainly Chiang Mai province) and in August–September 2006 in Malaysia (Pasoh National Forest Reserve, Negeri Sembilan province). The collection data are listed in Table 2.1. All examined collections are deposited in the herbarium of Ghent University (GENT); duplicates of the Thai materials are in the Mae Fah Luang University Herbarium (MFLU) and the Herbarium of the Department of Biology, Chiang Mai University (CMU); duplicates of the Malaysian materials are in the herbarium of the Forest Research Institute Malaysia (KEP).

Morphological study

Specimens were described and photographed in fresh condition. The collections were documented as described by Le et al. (2007a). The colour of fresh basidiocarps and latex was described in daylight conditions using the colour code of Kornerup & Wansher (1978). Latex colour and changes were noted when latex was exuded from the fruiting body, from a drop of latex on white tissue paper and a white handkerchief as well as from a drop placed on a glass slide with KOH (10%). For terminology of macroscopic features, I refer to Vellinga (1988). Lamella density was measured by counting the number of lamellae (L) and lamellulae (l) per cm at pileus mid-radius ((L+l)/cm) (Stubbe et al. 2011).

Microscopic features were studied from dried materials. All elements of the hymenium, pileipellis and stipitipellis were observed in 1% Congo Red in L4 (Clémençon 1973). Line drawings of hymenium elements and pileipellis structure were made using an Olympus CX21 microscope with the aid of a drawing tube. Basidia length excludes sterigmata. Basidiospores were observed in Melzer's reagent. Spore measurements are based on at least 20 spores for each collection using a Zeiss Axioskop 2 microscope and drawings were made using a drawing tube at 6000× magnification. Spores were measured in side view, excluding the height of ornamentation. For details on terminology, I refer to Verbeken (1996, 1998). Measurements are given as (MINa) [Ava-2×SD]–Ava–Avb–[AVb+2×SD] (MAXb), where Ava = lowest mean value for the measured collections, MINa = the minimum value corresponding with this mean value, AVb = highest mean value, MAXb = the maximum value corresponding with this mean value, and SD = standard deviation calculated for the measurements of one collection (minimum and maximum values are only given if not in the 2×SD-interval). Q corresponds to spore "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 specimens, respectively. All line drawings and measurements were done by the first author.

DNA extraction, amplification and sequencing of the ITS region

Genomic DNA was extracted from fresh material using a CTAB-based method (Nuytinck & Verbeken 2003, Van de Putte et al. 2010). In the present study I amplified the ITS region using the ITS1-F and ITS 4 primers (White et al. 1990). When amplification failed, intermediate primers ITS2 and ITS3 (White et al. 1990) were used. The PCR program was initiated by a 5 min denaturation step at 94 ºC, followed by 35 cycles of 30 sec at 94 ºC, 30 sec at 55 ºC and 45 sec at 70 ºC. Polymerisation was completed by an incubation of 7 min at 70 ºC.

Sequence alignment and phylogenetic approach

The program Sequencher™ v5.0 (GeneCodes Corporation Ann Arbor, Michigan, U.S.A.) was used to assemble forward and reverse sequences into contigs, inspect ABI chromatograms and edit nucleotides where needed. Afterwards, initial alignment was carried out using the on-line version of MAFFTv7 (Katoh & Standley 2013) with settings E-INS-I. The alignment was then manually edited with MEGA5 (Tamura et al. 2011). All sequences are deposited in GenBank (as shown in Table 2.1). Gblocks v0.91b (Castresana 2000) was used to eliminate poorly aligned positions in the alignment. The program settings allowed results with smaller blocks, gaps within these blocks and less strict flanking positions.

The Alignment Transformation Environment (ALTER) was used to transform multiple alignment formats (Glez-Peña et al. 2010). Maximum Likelihood analysis (ML) was executed with the program RAxMLGUI (Silvestro & Michalak 2011). The Rapid Bootstrapping algorithm was implemented for 500 replicates, followed by an ML tree search (Stamatakis et al. 2008). Branch supports were visualised using TreeGradients v.1.0.4 (http://www.phycoweb.net/software/TreeGradients/index.html).

TABLE 2.1. List of specimens and GenBank accession number of sequences used in the molecular phylogenetic analysis. Newly described species in this study are in boldface.

Species	Voucher collection	Origin	ITS accession no.
Lactarius			
***L. laccarioides* sp. nov.**	LTH10 (GENT, CMU)	Thailand	KF432989
***L. laccarioides* sp. nov.**	LTH372 (GENT, CMU)	Thailand	KF432990
***L. laccarioides* sp. nov.**	KW336 (GENT, MFLU)	Thailand	KF432991
***L. laccarioides* sp. nov.**	KW360 (GENT, MFLU)	Thailand	KF432992
***L. sublaccarioides* sp. nov.**	KW011(GENT, MFLU)	Thailand	KF432999
***L. sublaccarioides* sp. nov.**	KW300 (GENT, MFLU)	Thailand	KF432996
***L. sublaccarioides* sp. nov.**	KW323 (GENT, MFLU)	Thailand	KF432997
***L. sublaccarioides* sp. nov.**	KW332 (GENT, MFLU)	Thailand	KF432998
***L. pasohensis* sp. nov.**	DS06-231 (GENT, KEP)	Malaysia	KF432987
***L. pasohensis* sp. nov.**	DS06-245 (GENT, KEP)	Malaysia	KF432986
***L. pasohensis* sp. nov.**	KW355 (GENT, MFLU)	Thailand	KF432988
L. chichuensis	KW012 (GENT, MFLU)	Thailand	KF433008
L. chichuensis	KW359 (GENT, MFLU)	Thailand	KF433009
L. chichuensis	Wang 1236 (GENT)	China	KF475766
L. hirtipes	Wang 1243 (GENT)	China	KF433007
L. cfr. Subzonarius	LTH145 (GENT, CMU)	Thailand	KF433006
L. cfr. Subzonarius	LTH324 (GENT, CMU)	Thailand	KF433005
L. gracilis	KW006(GENT, MFLU)	Thailand	KF433015
L. gracilis	KW102 (GENT, MFLU)	Thailand	KF433016
L. gracilis	KW334 (GENT, MFLU)	Thailand	KF433017
L. sp. subg. *Russularia*	KW108 (GENT, MFLU)	Thailand	KF433012
L. sp. subg. *Russularia*	KW109 (GENT, MFLU)	Thailand	KF433013
L. sp. subg. *Russularia*	KW110 (GENT, MFLU)	Thailand	KF433014
L. sp. subg. *Russularia*	KW042 (GENT, MFLU)	Thailand	KF433010
L. sp. subg. *Russularia*	KW294 (GENT, MFLU)	Thailand	KF433011
L. sp. subg. *Russularia*	KW111 (GENT, MFLU)	Thailand	KF433021
L. sp. subg. *Russularia*	KW221 (GENT, MFLU)	Thailand	KF433022
L. sp. subg. *Russularia*	AV12-044 (GENT)	Thailand	KF432985
L. sp. subg. *Russularia*	KW331 (GENT, MFLU)	Thailand	KF433020
L. sp. subg. *Russularia*	KW351 (GENT, MFLU)	Thailand	KF433019
L. sp. subg. *Russularia*	KW396 (GENT, MFLU)	Thailand	KF433018
L. sp. subg. *Russularia*	LTH98 (GENT, CMU)	Thailand	KF433004

TABLE 2.1 Continued.

Species	Voucher collection	Origin	ITS accession no.
L. sp. subg. *Russularia*	LTH256 (GENT, CMU)	Thailand	KF433003
L. sp. subg. *Russularia*	KW100 (GENT, MFLU)	Thailand	KF433001
L. sp. subg. *Russularia*	LTH261 (GENT, CMU)	Thailand	KF433002
L. sp. subg. *Russularia*	KW016(GENT, MFLU)	Thailand	KF433000
L. sp. subg. *Russularia*	KW033(GENT, MFLU)	Thailand	KF432994
L. sp. subg. *Russularia*	KW034(GENT, MFLU)	Thailand	KF432995
L. sp. subg. *Russularia*	KW346(GENT, MFLU)	Thailand	KF432993
L. sp. subg. *Russularia*	KW231(GENT, MFLU)	Thailand	KF432984
L. camphoratus	AV10-40 (GENT)	Norway	KF432971
L. serifluus	RW1431 (GENT)	Belgium	AY332558
L. subumbonatus	RC-KVP 10-002	Belgium	KF432981
L. atlanticus	LAC11121201 (GENT)	Spain	KF432976
L. atlanticus	MCVE1810(MCVE)	Italy	JF908297
L. decipiens	AV2000-137 (GENT)	Italy	KF432973
L. sphagneti	JKLAC11091502 (GENT)	Germany	KF432975
L. rubrocinctus	EDC12-210 (GENT)	Germany	KF432977
L. fulvissimus	JKLAC10082001 (GENT)	Germany	KF432970
L. badiosanguineus	AV04-235 (GENT)	France	KF432983
L. hepaticus	JN02-049 (GENT)	Belgium	KF432980
L. subdulcis	KW134 (GENT)	Belgium	KF432969
L. obscuratus	LVL 02-006 (GENT)	Belgium	KF432978
L. aurantiacus	JN 2001-60 (GENT)	Slovakia	KF432974
L. tabidus	KW136 (GENT)	Belgium	KF432979
L. lacunarum	JKLAC11092901 (GENT)	Belgium	KF432982
L. quietus	KW131 (GENT)	Belgium	KF432972
L. purpureus	FH12-008 (GENT, MFLU)	Thailand	KF432966
L. scrobiculatus	JN01-058 (GENT)	Slovakia	KF432966
L. hatsudake	JN2011-065 (GENT)	Vietnam	KF432967
L. austrozonarius	FH12-007 (GENT, MFLU)	Thailand	KF432965
L. pterosporus	JN2012-037 (GENT)	Germany	KF432963
L. acris	BG2011-31 (GENT)	Belgium	KF432962
L. friabilis	FH12-103 (GENT, MFLU)	Thailand	KF432961
L. romagnesii	BG2011-32 (GENT)	Belgium	KF432964
Lactifluus			
Lf. longipilus	FH12-131 (GENT, MFLU)	Thailand	KF432958

TABLE 2.1 Continued.

Species	Voucher collection	Origin	ITS accession no.
Lf. distantifolius	FH12-043 (GENT, MFLU)	Thailand	KF432959
Lf. leae	FH12-013 (GENT, MFLU)	Thailand	KF432957
Multifurca			
M. zonaria	FH12-009 (GENT, MFLU)	Thailand	KF432960
Russula			
R. chloroides	RUS 12091401 (GENT)	Ireland	KF432954
R. delica	FH 12-272 (GENT)	Belgium	KF432955
R. cyanoxantha	FH 12-201 (GENT)	Germany	KF432956

[1]MUVE: Venice Museum of Natural History

Results

Nucleotide alignment

The ITS multiple alignment consists of 72 sequences of 874 bases (including gaps). Gblocks retained 96% of the original alignment. The excluded regions (about 31 bases) came from the beginning and the end of the ITS alignment.

Maximum likelihood analysis

The Maximum Likelihood analysis of the ITS region is shown in Figure 2.1. It includes three of the four species that I here describe as new from Southeast Asia: *L. pasohensis* (clade 5), *L. laccarioides* (clade 8) and *L. sublaccarioides* (clade 12). Each of these species is supported by a high bootstrap value and is well supported. Unfortunately, we could not include *L. stubbei* sequence in this analysis since the quality of the obtained ITS sequence was very poor. Additional collections are necessary to confirm the phylogenetic position of this species. Sixteen well-known European species are also included in this analysis. Most European species appear to be closely related to one another and graded from some clades of Southeast Asian taxa (clade 11, 12, 13 and 14). *Lactarius atlanticus*, *L. decipiens*, *L. camphoratus*, *L. serifluus* and *L. subumbonatus* are nested in clades of tropical Asian taxa. The distance between two representatives of *L. atlanticus* resulted from a missing region of LAC11121201. Some known tropical Asian species are placed in distinct clades: *L. chichuensis* (clade 2), *L. gracilis* (clade 6) and *L. hirtipes* (clade 7). The two specimens of *L.* cfr. *subzonarius* group in clade 9. Several remaining undescribed Southeast Asian species are scattered in different clades (clade 1, 3, 4, 10, 11, 13, 14 and 15).

Identification key to *Lactarius* species with distant gills

1. Species with pleuromacrocystidia and cheilocystidia……………………………………………………………………………………(2)

1. Species without pleuromacrocystidia and cheilocystidia……………………………………………………………………………..(3)

2. Pleuromacrocystidia and cheilocystidia very distinctly emergent; spore ornamentation a complete reticulum; spore on average 8.2−8.3 × 7.1−7.2 µm..***Lactarius sublaccarioides* sp. nov.**

2. Pleuromacrocystidia and cheilocystidia not emergent; spore ornamentation an incomplete reticulum; spore on average 7.1 × 6.6 µm……………………………………………………………………………….…………***Lactarius stubbei* sp. nov.**

3. Pileus surface non-velutinous; stipe rather long, 26−71 mm in length; lower half hispid; basidia with 2 or 4 sterigmata; pileipellis a trichopalisade…………………………………………………………………***Lactarius laccarioides* sp. nov.**

3. Pileus surface minutely velutinous; stipe short, 9−22 mm in length; hairs only at base; basidia mostly with 4 sterigmata; pileipellis a trichoepithelium……………………………….…………………………….***Lactarius pasohensis* sp. nov.**

Taxonomy

Lactarius laccarioides Wisitrassameewong & Verbeken, sp. nov. (Figure 2.2, 2.6a and 2.7a)

MycoBank: MB 804541

Diagnosis: *Lactarius laccarioides* differs from the other distant-gilled species of *Lactarius* subg. *Russularia* by this combination of characters: pleurocystidia and cheilocystidia absent, pileus surface non-velutinous; basidia 2- or 4-spored; pileipellis a trichopalisade.

Etymology: 'laccarioides' means resembling a *Laccaria* species.

Typus: Thailand, Chiang Mai Province, Muang district, Doi Suthep-Pui National Park, Sangasabhasri Lane to Huai Kok Ma village, 1145 m elev., N18º48.62' E98º54.60', 5/7/2012, KW336 (holotypus MFLU!, isotypus GENT!).

Pileus 9–78 mm diam., at first plano-convex, later with expanding margin, slightly infundibuliform to infundibuliform with more depressed center, papillate at center; surface dry, smooth, slightly greasy, hygrophanous, deeply veined to center, inflexed to straight; edge crenulate or irregularly undulate, brownish yellow (5C7), pale brown (6D7) to brown (7E8), reddish brown (8E8) to reddish brown (9D8). *Lamellae* subdecurrent to decurrent, 1.5–3.5 mm broad, distant (5–6 L+l/cm), with 2–4 series of lamellulae, pale orange (5B5) to pale brown (5D5). *Stipe* 26–71 × 2–8 mm, cylindrical, rather long, central, surface dry, glabrous, pale orange (5A4) to golden brown (5D7) to reddish (6C7), sometimes yellowish brown (5E7); base hispid, 1/3 or almost a half of stipe covered by 2–3 mm long hairs.

FIGURE 2.1. The ML phylogeny based on ITS regions, resulting from a 500 replicates rapid bootstrapping algorithm and a heuristic ML tree search by RAxML. The bootstrap value ≥ 75% are shown. Species names in boldface are newly proposed species. Numbered clades are described in the text.

Context in pileus very thin, 0.5–1 mm, hollow in stipe, concolourous with stipe surface, light brown (6D5) with 10% KOH, unchanging with $FeSO_4$; smell sweetish; taste mild. *Latex* watery white, abundant, unchanging when isolated, unchanging on tissue paper, handkerchief or with 10% KOH; taste mild.

Basidiospores 6.7–7.2–7.7–8.3 (–8.5) × 6.0–6.5–6.9–7.7 µm, Q = 1.03–1.10–1.11–1.20 (n = 60), globose to subglobose to ellipsoid; ornamentation amyloid, forming an incomplete reticulum, never forming a complete reticulum, nodes subacute to acute, irregular up to 1 µm high, often with disconnected thin ridges, sometimes forming parallel stripes; isolated low warts numerous; plage distally amyloid. *Basidia* 39–53 × 9–14 µm, 2- or 4-spored, subclavate, sometimes subcylindrical, very often with guttulate contents, hyaline, thin-walled; sterigmata 9–11 × 2–3 µm. *Pleuromacrocystidia* absent. *Pleuropseudocystidia* 4–7 µm diam., abundant, emergent, irregularly cylindrical, slightly flexuous, with obtuse apex. *Lamella edge* sterile; marginal cells sometimes covered with a slightly refringent and gelatinized thin layer, clavate to lageniform; cheilocystidia absent. *Hymenophoral trama* with abundant lactifers, intermixed with rosettes of sphaerocytes. *Pileipellis* a trichopalisade 40–80 µm thick, suprapellis with repent cylindrical hyphae 24–42 × 5–8 µm partly with isodiametric elements; subpellis dominated by isodiametric to irregularly globose cells. Stipitipellis a cutis composed of repent and inflated hyphae, thin-walled 29–50 × 4–9 µm.

Habitat: gregarious on ground or decayed wood in deciduous forest, dominated by *Castanopsis* sp. and *Lithocarpus* sp.

Studied material: Thailand. Chiang Mai Province, Muang district, Doi Suthep-Pui National Park, Sangasabhasri Lane to Huai Kok Ma village, 1145 m elev., N18º48.62' E98º54.60', 5/7/2012, KW336 (holotype MFLU!, isotype GENT!); ibid., 16/7/2012, LTH10 (GENT!, CMU!); ibid., 4/7/2002, KW360 (MFLU!, GENT!), Chiang Mai province, Mao-on district, Huay Kaew sub-district, Ban Mae Kampong, 1450 m elev., N18°51.43' E99°22.09', 15/7/2012, KW357 (MFLU!, GENT!); Chiang Mai Province, Mae Taeng district, Ban Mae Sae village, on Hwy 1095 near 50 km marker, 962m elev., N19º14.59' E98º39.45', 3/6/2006, LTH372 (GENT!, CMU!).

Comment: In the field, the species is easily characterized by its habitus that is typical of a *Laccaria* species: the orange-reddish colours, the thin pileus and the striate to sulcate margin showing the rather distant lamellae in translucency. It has a look-alike though, as *L. sublaccarioides* (see below) looks very similar. For differences, see under that species.

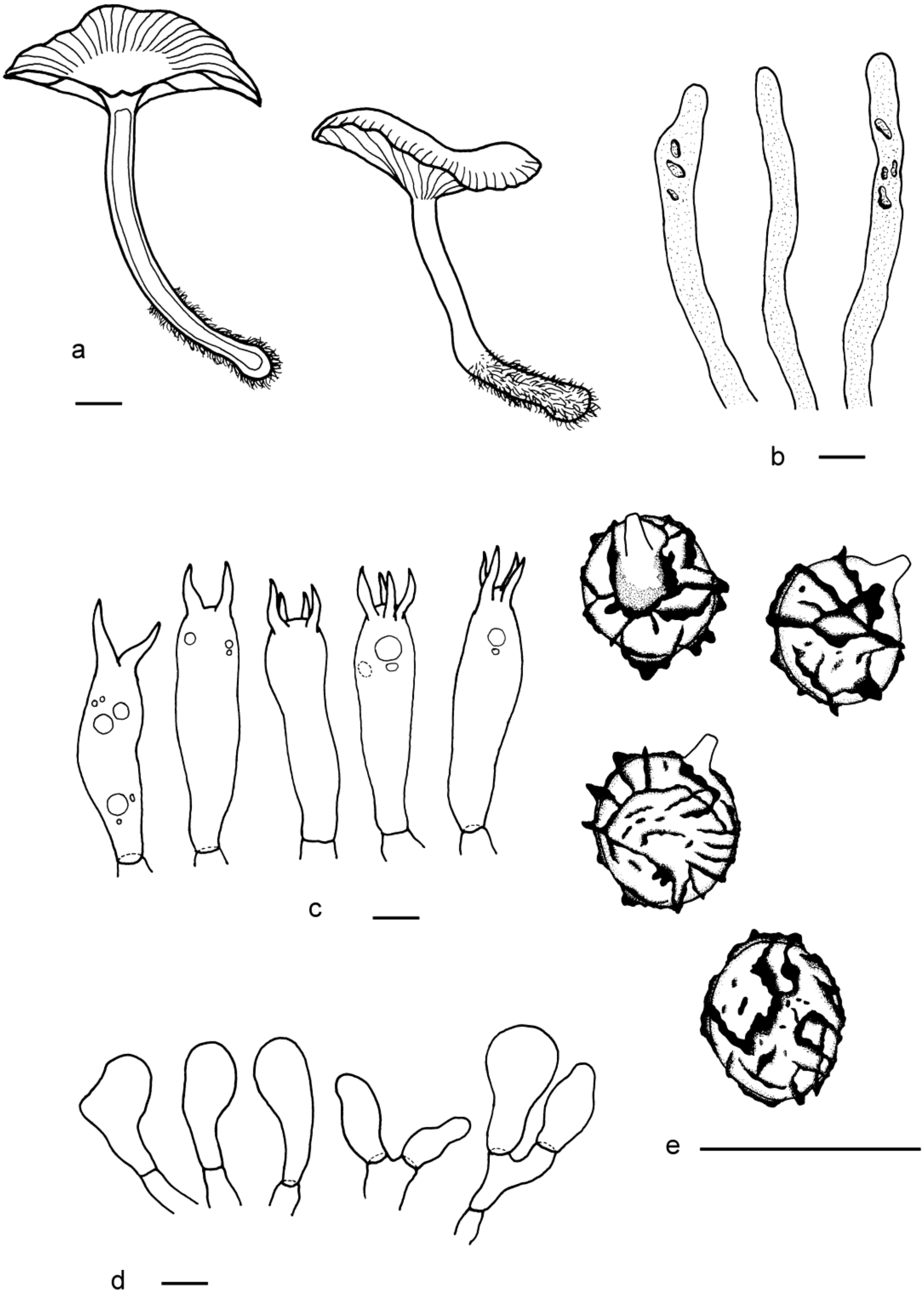

FIGURE 2.2. *Lactarius laccarioides* (KW336, holotype): a. basiodiocarp, b. pseudocystidia, c. basidia, d. marginal cell, e. basidiospore (a: scale bar = 1 cm, b-e: scale bar 10 = µm).

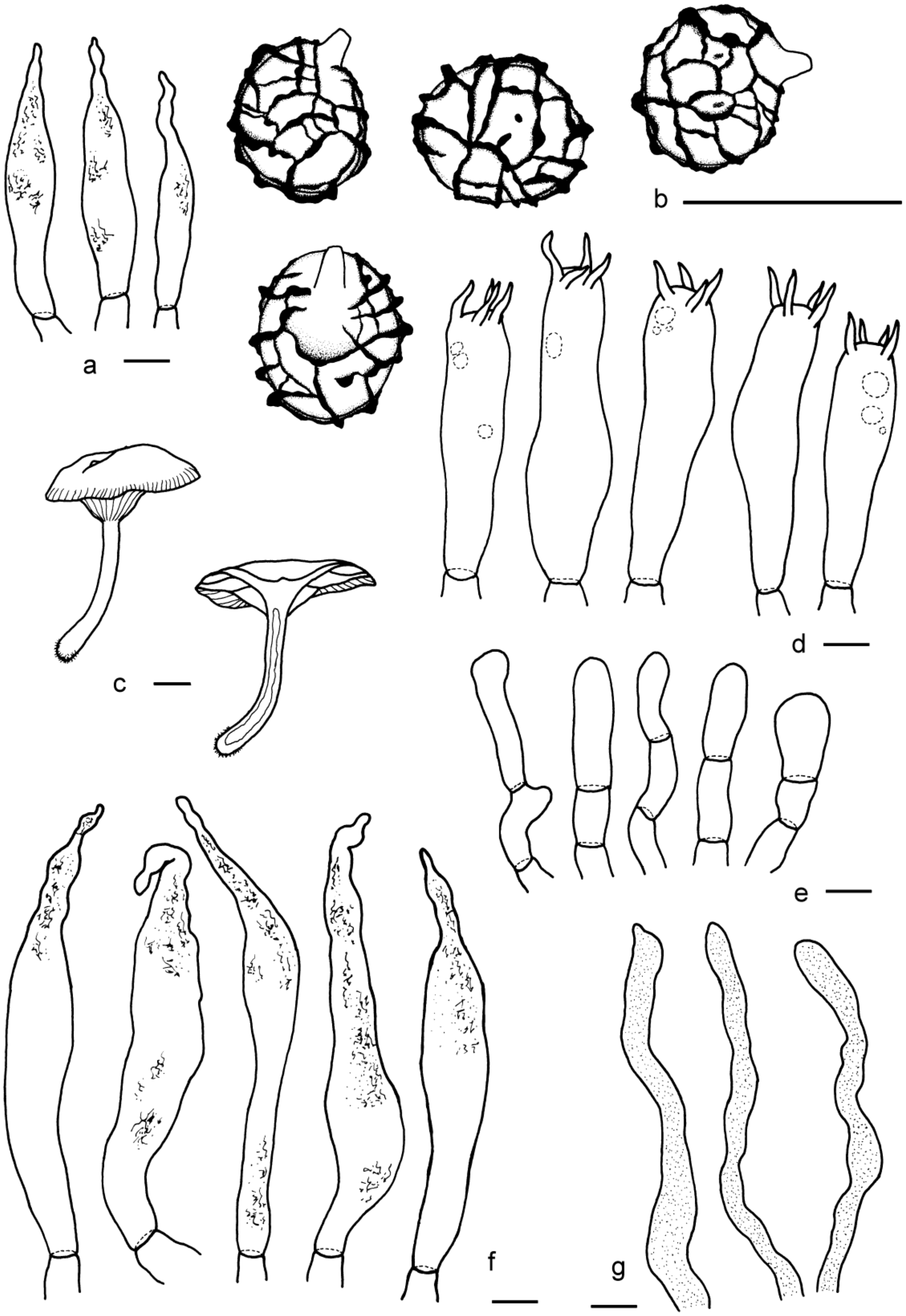

FIGURE 2.3. *Lactarius sublaccarioides*: a. cheilocystidia (KW300, holotype), b. basidiospore (KW300, holotype), c. basidiocarp (KW323), d. basidia (KW300, holotype), e. marginal cell (KW300, holotype), f. pleuromacrocystidia (KW300, holotype), g. pseudocystidia (KW300, holotype) (a–b and d–g: scale bar = 10 µm, c: scale bar = 1 cm).

Lactarius sublaccarioides Wisitrassameewong & Verbeken, sp. nov. (Figure 2.3, 2.6b and 2.7b)

MycoBank: MB 804542

Diagnosis: *Lactarius sublaccarioides* differs from the other distant-gilled species of *Lactarius* subg. *Russularia* by the presence of very distinct and emergent pleurocystidia and cheilocystidia.

Etymology: 'sublaccarioides' means macromorphologically very similar to *L. laccarioides*.

Typus: Thailand, Chiang Mai province, Mae Taeng District, Pa Pae sub-district, Bahn Pa Dheng, Mushroom Research Center, 900 m elev., N19°07.20' E98°44.044', 21/6/2012, KW300 (holotypus MFLU!, isotypus GENT!).

Pileus 11–44 mm diam., at first plano-convex, later with expanding margin, becoming applanate to slightly infundibuliform, with acute to blunt papilla in center, becoming wavy to irregularly crenulate at margin; surface dry, hygrophanous, finely radially wrinkled especially in the center; inflexed, translucently striate to sulcate nearly up to the center, reddish brown (8D8), reddish orange, not always unicolourous, sometimes unevenly light brown (6D5–D7), paler and greyish orange (6B4–B5) near margin, locally more reddish to brownish red (8C8). *Lamellae* subdecurrent to decurrent, 1–7 mm broad, subdistant (6–7 L+l/cm), 2–3 series of lamellulae, intervenose when old, buff to greyish orange (5B4) to brownish orange (6C5). *Stipe* 27–48 × 2-4 mm, cylindrical, slender, eccentric to central; surface dry, smooth, pale orange, pale brown (7D7) to brown (7E7), sometimes greyish orange (5B4); base slightly strigose. *Context* in pileus very thin, 0.5–1 mm, pale yellow (3A3), fragile, hollow in stipe, greyish green (26D7) with 10% KOH, unchanging with $FeSO_4$; smell sweetish, rather strong; taste mild. *Latex* watery white, abundant, unchanging when isolated, unchanging on tissue paper, handkerchief or with 10% KOH; taste faintly bitter after a while.

Basidiospores 7.5–8.2–8.3–9.1 × 6.3–7.1–7.2–8.1 (–8.5) µm, Q = 1.06–1.16-1.19–1.36 (n=60); subglobose to broadly ellipsoid or ellipsoid; ornamentation amyloid, forming a complete reticulum with thick and irregular ridges up to 1 µm high, interconnected by thin lines; few isolated warts present; plage inamyloid to distally amyloid. *Basidia* 56–66 × 12–15 µm, 4-spored, rarely 1-spored, subclavate to subcylindrical, thin-walled, often with guttulate contents; sterigmata 4–7 × 1–2 µm. *Pleuromacrocystidia* abundant, 88–102 × 8–15 µm, distinctly emergent, subfusiform with moniliform apex, occasionally with tortuous apex, thin-walled, mostly with granular and needle-liked contents. *Pleuropseudocystidia* abundant, 3–9 µm diameter, slightly emergent, cylindrical to tortuous with obtuse apex, occasionally capitate. *Lamella edge* sterile, composed of marginal cells and cheilocystidia; marginal cells, 20–25 × 10–12 µm, cylindrical to subclavate; thin-walled; cheilocystidia scarce to moderately abundant, 51– 59 × 8–10 µm, narrowly fusiform, mostly with moniliform apex, thin-walled, partially filled with granular and needle-liked contents. *Hymenophoral trama* with abundant lactifers intermixed with rosettes of sphaerocytes. *Pileipellis* a rather thin but distinct and compact layer of globose cells with some terminal elements on top, 35–50 µm thick, terminal elements oblique to parallel cylindrical, 14–28 × 3–7 µm, cylindrical to subfusiform. *Stipitipellis* a trichoderm with transitions to a cutis; terminal elements oblique or erect, cylindric to clavate; subpellis of mainly parallel, frequently septate hyphae which are sometimes inflated, 2.5–5 µm diam. and thin-walled.

Habitat: gregarious on ground in deciduous forest, dominated by *Castanopsis* sp. and *Lithocarpus* sp.

Studied material: Thailand, Chiang Mai Province, Mae Taeng District, Pa Pae sub-district, Bahn Pa Dheng, Mushroom Research Center, 900 m elev., N19°07.20' E98°44.044', 21/6/2012, KW300 (holotype MFLU!, isotype GENT!); ibid., 30/6/2012, KW323 (MFLU!, GENT!); ibid., 3/7/2012, KW332 (MFLU!, GENT!).

Comment: *Lactarius sublaccarioides* has again features reminiscent of the genus *Laccaria* such as the orange colours and the thin and striate pileus showing the distant gills in transparency. The similar species *L. laccarioides* differs by the hirsute stipe with hairs of 2–3 mm long that are present all over the surface of the lower half of the stipe while *L. sublaccarioides* has at most a slightly strigose base. Microscopically the distinction between the two species is easily made: *L. sublaccarioides* has abundant pleuromacrocystidia and at least some cheilocystidia while pleuro- and cheilocystidia are completely lacking in *L. laccarioides*.

Lactarius pasohensis Wisitrassameewong & Stubbe, sp. nov. (Figure 2.4, 2.6c and 2.7c)

MycoBank: MB 804543

Diagnosis: *Lactarius pasohensis* differs from the other distant-gilled species of *Lactarius* subg. *Russularia* by this combination of characters: pleurocystidia and cheilocystidia absent, pileus minutely velutinous, basidia mostly 4-spored and pileipellis a trichoepithelium.

Etymology: 'pasohensis' refers to the collecting site, Pasoh National Forest Reserve in Malaysia.

Typus: Malaysia, Negeri Sembilan, Pasoh National Forest Reserve, along main trail, not far from stream, 98m elev., N02°58.82' E102°18.23', 19/9/2006, DS06-231(KEP!, GENT!); ibid., 19/9/2006, DS06-245 (holotypus KEP!, isotypus GENT!).

Pileus 7–58 mm diam., convex when young becoming applanate and concave with irregularly waving margin; center acutely papillate, rugulose to wrinkled in central area, often slightly grooved and translucently striate towards the margin; margin smooth to weakly and irregularly crenate; surface minutely velutinous, brownish orange (6CD4–8), darker in center and paler towards the margin. *Lamellae* sinuate to decurrent (3–4 L+l/cm), moderately distant, rather narrow; orange-white when young, becoming slightly more orange-brown (5A3, 5B4) when maturing; edge smooth, concolourous. *Stipe* 9–22 × 1.5–4 mm, cylindrical to somewhat compressed, equal or slightly tapering downwards, central to eccentric; dry, velutinous to almost tomentose, concolourous with cap but often slightly darker (up to 6E5–8) or slightly paler and in a more whitish shade (6C3), with long and bristle-like brownish orange hairs at base and attachment to substrate. *Context* rather thin in pileus, hollow in stipe, orange-white (5A2–3) to concolourous with surface; smell faint, pleasant, sweetish fungoid; taste mild. *Latex* watery white, unchanging, moderately abundant to rather abundant; taste mild.

Basidiospores 6.3–6.7–7.1–7.7 × 5.7–6.1–6.5–7.2 µm, Q = 1.04–1.09–1.16 (n=60); globose to subglobose to ellipsoid, ornamentation amyloid, forming an incomplete reticulum, sometimes almost a reticulum, irregular ridges up to 1 µm high, often with discontinuous ridges, slightly acute to acute, isolated warts between main ridges present; plage inamyloid or rarely slightly amyloid distally. *Basidia* abundant, 38–62 × 11–19 µm; generally 4-spored, sometimes 1- or 2-spored, subclavate, thin-walled; with sterigmata 8–17 × 1.5–3 µm. *Pleuromacrocystidia* absent. *Pleuropseudocystidia* rather abundant 5–7 µm diam., slightly emergent, cylindrical, sometimes a bit bent near apex; apex obtuse. *Lamella edge* sterile; cheilocystidia absent; marginal cells 14–30 × 9–10 µm, cylindrical with obtuse to slightly acute apex. *Hymenophoral trama* with abundant lactifers, rosettes of sphaerocytes and hyphae. *Pileipellis* a trichoepithelium, partly hyphoepithelium; suprapellis composed of erect, oblique, bent and repent, cylindrical elements; subpellis a layer of isodiametric cells 80–110 µm thick, thick-walled. *Stipitipellis* a cutis; terminal elements oblique or repent, cylindrical; subpellis composed of compact layer of septate hyphae,

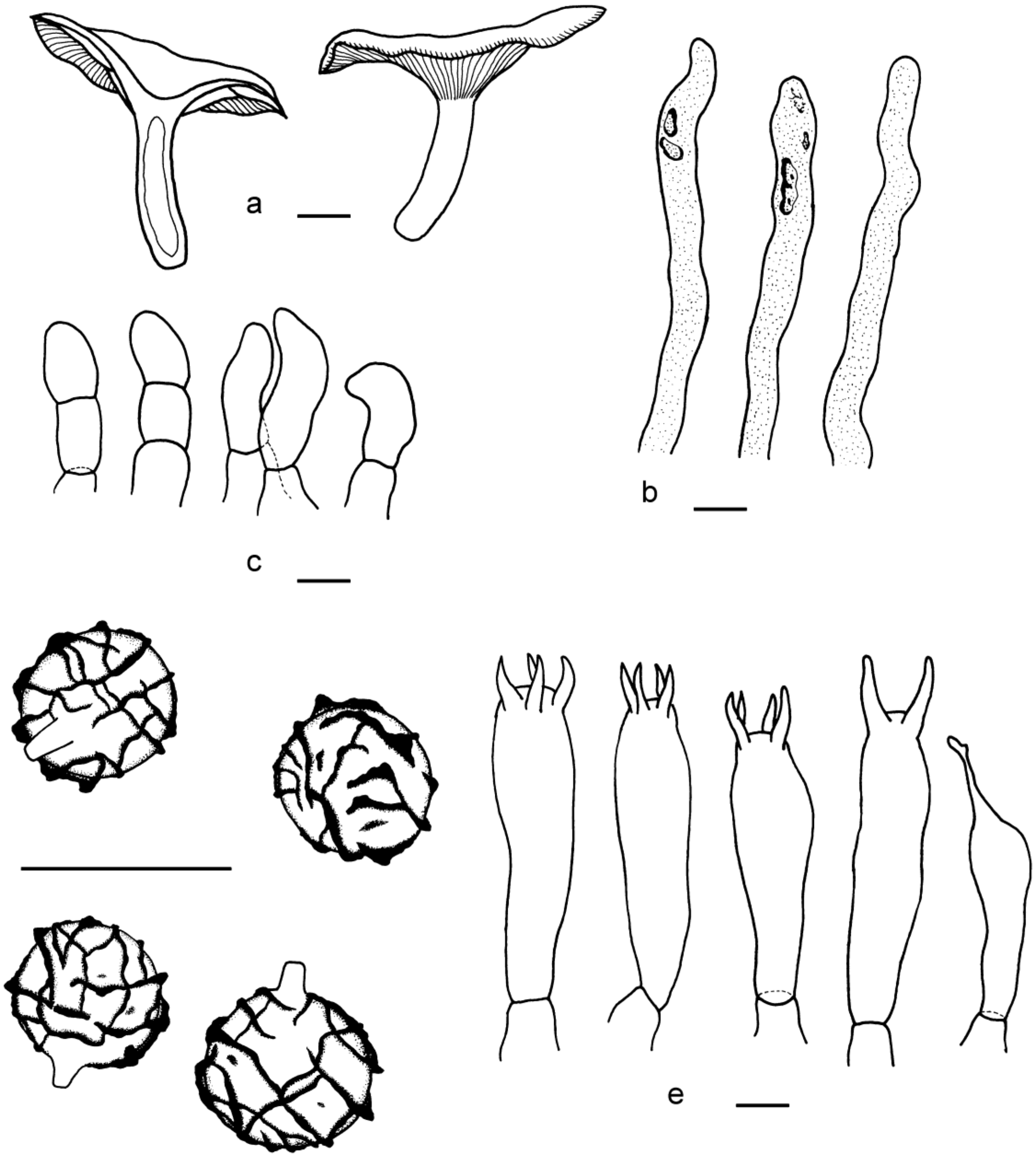

FIGURE 2.4. *Lactarius pasohensis* (DS06-245, holotype): a. basidiocarp, b. pseudocystidia, c. marginal cell, d. basidiospore, e. basidia (a: scale bar = 1 cm, b-e: scale bar = 10 µm).

mainly parallel, 4–6 µm diam. and thin-walled.

Habitat: gregarious on ground, dry Dipterocarp forest, with *Shorea* sp.

Studied material: Malaysia, Negeri Sembilan, Pasoh National Forest Reserve, along main trail, not far from stream, 98 m elev., N02°58.82' E102°18.23', 19/9/2006, DS06-231(KEP!, GENT!); ibid., 19/9/2006, DS06-245 (holotype KEP!, isotype GENT!), Thailand, Chiang Mai province, Mao-on district, Huay Kaew sub-district, Ban Mae Kampong, 1450 m elev., N18°51.43' E99°22.09', 15/7/2012, KW355 (MFLU!, GENT!).

Comment: In the field this species can be distinguished by its brownish orange colour, widely spaced gills and minutely velutinous aspect of pileus and stipe. The species resembles *L. laccarioides* which differs by a more deeply striated cap and a longer and more hispid stipe. *Lactarius pasohensis* differs clearly from *L. sublaccarioides* by the absence of pleuro- and cheilocystidia. An important microscopic character of *L. pasohensis* is the pileipellis structure: a trichoepithelium to hyphoepithelium with erect or oblique terminal elements.

Lactarius stubbei Wisitrassameewong & Verbeken, sp. nov. (Figure 2.5, 2.6d and 2.7d)

MycoBank: MB 804544

Diagnosis: *Lactarius stubbei* differs from the other distant-gilled species of *Lactarius* subg. *Russularia* by the pleuromacrocystidia which are not emergent (67–72 × 13–15 µm) and the cheilocystidia which are not emergent but abundant (29–44 × 8–10 µm).

Etymology: ‘stubbei’ refers to the name of collector, Dr. Dirk Stubbe.

Typus: Malaysia, Negeri Sembilan, Pasoh National Forest Reserve, along trail south of experimental plots, in formerly completely logged-over area, now regenerating rainforest, on and near decaying wood and leaf litter, 136 m elev., N02°58.18' E102°17.65', 25/6/2006, DS06-299 (holotypus KEP!, isotypus GENT!).

Pileus 10–39 mm diam., convex to irregularly infundibuliform, center depressed to deeply depressed, with acute papilla, central area rugulose, smoother towards the margin but becoming slightly, or sometimes strongly, grooved, sometimes concentrical wrinkles present near margin, margin smooth to faintly crenate; surface dry, smooth; warm brown (7E6–8), in central area often darker, up to almost liver brown (8F6–8); often translucently striate towards the margin. *Lamellae* decurrent, distant (5–6 L+l/cm), moderately broad (2–4 mm), whitish pink (6A2, 6B3); edge smooth, concolourous. *Stipe* 15–25 × 2–5 mm, cylindrical, more or less equal, central to slightly eccentric; surface dry, smooth, subtomentose; pale brownish pink (7–8C3, but with slightly more brownish tinges). *Context* thin in pileus, solid to stuffed in stipe; whitish, unchanging or locally and very faintly getting a pale yellowish tinge; smell pleasantly sweetish (with a scent of burnt sugar); taste mild to slightly bitter, after a while faintly acrid. *Latex* rather abundant, watery white, colour fading when drying on context or lamellae, or sometimes with a hardly noticeable, pale yellowish tinge, latex drying with a clearly visible pale yellow tinge (1A2–3) when isolated on a glass slide.

Basidiospores 6.4–7.1–7.8 × 6.1–6.6–7.1(–7.2) µm, Q = 1.04–1.08–1.17 (n=20); globose to subglobose to ellipsoid, ornamentation amyloid, forming an incomplete reticulum, thick and high ridges up to 1.2 µm high, typically with high obtuse warts in between main ridges, sometimes thin lines interconnecting between main ridges; plage inamyloid, sometimes distally amyloid. *Basidia* abundant, 58–73 × 11–15 µm; generally 4-spored, 2-spored basidia also present, subclavate to irregularly subcylindrical, rather slender, thin-walled, with granular contents; sterigmata 4–10 × 1.5–2.5 µm. *Pleuromacrocystidia* scarce to abundant, 67–72 × 13–15 µm, not emergent, subfusiform, with a mucronate apex, thin-walled. *Pleuropseudocystidia* abundant, 4–6 µm diam., not emergent, cylindrical with an obtuse apex. *Lamella edge* fertile, composed of marginal cells, cheilocystidia and some basidia; marginal cells 17–23 × 6–13 µm, subclavate, clavate to broadly clavate; cheilocystidia abundant, 29– 44 × 8–10 µm, subcylindrical to subfusiform or lanceolate, with a mucronate apex, thin-walled, not emergent. *Hymenophoral trama* with abundant lactifers intermixed with rosettes of sphaerocytes. *Pileipellis* a trichoepithelium, compact layer of globose cells with some terminal elements on top, 60–100 µm thick; terminal elements oblique to parallel, cylindrical. *Stipitipellis* a cutis; terminal elements repent and oblique, cylindrical; subpellis composed of compact, parallel and septate hyphae, 3–6 µm diam., thin-walled.

Habitat: gregarious on ground in deciduous forest.

Studied material: Malaysia, Negeri Sembilan, Pasoh National Forest Reserve, along trail south of experimental plots, in formerly completely logged-over area, now regenerating rainforest, on and near decaying wood and leaf litter, 136 m elev., N02°58.18' E102°17.65', 25/6/2006, DS06-299 (holotype KEP!, isotype GENT!).

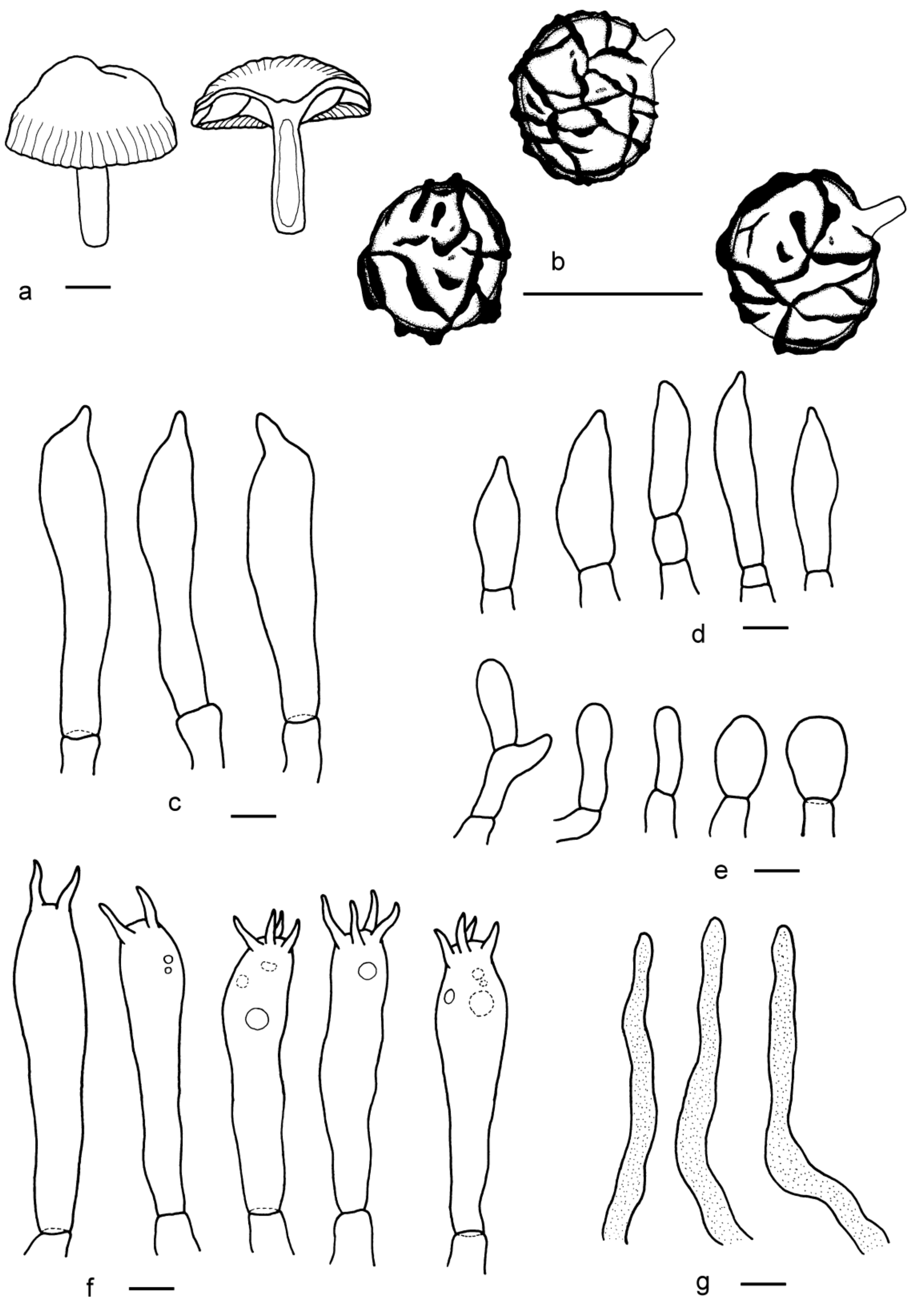

FIGURE 2.5. *Lactarius stubbei* (DS06-299, holotype): a. basidiocarp, b. basidiospore, c. pleuromacrocystidia, d. cheilocystidia, e. marginal cell, f. basidia, g. pseudocystidia (a: scale bar = 1 cm, b-g: scale bar = 10 µm).

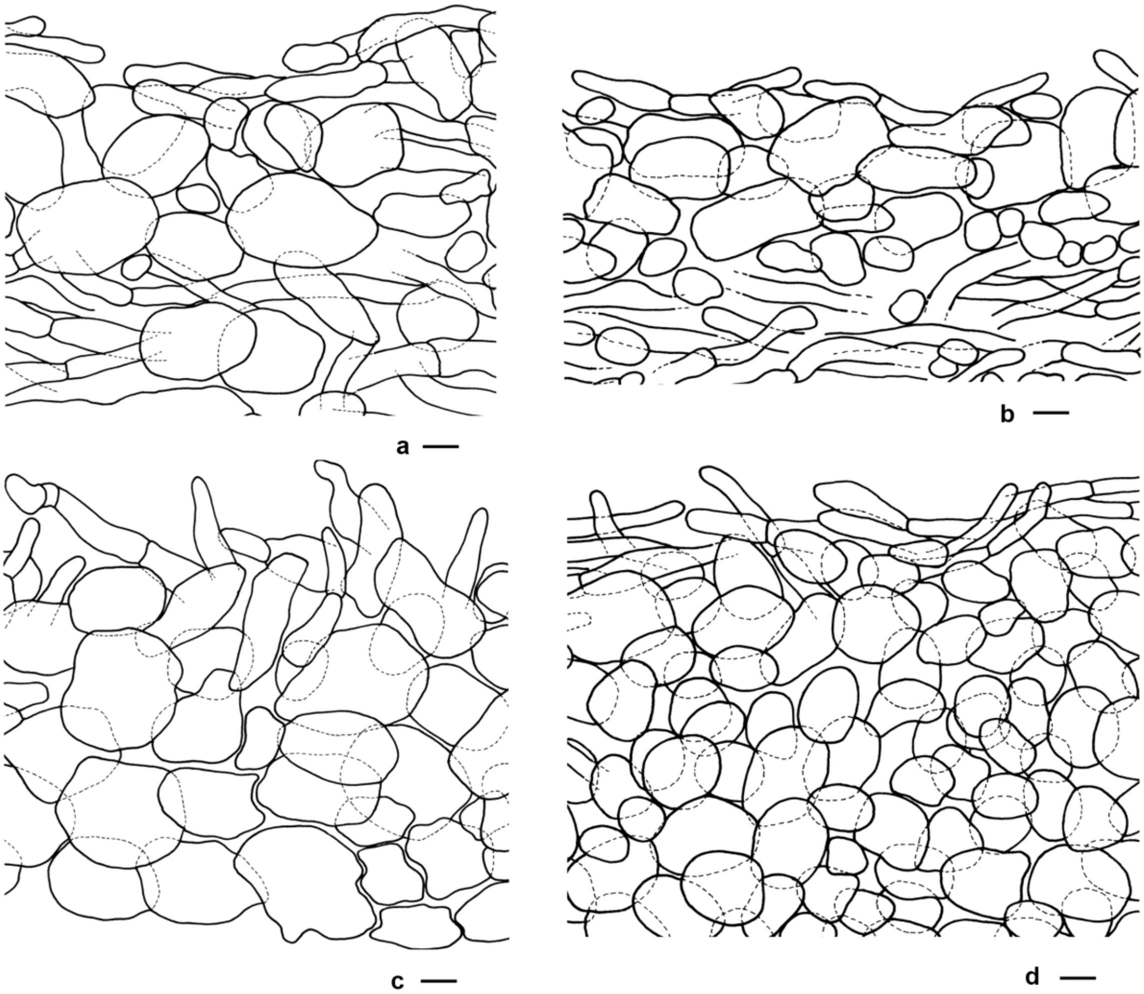

Figure 2.6. Pileipellis structure: a. *Lactarius laccarioides* (KW336, holotype), b. *Lactarius sublaccarioides* (KW300, holotype), c. *Lactarius pasohensis* (DS06-245, holotype) and d. *Lactarius stubbei* (DS06-299, holotype) (scale bar = 10 µm).

Comment: Of the four species with distant gills, *L. stubbei* is recognisable by its latex colour (white turning pale yellow when isolated on a glass slide). The stipe is rather short and firm, has a subtomentose surface with a pale brownish pink colour. Pleuro- and cheilocystidia are present and embedded or slightly emergent from the hymenium. Basidiospores are ornamented with firm ridges that are up to 1.2 µm high and form an incomplete reticulum.

Discussion

In temperate regions, *L.* subgenus *Russularia* is known to have some groups of very closely related species that are morphologically very difficult to separate, such as for example *L. aurantiacus*/*L. aurantiofulvus*/*L. mitissimus* and *L. serifluus*/*L. subumbonatus* (Heilmann-Clausen et al. 1998). Some morphologically very similar species have been described from Asia and have been a source for confusion. Wang and Liu (2002) discussed the problem of *L. hirtipes* and *L. chichuensis* in China. Our phylogeny reveals that both Chinese species are not sister species. Apparently some characters and character states of *L.* subg. *Russularia* are more difficult to define and to distinguish. In the present study we also deal with a group of species that can easily be confused in the field because they all have distant gills and a translucent-striate pileus margin (often giving them the aspect of a *Laccaria* species), a combination of characters which is very striking in the subgenus because most species in *L.* subgenus *Russularia* have crowded or less distant gills. The study of microscopic characters and the use of molecular tools are necessary to distinguish the different distant-gilled species, and to check whether they form a natural group.

We hypothesised that the new species might form a separate clade from the other *L.* subg. *Russularia* species because of their shared characters and different appearance. In spite of their strikingly similar macroscopic features, the phylogenetic results show that the species with distant gills do not form a separate clade in the ITS phylogeny. On the contrary, they are distributed in different clades and nest with other *L.* subg. *Russularia* species. In addition, intercontinental conspecificity is absent between Europe and Southeast Asia which corresponds with the findings of Stubbe et al. (2010). *Lactarius sublaccarioides* is without doubt a distinct new species and separate from the other new species in the tree. It is characterized by its projecting macrocystidia with an irregularly moniliform apex and its resemblance to a *Laccaria* species. During microscopic examination, this species was examined together with *L. laccarioides*, with which we first assumed to be conspecific. Macroscopically, there are only slight differences among these taxa, e.g. the length of stipe and the degree of strigose hairs at the stipe base. *Lactarius pasohensis* and the two European species *L. serifluus* and *L. subumbonatus* evolved from the same internal node, together with Thai specimens in clade 4; AV12-044, KW111 and KW221. However, a thorough microscopic examination revealed that clade 4 actually comprises two species, *L.*

fuscomaculatus and *L. rubrobrunneus* (see more details in Chapter 4).

With microscopic examination, all four species have a hyphoepithelium in the pileipellis and almost complete reticulum in spore ornamentation. *L. serifluus*, *L. subumbonatus* and *L. pasohensis* lack pleuro- and cheilocystidia, except for the unknown taxon that possesses pleuromacrocystidia with a mucronate apex. *L. laccarioides* and *L. atlanticus* were derived from the same internal node. Both species are often remarkably strigose at the stipe base and lack macrocystidia. Apparently, *L. atlanticus* differs from *L. laccarioides* by the absence of a strongly striated margin, often with a shiny surface to the cap and much less distant gills.

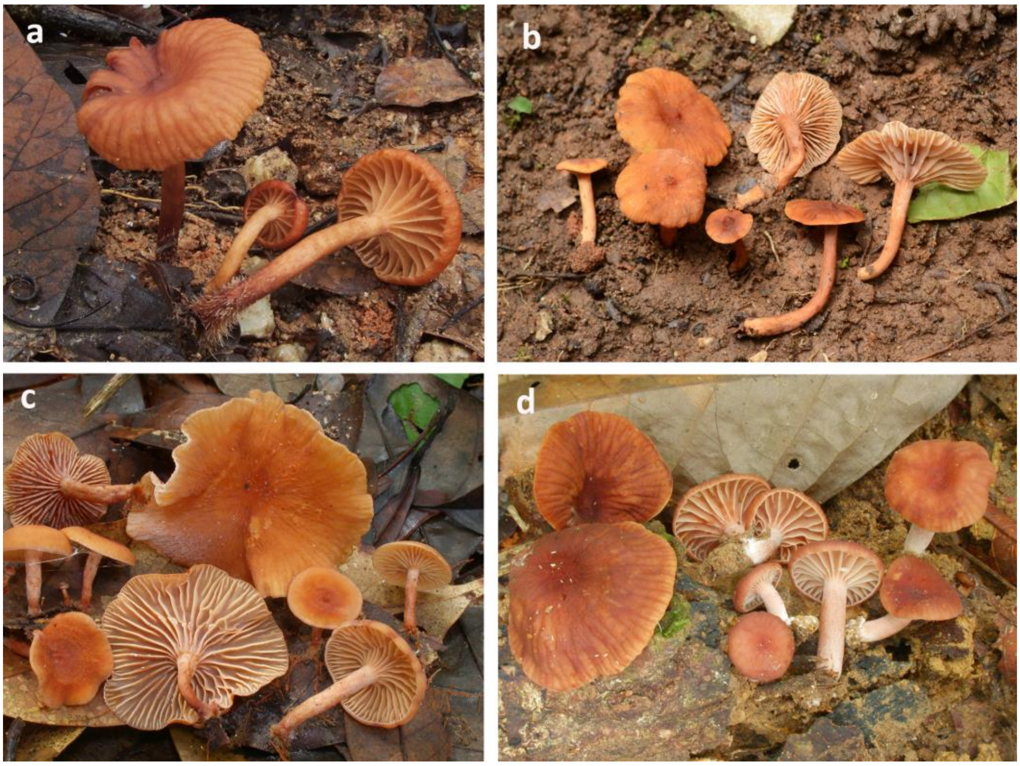

FIGURE 2.7. basidiocarps; a: *L. laccarioides* (Photo by Felix Hampe), b: *L. sublaccarioides*, c: *L. pasohensis* (Photo by Dirk Stubbe), d: *L. stubbei* (Photo by Dirk Stubbe).

The present study also shows some interesting clades which need further molecular investigation. An undescribed collection, KW231, groups together with the European *L. quietus* in clade 15, however, when more *L. quietus* sequences are included in the analysis, the European taxon formed a separate clade from KW231 (result not shown). The remaining undescribed species in the phylogenetic tree will be discussed later in this series of publications. Since the knowledge of the diversity of *L*. subgenus *Russularia* in this region is still poor, further explorations with additional specimens for the molecular analyses are necessary for a comprehensive knowledge of the tropical representatives of this subgenus.

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CHAPTER 3

SPECIES WITH REMARKABLY SMALL BASIDIOCARPS

Abstract

This paper is the second in a series of biodiversity papers on *Lactarius* subgenus *Russularia* in tropical forests of Southeast Asia. This study is based on extensive mycological exploration, especially in Northern Thailand, during the past ten years. In this paper we consider some species that are characterized by remarkably small basidiocarps i.e. with an average pileus diameter that is smaller than 20 mm. One of the most common species in Northern Thailand with dwarf basidiocarps is *L. gracilis*, originally described from Japan. We introduce the new species *L. crenulatulus*, *L. perparvus* and *L. glabrigracilis* with morphological descriptions and illustrations. Molecular evidence based on the ITS sequence analysis supports the classification and novel status of the taxa. All species are associated with trees belonging to the Fagaceae. These are the first reported collections of small basidiocarps of *L.* subg. *Russularia* in Southeast Asia. In this paper we compare these new species with small basidiocarp with similar representatives from Europe and other Asian regions.

Keywords: milkcaps, taxonomy, molecular phylogeny

Modified from: Wisitrassameewong, K., Nuytinck, J., Hampe, F., Hyde, K.D. & Verbeken, A. (2014b). *Lactarius* subgenus *Russularia* (Russulaceae) in South-East Asia: 2. Species with remarkably small basidiocarps. *Phytotaxa* 188(4): 181−197.

Introduction

In the recent classification of *Lactarius* Pers. (Buyck *et al.* 2010), three subgenera, *L.* subg. *Lactarius* (Fr. ex J. Kickx f.) Kauffman, *L.* subg. *Russularia* (Fr.) Kauffman and *L.* subg. *Plinthogalus* (Berk.) Hesler & A.H. Sm. were recognized. Among these three subgenera, *L.* subg. *Russularia* is assumed to be a taxonomically complex group which is widely distributed in temperate and tropical regions, but poorly represented in tropical Africa and South America (Verbeken 1996, Verbeken & Walleyn 2010; Wisitrassameewong et al. 2014). In Southeast Asia, 13 species have been reported in *L.* subg. *Russularia*, mainly from Indonesia (Verbeken et al. 2001), Southern China (Wang 2000, Wang & Liu 2002) and Malaysia and Thailand (Wisitrassameewong et al. 2014). Studies have also reported and described taxa from subg. *Russularia* in other regions of Asia, e.g. in Japan by Hongo (1957a, 1957b, 1971), in Papua New Guinea (Verbeken & Horak 2000), in the Himalaya region, India by Das et al. (2004) and Joshi et al. (2012). Chinese type specimens, including representatives of this subgenus, have been re-evaluated because European and American names were misapplied (Wang 2007). *Lactarius squamulosus* Z.S. Bi & T.H. Li was considered a synonym of *L. gracilis* Hongo. Up to now all species known in Asia are endemic for the continent and the use of American and European names could not be justified.

Macromorphologically, most members of *L.* subg. *Russularia* can be recognized by their dry pileus surface and colours that are typically dominated by shades of orange and reddish brown to brown. The latex colour and colour changes are not as variable as in the other subgenera of *Lactarius*, with most species having white or whitish and unchanging latex. In a few species however, the latex is yellow (Verbeken et al. 2001, Das et al. 2004) and in some the latex changes from white to cream or yellow. An exceptional species is *L. sulphurescens* Verbeken & E. Horak from Java, Indonesia (but also occurring in Malaysia), that has abundant, spectacular, golden yellow latex (Verbeken et al. 2001). In some species, such as *L. stubbei* Wisitrassameewong & Verbeken, the colour change only becomes visible when the latex is drying, then showing a clear pale yellow tinge (Wisitrassameewong et al. 2014). Spore ornamentation and pileipellis structure are microscopic characters that discriminate between species. The spore ornamentation is typically an incomplete to complete reticulum while spiny or obtuse isolated warts occur in some species. The pileipellis structure shows a wide variety, ranging from an epithelium, hyphoepithelium or trichopalisade to a cutis (Heilmann-

Clausen et al. 1998, Das & Sharma 2005, Wisitrassameewong et al. 2014). The presence or absence of pleuromacro- and cheilocystidia are often useful for species identification. Many representatives possess subcylindrical or narrowly fusiform or fusiform cystidia, but some lack this character state.

The diversity of *L.* subg. *Russularia* in Thailand is high due to the presence of many families of ectomycorrhizal trees e.g. Betulaceae, Dipterocarpaceae, Fagaceae, and Pinaceae and a strongly seasonal climate (Yuwa-Amornpitak et al. 2006, Stubbe et al. 2007, Le et al. 2007, Phosri et al. 2012). In addition, rainfall and humidity are optimal for the growth of mycelium and fructification (Le et al. 2007). On many excursions, representatives of *L.* subg. *Russularia* were the dominant basidiocarp-formers. However, the knowledge of their diversity is limited and only based on the work of Le (2007) and Wisitrassameewong et al. (2014). So far, only four species are known from Thailand: *L. gracilis, L. chichuensis* Chiu, first described from China, and *L. laccarioides* Wisitrassameewong & Verbeken and *L. sublaccarioides* Wisitrassameewong & Verbeken, both described from Thailand.

In the present study, we describe and illustrate three new species: *L. crenulatulus*, *L. perparvus* and *L. glabrigracilis*. The three species share a striking field character in having remarkably small basidiocarps. The maximum pileus diameter is 40 mm (in *L. glabrigracilis*), but mostly in the range of 2–20 mm. Dwarf species with a small pileus and relatively slender stipe are more common in *L.* subg. *Russularia* than in the other two subgenera. In Europe, this group with very small basidiocarps is associated with *Alnus* e.g. *L. obscuratus* (Lasch: Fr.) Fr., *L. cyathuliformis* Bon and *L. omphaliformis* Romagn., whereas *L. rostratus* Heilmann-Clausen is found in *Fagus* forest (Heilmann-Clausen et al. 1998). In tropical Asia, representatives with small basidiocarps occur mainly in broad-leaved forests dominated by members of the Fagaceae. All species have striking features. For example, *L. gracilis* appears to be very common in many Asian countries, e.g. China, Japan and Thailand, and is easily recognizable by the flocks at the pileus margin. *Lactarius liliputianus* Verbeken & E. Horak, found in Papua New Guinea and Indonesia, is a species with spores with isolated and blunt warts that recall the typical aspect of *Russula* spore ornamentation. *Lactarius perconicus* Verbeken & E. Horak is the smallest representative of *Lactarius* in Papua New Guinea (8 mm pileus diam.); it has a non-striate pileus. *Lactarius inamyloideus* Verbeken & E. Horak seems to have the weakest amyloidity of spores ever encountered in the genus.

Lactarius pennulatus Verbeken & E. Horak has reddish basidiocarps with pure watery latex (Verbeken & Horak 2000).

This study reports on the novel diversity of *L.* subg. *Russularia* from Thailand, using a combination of morphological and phylogenetic data. A phylogenetic analysis based on ITS sequences and including species from other continents was performed to evaluate the status of the new species and their position in the subgenus.

Material and Methods

Collections

Collections were made from June to July 2004 to 2012 in Northern Thailand (mainly Chiang Mai and Chiang Rai provinces). All collections are deposited in the herbarium of Gent University (GENT), Belgium; Mae Fah Luang University herbarium (MFLU) and Chiang Mai University (CMU), Thailand; and/or San Francisco State University, USA (SFSU).

Morphological features

The macroscopic study is based on fresh material. Colour codes refer to Kornerup and Wanscher (1978). Latex colour and changes are documented as previously described by Wisitrassameewong et al. (2014). Colour change of latex needs careful observation, sometimes the colour change is best observed on a white handkerchief or white tissue or in contact with potassium hydroxide (10%). For terminology of macro- and micro-morphology, we refer to Vellinga (1988) and Verbeken (1996). Microscopic studies were performed using the exsiccata of mature specimens. Basidiospores were measured and described as observed in Melzer’s reagent. Spore sizes were measured at least 20 spores per collection in lateral view, using a Zeiss Axioscop 2 microscope and a drawing tube at 6,000× magnification. Measurements were made excluding the height of ornamentation. The spore measurement are given as {(MIN) [AV-2×SD] –*AV*– [AV+2×SD] (MAX)} length × {(MIN) [AV-2×SD] –*AV*–[AV+2×SD] (MAX)} width in which AV = mean value for the measured collection and SD = standard deviation. Q corresponds to spore “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 specimens, respectively. All other elements of the hymenium, pileipellis and stipitipellis structure were observed in Congo-Red in L4, using an Olympus CX31 microscope and drawing tube at a magnification of 1,600×. Basidia length excludes sterigmata length.

Molecular approach

DNA extraction of collections was done using fresh tissue and a CTAB-based method (Nuytinck & Verbeken 2003, Van de Putte et al. 2010). The ITS gene was amplified and sequenced using ITS1-F and ITS4 primers (White et al. 1990, Gardes & Bruns 1993). The obtained trace files were assembled to contigs and manually edited using the software Sequencer™ v5.0 (GeneCodes Corporation Ann Arbor, Michigan, U.S.A.). In order to obtain a reliable multiple alignment, we used the online MAFFT v7 (Katoh &Standley 2013) with settings E-INS-I, edited using MEGA5 (Tamura et al. 2011) and later excluded the poorly aligned regions using Gblocks v0.91b (Castresana 2000). The minimum number of sequences for a conserved position and for a flank position was set to half the number of sequences, the number of contiguous non-conserved positions was set to 10, the minimum length of a block after gap cleaning to 5 and positions with gaps were not treated differently from other positions.

Table 3.1 lists the specimens and GenBank accession numbers of sequences used in the molecular phylogenetic analyses. Apart from the sequences that were newly generated for this study, we also included GenBank sequences of closely related species. The ITS sequences were partitioned in the partial ribosomal gene 18S, the first spacer region ITS1, the ribosomal gene 5.8S, the second spacer region ITS2 and the partial ribosomal gene 28S. Maximum likelihood analysis (ML) was executed using RAxML v7.0.3, applying Maximum likelihood search and rapid bootstrapping for 500 replicates (Stamatakis 2006). Bayesian inference analysis (BI) was executed using MrBayes v3.1.2 (Ronquist & Huelsenbeck 2003) applying the GTR+I+G model of character evolution as determined by MrModeltest v2.3 (Nylander 2004). Five independent runs with four chains each were run for 20 million generations. Sample 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).

TABLE 3.1. List of specimens and GenBank accession number of sequences used in the molecular phylogenetic analysis. Newly described species in this study are in boldface.

Species	**Voucher collection**	**Origin**	**ITS accession no.**
Lactarius			
L. crenulatulus **sp.nov.**	KW368 (GENT, MFLU)	Thailand	KJ458979
L. crenulatulus **sp.nov.**	KW382 (GENT, MFLU)	Thailand	KJ458980
L. perparvus **sp.nov.**	KW320 (GENT, MFLU)	Thailand	KJ458981
L. perparvus **sp.nov.**	KW337 (GENT, MFLU)	Thailand	KJ458982
L. glabrigracilis **sp.nov.**	KW095 (GENT, MFLU)	Thailand	KJ458983
L. glabrigracilis **sp.nov.**	KW230 (GENT, MFLU)	Thailand	KJ458984
L. glabrigracilis **sp.nov.**	KW335 (GENT, MFLU)	Thailand	KJ458985
L. laccarioides	LTH10 (GENT, CMU)	Thailand	KF432989
L. laccarioides	LTH372 (GENT, CMU)	Thailand	KF432990
L. laccarioides	KW336 (GENT, MFLU)	Thailand	KF432991
L. laccarioides	KW360 (GENT, MFLU)	Thailand	KF432992
L. sublaccarioides	KW011(GENT, MFLU)	Thailand	KF432999
L. sublaccarioides	KW300 (GENT, MFLU)	Thailand	KF432996
L. sublaccarioides	KW323 (GENT, MFLU)	Thailand	KF432997
L. sublaccarioides	KW332 (GENT, MFLU)	Thailand	KF432998
L. pasohensis	DS06-231 (GENT, KEP)	Malaysia	KF432987
L. pasohensis	DS06-245 (GENT, KEP)	Malaysia	KF432986
L. pasohensis	KW355 (GENT, MFLU)	Thailand	KF432988
L. chichuensis	KW359 (GENT, MFLU)	Thailand	KF433009
L. chichuensis	Wang 1236 (GENT)	China	KF475766
L. hirtipes	Wang 1243 (GENT)	China	KF433007
L. cfr. subzonarius	LTH145 (GENT, CMU)	Thailand	KF433006
L. cfr. subzonarius	LTH324 (GENT, CMU)	Thailand	KF433005
L. gracilis	KW006(GENT, MFLU)	Thailand	KF433015
L. gracilis	KW102 (GENT, MFLU)	Thailand	KF433016
L. gracilis	KW334 (GENT, MFLU)	Thailand	KF433017
L. sp. subg. *Russularia*	KW108 (GENT, MFLU)	Thailand	KF433012
L. sp. subg. *Russularia*	KW109 (GENT, MFLU)	Thailand	KF433013
L. sp. subg. *Russularia*	KW110 (GENT, MFLU)	Thailand	KF433014
L. sp. subg. *Russularia*	KW042 (GENT, MFLU)	Thailand	KF433010
L. sp. subg. *Russularia*	KW294 (GENT, MFLU)	Thailand	KF433011
L. sp. subg. *Russularia*	KW111 (GENT, MFLU)	Thailand	KF433021
L. sp. subg. *Russularia*	KW221 (GENT, MFLU)	Thailand	KF433022

TABLE 3.1. Continued.

Species	Voucher collection	Origin	ITS accession no.
L. sp. subg. *Russularia*	KW331 (GENT, MFLU)	Thailand	KF433020
L. sp. subg. *Russularia*	KW351 (GENT, MFLU)	Thailand	KF433019
L. sp. subg. *Russularia*	KW396 (GENT, MFLU)	Thailand	KF433018
L. sp. subg. *Russularia*	LTH98 (GENT, CMU)	Thailand	KF433004
L. sp. subg. *Russularia*	LTH256 (GENT, CMU)	Thailand	KF433003
L. sp. subg. *Russularia*	KW100 (GENT, MFLU)	Thailand	KF433001
L. sp. subg. *Russularia*	LTH261 (GENT, CMU)	Thailand	KF433002
L. sp. subg. *Russularia*	KW016(GENT, MFLU)	Thailand	KF433000
L. sp. subg. *Russularia*	KW033(GENT, MFLU)	Thailand	KF432994
L. sp. subg. *Russularia*	KW034(GENT, MFLU)	Thailand	KF432995
L. sp. subg. *Russularia*	KW346(GENT, MFLU)	Thailand	KF432993
L. sp. subg. *Russularia*	KW231(GENT, MFLU)	Thailand	KF432984
L. camphoratus	AV10-40 (GENT)	Norway	KF432971
L. serifluus	RW1431 (GENT)	Belgium	AY332558
L. subumbonatus	RC-KVP 10-002	Belgium	KF432981
L. atlanticus	LAC11121201 (GENT)	Spain	KF432976
L. atlanticus	MCVE1810(MCVE[1])	Italy	JF908297
L. decipiens	AV2000-137 (GENT)	Italy	KF432973
L. sphagneti	JKLAC11091502 (GENT)	Germany	KF432975
L. rubrocinctus	EDC12-210 (GENT)	Germany	KF432977
L. fulvissimus	JKLAC10082001 (GENT)	Germany	KF432970
L. badiosanguineus	AV04-235 (GENT)	France	KF432983
L. hepaticus	JN02-049 (GENT)	Belgium	KF432980
L. subdulcis	KW134 (GENT)	Belgium	KF432969
L. obscuratus	LVL 02-006 (GENT)	Belgium	KF432978
L. aurantiacus	JN 2001-60 (GENT)	Slovakia	KF432974
L. tabidus	KW136 (GENT)	Belgium	KF432979
L. tabidus	BB22-104 (ZALF[2])	Germany	HM189833
L. lacunarum	JKLAC11092901 (GENT)	Belgium	KF432982
L. quietus	KW131 (GENT)	Belgium	KF432972
L. purpureus	FH12-008 (GENT, MFLU)	Thailand	KF432966
L. scrobiculatus	JN01-058 (GENT)	Slovakia	KF432966
L. hatsudake	JN2011-065 (GENT)	Vietnam	KF432967
L. austrozonarius	FH12-007 (GENT, MFLU)	Thailand	KF432965
L. pterosporus	JN2012-037 (GENT)	Germany	KF432963

TABLE 3.1. Continued.

Species	Voucher collection	Origin	ITS accession no.
L. acris	BG2011-31 (GENT)	Belgium	KF432962
L. friabilis	FH12-103 (GENT, MFLU)	Thailand	KF432961
L. romagnesii	BG2011-32 (GENT)	Belgium	KF432964
Lactifluus			
Lf. longipilus	FH12-131 (GENT, MFLU)	Thailand	KF432958
Lf. distantifolius	FH12-043 (GENT, MFLU)	Thailand	KF432959
Lf. leae	FH12-013 (GENT, MFLU)	Thailand	KF432957
Multifurca			
M. zonaria	FH12-009 (GENT, MFLU)	Thailand	KF432960
Russula			
R. chloroides	RUS 12091401 (GENT)	Ireland	KF432954
R. delica	FH 12-272 (GENT)	Belgium	KF432955
R. cyanoxantha	FH 12-201 (GENT)	Germany	KF432956

[1] MUVE: Venice Museum of Natural History

[2] ZALF: The Leibniz Centre for Agricultural Landscape Research

Outgroup and closely related genera

According to the recent molecular phylogeny of the family Russulaceae (Buyck *et al.* 2008), three genera are related to *Lactarius*: *Russula* Pers., *Multifurca* Buyck & V. Hofst. and *Lactifluus* (Pers.) Roussel. Representatives from these three genera were included in this study. Three *Russula* species, *R. cyanoxantha* (Schaeff.) Fr., *R. delica* Fr. and *R. chloroides* (Krombh.) Bres., were chosen as an outgroup for all phylogenetic analyses. *Multifurca zonaria* (Buyck & Desjardin) Buyck & V. Hofst. is the representative of *Multifurca*. *Lactifluus (Lf.) distantifolius* (Van de Putte, Stubbe & Verbeken) Van de Putte, *Lf. longipilus* (Van de Putte, H.T. Le & Verbeken) Van de Putte and *Lf. leae* (Stubbe & Verbeken) Verbeken are the representatives of *Lactifluus*. Additionally, representatives from *L.* subg. *Lactarius* and *L.* subg. *Plinthogalus,* are included in this study (Table 3.1).

Result

Molecular analyses

The ITS alignment includes 76 sequences, 61 of which belong to *L.* subg. *Russularia*. After exclusion of ambiguously aligned regions, 878 characters were retained from the original alignment (96% of the original 906 characters). Sixteen European species of *L.* subg. *Russsularia* are included in these analyses.

Tree topologies of ML and BI analyses are highly similar. A ML phylogram with posterior probability values of the BI analysis is shown in Figure 3.1. The genera in Russulaceae are well-supported. *Lactarius* subg. *Russularia* appears to be monophyletic but has low bootstrap and posterior probability values. The European and Asian species of *L.* subg. *Russularia* do not form separate clades. The Asian representatives with small basidiocarps form four well-supported clades, and are thus supported as separate species because each of these clades receives a high bootstrap support and posterior probability value. Species 1, here proposed as *L. crenulatulus,* clusters with *L. pasohensis* Wisitrassameewong & Stubbe (clade 1), which has larger basidiocarps. Although both species in clade 1 are very different macromorphologically, some similarities were found: 1) the absence of pleuromacrocystidia and cheilocystidia, 2) the spore size (*L. crenulatulus* 6.4–6.6 × 7.1 µm in average and *L. pasohensis* 6.1–6.5 × 6.7–7.1 µm in average). However, the spore ornamentation is different with *L. crenulatulus* having a complete reticulum, and a thicker and denser ornamentation than *L. pasohensis*. The other two newly proposed species, *L. perparvus* and *L. glabrigracilis*, are in clade 8, together with *L. gracilis*, which is also a species with small basidiocarps. Features that differentiate between these three species are described in the taxonomy part below. Clade 10 contains two species, *L. laccarioides* and *L. atlanticus* Bon. Both species are remarkably strigose at the base of the stipe and lack macrocystidia, but the European taxon has more crowded gills and no strong striation at the pileus margin. *Lactarius laccarioides* has the typical aspect of a *Laccaria* species, with thin flesh in the pileus and distant gills (Wisitrassameewong *et al.* 2014). It has been described together with *L. sublaccarioides* (clade 14) because of subtle differences in macro- morphology. It differs from the latter species by the lack of true cystidia, and the very hispid and much longer stipe. The specimens in clade 12 are labeled *L.* cfr. *subzonarius* because they are morphologically similar to *L. subzonarius* Hongo (Le 2007). This species is characterized by the zonation of the pileus, the moist pileus surface, reddish brown cap and trichoderm pileipellis structure.

Taxonomy

Lactarius crenulatulus Wisitrassameewong & Verbeken sp. nov. (Figure 3.2, 3.6a and 3.6b)

Mycobank: MB808627

Diagnosis: this species differs from the other small species by the warm orange colour, the base of stipe being slightly strigose and white, microscopically by the dense and complete reticulum on the spores and the absence of true cystidia.

Etymology: 'crenulatulus' means small and with crenulate margin.

Typus: Thailand, Chiang Rai province, Muang district, Thasai sub-district, forest at Doi Pui Reverse Signal Station, Doi Pui, 740 m elev., N19°49.00 E99°52.03, KW383 (holotypus GENT!, isotypus MFLU!).

Pileus small, 2–15 mm diam., convex to broadly convex, with a papilla when young, later infundibuliform without or with inconspicuous papilla; margin striate up to halfway the pileus, inflexed to slightly reflexed, wavy, crenulate; surface dry, smooth, olive brown (4D6–D7) when young, generally greyish yellow (4B5) to blond (4C6), orange, with paler patches (pale yellow, 4A3) in some parts of pileus, often with light brown (5D7) spots, light brown (5D7) when old.$_4$ *Lamellae* adnate to decurrent, 0.5–3 mm broad, subdistant, with 3 series of lamellulae, pale yellow (3A3 to 4A3), greyish brown (6D3) when old; edge crenulate, concolourous. *Stipe* 10–19 × 1–2 mm, cylindrical, central; surface dry, smooth, mostly concolourous with pileus colour, except at the base which is white and slightly pruinose to strigose. *Context* in pileus very thin, 0.5 mm broad, hollow in stipe, pale orange (5A3 or 6A3), unchanging with 10%KOH, unchanging with FeSO; smell faint, reminding of European *L. quietus* (like bugs); taste mild. *Latex* watery to watery white, sparse, unchanging on exposure, unchanging on white paper, unchanging with 10%KOH; taste mild.

Basidiospores globose to subglobose to ellipsoid, 6.3–*7.1*–7.8(–7.9) × 5.8–*6.4–6.6*–7.1(–7.2) µm, Q = 1.02–*1.07– 1.11*–1.21 (n=60); ornamentation amyloid, forming a complete and dense reticulum with small meshes, consisting of thick and irregular ridges which are up to 1 µm high and obtuse; isolated warts rare; plage inamyloid to distinctly distally amyloid. *Basidia* 41–61 × 11–14 µm, rather slender, mostly subclavate, sometimes clavate, 4-spored,

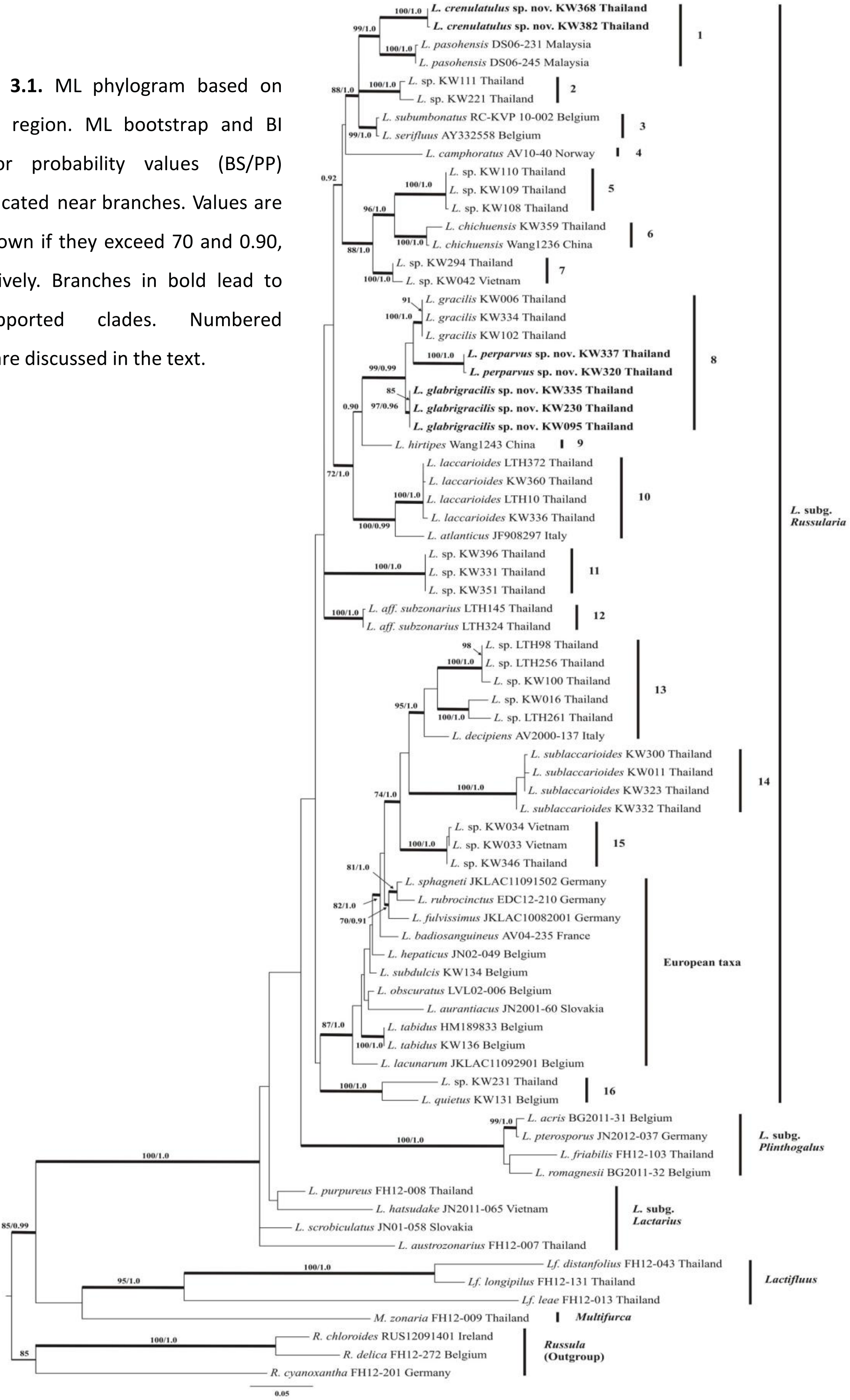

FIGURE 3.1. ML phylogram based on the ITS region. ML bootstrap and BI posterior probability values (BS/PP) are indicated near branches. Values are only shown if they exceed 70 and 0.90, respectively. Branches in bold lead to well-supported clades. Numbered clades are discussed in the text.

rarely 2−spored, mostly with guttate contents, with sterigmata 5–8 × 1–2 µm. *Pleuromacrocystidia* absent. *Pleuropseudocystidia* 5–10 µm diam., numerous, emergent, aseptate, cylindrical to irregular, with obtuse apex. *Lamellae edge* sterile; marginal cells abundant, irregularly cylindrical to subclavate; cheilocystidia absent. *Hymenophoral trama* with abundant lactifers intermixed with rosettes of sphaerocytes. *Pileipellis* an epithelium, with transition to a palisade, 30–80 µm thick; suprapellis consisting of globose elements; subpellis composed of recumbent cylindrical hyphae. *Stipitipellis* a cutis, 30–45 µm thick, composed of cylindrical hyphae which are septate, thin-walled, forming a distinct layer; subpellis composed of slightly thick-walled isodiametric cells.

Habitat: gregarious on soil between leaf litters in montane broad-leaved forest dominated by *Castanopsis armata* and *Quercus aliena*.

Studied material: Thailand, Chiang Mai province, Mae Taeng District, Pa Pae sub-district, Bahn Pha Deng Village, Pathummikaram Temple, 1050 m elev. N19°06.28 E98°44.47, forest trail, 25/6/2012, KW312 (GENT!, MFLU!), Thailand, Chiang Rai province, Muang district, Thasai sub-district, forest at Doi Pui Reverse Signal Station, Doi Pui, 740 m elev., N19°49.00 E99°52.03, 25/7/2012, KW368 (GENT!, MFLU!);−ibid., 25/7/2012, KW369 (GENT!, MFLU!);−ibid., 31/7/2012, KW382 (GENT!, MFLU!);−ibid., 31/7/2012, KW383 (holotype GENT!, isotype MFLU!);−ibid., 31/7/2012, KW387 (GENT!MFLU!), Thailand, Chiang Mai Prov., Mae Taeng Distr., Highway 1095 at 22 km marker, 750 m elev., N19°07.57' E98°45.65', forest dominated by *Castanopsis* sp., *Dipterocarpus* spp. and partly mixed with *Pinus kesiya*, 05/7/2004, LTH183 (GENT!, CMU!, SFSU!).

Comment: This species was mentioned in Le (2007) as *Lactarius* sp. 19. It is recognized in the field by the small fruitbody with deeply striate pileus, strikingly crenulate margin and mostly a whitish pruinose base. It is most similar to *L. perparvus*, growing in the same habitats, because of the small size, the strongly striate margin, and the general stature. The colour however is different because *L. perparvus* has less pure orange tinges and is dominantly greyish brown. One has to pay attention to the stipe base: the pruinose white layer is sometimes scarcely developed but usually present in *L. crenulatulus*, and absent in *L. perparvus*, where some strigose hairs may be present but they are never purely white. Microscopically, the completely reticulate spores and the lack of pleurocystidia and cheilocystidia make the species very different from *L. perparvus*.

Lactarius perparvus Wisitrassameewong & F. Hampe sp. nov. (Figure 3.3 and 3.6c).

MycoBank: MB808628

Diagnosis: *Lactarius perparvus* is characterized by this combination of characters: extraordinary small size, strong pileus striation, incomplete reticulate spores and a cutis as pileipellis structure. See comment above.

Etymology: 'perparvus' means very small, very little.

Typus: Thailand, Chiang Mai province, Muang district, Doi Suthep-Pui National Park, Sangasabhasri Lane to Huai Kok Ma village, 1145 m elev., N18°48.62 E098°54.60, 29/6/2012, KW320 (holotypus GENT!, isotypus MFLU!).

Pileus 5–7 mm diam., plane to applanate to slightly infundibuliform, with or without inconspicuous papilla in center; surface dry to greasy, smooth; margin deeply striate up to the center, irregularly crenulate to wavy, mostly slightly inflexed; greyish orange (5B4) to brownish orange (5C4) to light brown (5D5), greyish [4]brown, turning brown (6E6) when old. *Lamellae* decurrent, 1–2 mm broad, subdistant, with 2–3 series of lamellulae, yellowish white (2A2) to pale yellow (3A3); edge concolourous. *Stipe* 9–12 × 1–2 mm, cylindrical to slightly broader at base, slender, centrally attached; surface greasy, smooth; brownish orange (6C5–C6) to light brown (6D5–7), slightly strigose at the base with some concolourous hairs. *Context* thin in pileus, thinner than 0.5 mm; yellowish white (2A2), fragile, hollow in stipe, pale brownish orange (5D5) with 10%KOH, unchanging with $FeSO_4$; smell indistinct; taste mild. *Latex* sparse, watery, taste mild.

Basidiospores subglobose to ellipsoid, 7.7–*8.2*–8.7(–8.8) × 6.8–*7.3*–*7.4*–7.9 µm, Q = 1.04–*1.12*–*1.16*–1.24 (n=40); ornamentation amyloid, mostly forming an incomplete reticulum, not forming closed meshes; ridges irregular, up to 1 µm high, subacute; isolated warts numerous and sometimes grouped; plage mostly inamyloid or slightly distally amyloid, sometimes distinctly distally amyloid. *Basidia* 35–46 × 15–18 µm, subclavate, 4−spored,

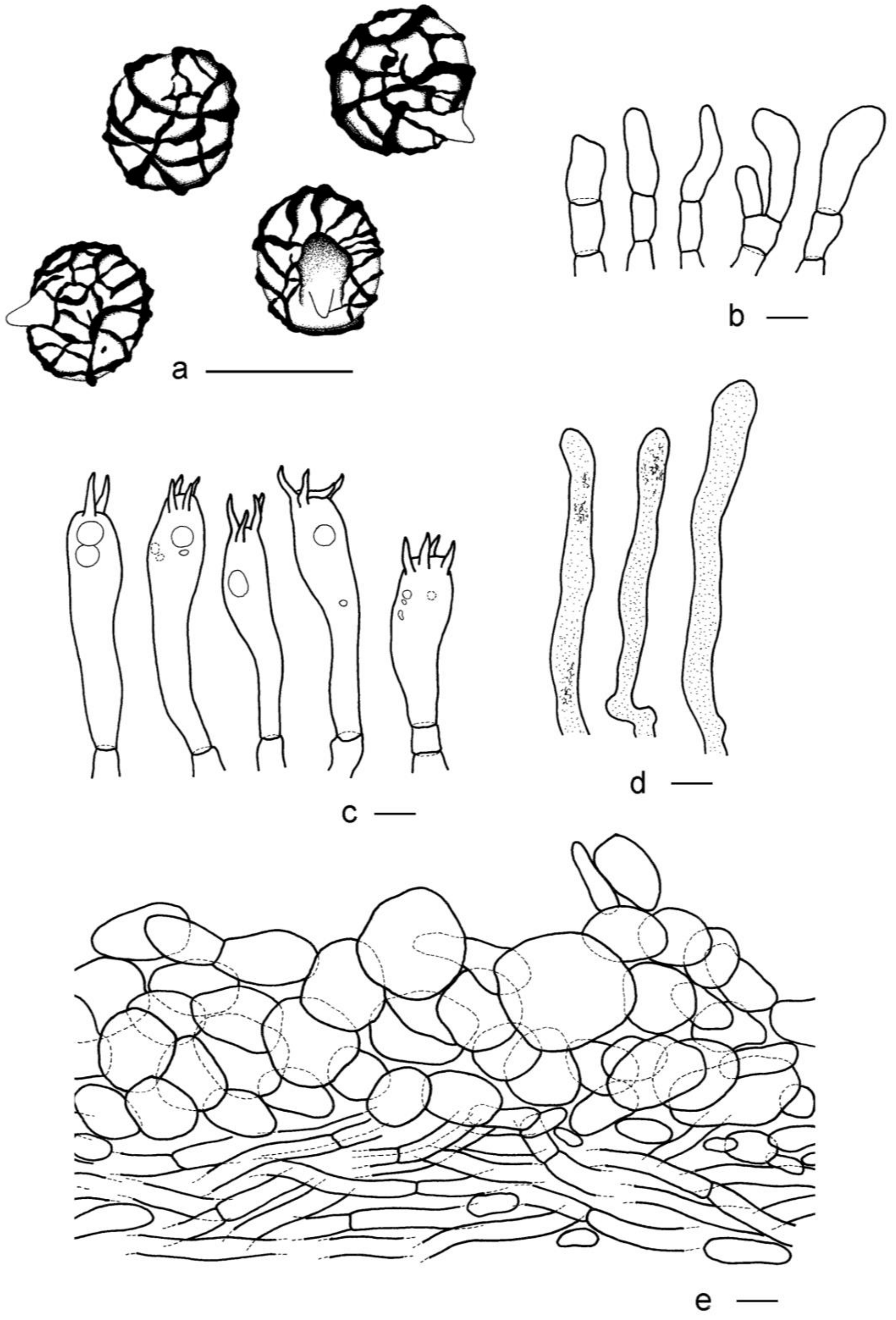

FIGURE 3.2. *Lactarius crenulatulus* (KW383): a. basidiospore, b. lamellae edge, c. basidia, d. pseudocystidia, e. pileipellis (scale bar = 10 µm).

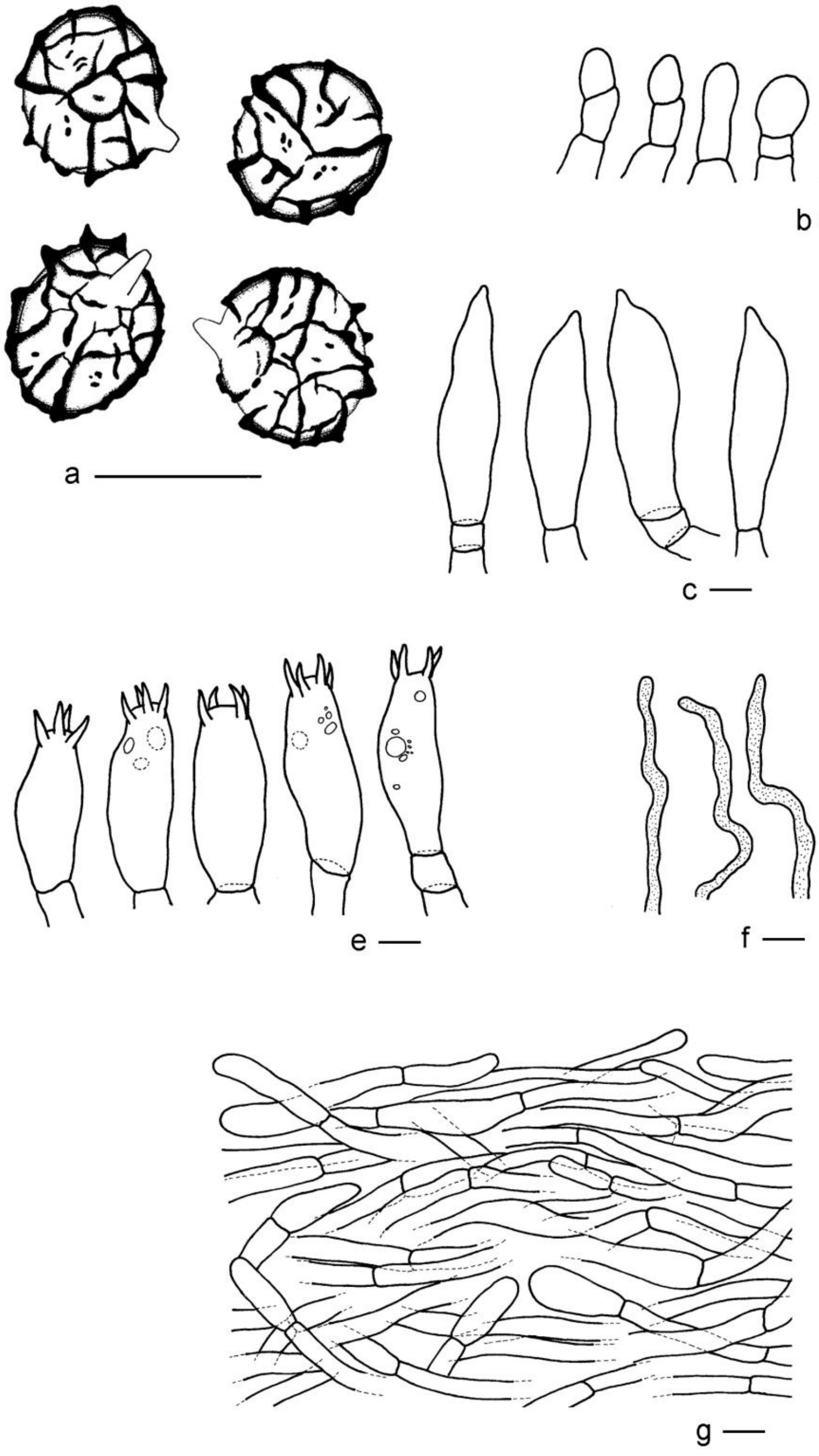

FIGURE 3.3. *Lactarius perparvus* (KW320): a. basidiospore, b. lamellae edge, c. pleuromacrocystidia, d. cheilocystidia e. basidia, f. pseudocystidia, g. pileipellis (scale bar = 10 µm).

rarely 2–spored, with guttulate contents, with sterigmata 6–9 × 1.5–3 µm. *Pleuromacrocystidia* infrequent to moderately abundant, 46–57 × 10–15 µm, not emergent, subclavate to subfusiform, with mucronate apex, thin-walled. *Pleuropseudocystidia* scarce, 2–3 µm diam., not emergent to slightly emergent, subcylindrical, tortuous. *Lamellae edge* sterile, with abundant marginal cells and rare cheilocystidia; marginal cells 9–23 × 8–13 µm, cylindrical to subclavate; cheilocystidia scarce, 24–42 × 6–10 µm, embedded, irregularly subfusiform. *Hymenophoral trama* with abundant lactifers intermixed with rosettes of sphaerocytes. *Pileipellis* a cutis, 30–70 µm thick, composed of parallel hyphae which are sometimes slightly inflated and septate. *Stipitipellis* a cutis, 70–90 µm thick, a compact layer of cylindrical, thin-walled hyphae.

Habitat: gregarious on soil between leaf litter, sometimes growing on a dried leaf, sometimes on steep slopes and bare soil, in montane broad-leaved forest dominated by *Castanopsis* spp. and *Lithocarpus* spp.

Studied material: Thailand, Chiang Mai province, Muang district, Doi Suthep-Pui National Park, Sangasabhasri Lane to Huai Kok Ma village, 1145 m elev., N18°48.62 E098°54.60, 29/6/2012, KW320 (holotype GENT!, isotype MFLU!);–ibid., 5/7/2012, KW337 (GENT!, MFLU!).

Comments: By extension of the whole genus *Lactarius*, *L. perparvus* is the smallest representative of *L.* subg. *Russularia* currently known in Thailand (note that in *Lactifluus*, the other genus of milkcaps, species with very small basidiocarps also occur). In the field this species could be confused with *L. crenulatulus* (see under this species). Besides the less reticulate spores, *L. perparvus* also has larger spores. Our phylogeny shows that the species is closely related to *L. gracilis* and *L. glabrigracilis*. Both species have a different stature: longer and very narrow stipe and a somewhat larger pileus with distinct and acute papilla and denser gills. *Lactarius gracilis* is also easily recognized by the tufts of hairs at the pileus margin.

Lactarius glabrigracilis Wisitrassameewong & Nuytinck (Figure 3.4 and 3.6d).

MycoBank: MB 808629

Diagnosis: this species is recognized by its small pileus with a very acute papilla and distinct striation, its very slender and long stipe and an epithelium as pileipellis structure.

Etymology: 'glabrigracilis' is reminiscent of *L. gracilis* but without hairs (on the pileus).

Typus: Thailand, Chiang Mai province, Muang district, Suthep sub-district, Doi Suthep-Pui National Park, Sangasabhasri Lane to Huai Kok Ma village, 1145 m elev., N18 48.62 E98 54.60, 2/7/2011, KW093 (holotypus GENT!, isotypus MFLU!).

Pileus 5–40 mm diam., at first broadly convex to applanate, infundibuliform with age, with a distinct papilla in the center; margin strongly striate, mostly inflexed, rarely reflexed, crenate to crenulate; surface moist to dry, translucent, slightly hygrophanous, brown (6E5) when young, light brown (7D7) to brown (7E7) to reddish brown (8D8 to 8E7) on papilla or in center, cinnamon, greyish orange (6B5) to brownish orange (6C5–C6) to reddish gold (6C7) to light brown (6D6–D7), paler towards margin, brownish orange (6C7) and darker in the grooves. *Lamellae* decurrent, subdistant, 0.5–1.5 mm broad, with 2–4 series of lamellulae, pale yellow (4A3–5A3), greyish orange (5A4–B4) to brownish yellow (5C5) when bruised. *Stipe* 10–50 × 0.5–3 mm, cylindrical, sometimes slightly broader at the base, slender; surface, smooth, brown (6D7–D8), often paler at top, orange yellow (4A3–A4); strigose but not whitish at base. *Context* in pileus very thin, 0.5–1 mm broad, hollow in stipe, yellowish white (4A2), greyish green (26D5) with 10%KOH, light brown (6D5) with $FeSO_4$; smell like the European *L. quietus;* taste mild. *Latex* often sparse, watery white, unchanging on exposure, unchanging on tissue paper, handkerchief and with 10%KOH; taste mild.

Basidiospores globose to subglobose to ellipsoid; 6.4–*7.0–7.5*–8.2 × 5.8–*6.3–6.6*–7.3; Q = 1.03–*1.11–1.13*–1.25 (n=80); ornamentation amyloid, composed of irregular ridges up to 1 µm high, often interconnected by thin lines, forming an incomplete reticulum, occasionally subreticulate; elongated to rounded isolated warts numerous, sometimes grouped; plage mostly slightly distally amyloid. *Basidia* 43–55 × 12–16 µm, mostly subclavate, rarely clavate, somewhat slender, generally 4−spored, sometimes 1−or 2−spored, mostly with granular or guttate contents; sterigmata 7–11 × 2–3 µm. *Pleuromacrocystidia* fairly abundant, 35–53 × 9–15 µm, not emergent, variable from narrowly fusiform to subclavate; apex short to long mucronate. *Pleuropseudocystidia* abundant, 3–5 µm diam., aseptate, slightly emergent to emergent, rather straight to tortuous, sometimes branching at apex. *Lamellae edge* sterile, composed of marginal cells and cheilocystidia; marginal cells abundant, 15–29 × 8–12 µm, mostly cylindrical, sometimes slightly broader at apex; cheilocystidia present but not emergent, 30–40 × 8–12 µm, narrowly irregularly fusiform; apex (sub)mucronate.

Hymenophoral trama consisting of sphaerocytes and lactifers. *Pileipellis* an epithelium, 30–60 µm thick; suprapellis consisting of round cells; subpellis consisting of cylindrical to inflated hyphae. *Stipitipellis* a cutis; composed of cylindrical to inflated hyphae which are mainly parallel, 3–20 µm diam., septate, thin-walled.

Habitat: gregarious on rotten wood or on soil under leaf litter in montane broad-leaved forest dominated by *Castanopsis* spp. and *Lithocarpus* spp.

Studied material: Thailand, Chiang Mai province, Muang district, Suthep sub-district, Doi Suthep-Pui National Park, Sangasabhasri Lane to Huai Kok Ma village, 1145 m elev., N18°48.62 E98°54.60, 2/7/2011, KW093 (holotype GENT!, isotype MFLU!);–ibid., 2/7/2011, KW095 (GENT!, MFLU!);–ibid., 2/7/2011, KW097 (GENT!, MFLU!);–ibid., 2/7/2011, KW101 (GENT!, MFLU!);–ibid., 29/6/2012, KW321 (GENT!, MFLU!);–ibid., 5/7/2012, KW335 (GENT!, MFLU!);–ibid., 16/7/2012, KW358 (GENT!, MFLU!), Thailand, Chiang Mai province, Mae-On district, Pa Mieang sub-district Thep Saded Waterfall, Mae Takraii National Park, 1150 m elev., N18°55.32 E99°21.31, 1/6/2012, KW230 (GENT!, MFLU!), Thailand, Chiang Mai Prov., Jomthong district, Doi Inthanon National Park, junction of Highway 1009 and road to Mae Chem district, 1700 m elev., N19°31.58′ E 98°29.64′, 25/6/2004, LTH141 (GENT!, CMU!, SFSU!);–ibid., 25/6/2004, LTH142 (GENT!, CMU!, SFSU!);–ibid., 27/6/2005, LTH327 (GENT!, CMU!, SFSU!).

Comment: This species was mentioned in Le (2007) as *Lactarius* cfr. *obscuratus*. Among the small species, it is one of the most commonly observed species in Thailand, together with *L. gracilis*. *Lactarius glabrigracilis* has the same stature and colours as *L. gracilis*, but differs by the lack of hairs on the margin of the pileus, a striking character for *L. gracilis*. Microscopically, *L. gracilis* differs from this new species in possessing a cutis as pileipellis, and emergent and larger pleuromacrocystidia.

Lactarius gracilis Hongo, J.Jap.Bot. 32:144. 1957. (Figure 3.5 and 3.6e).

Pileus 9–27 mm diam., broadly convex to convex when young, turning to plano-convex or applanate with umbonate or papillate at center; surface smooth, dry to greasy, in some specimens with slightly minutely cracked center; margin inflexed margin, bearing floccose tufts of hairs in young basidiocarps which become scarcer with age, dark yellowish brown to orange, pale orange or greyish orange (5C4) towards the margin, typically darker colour on

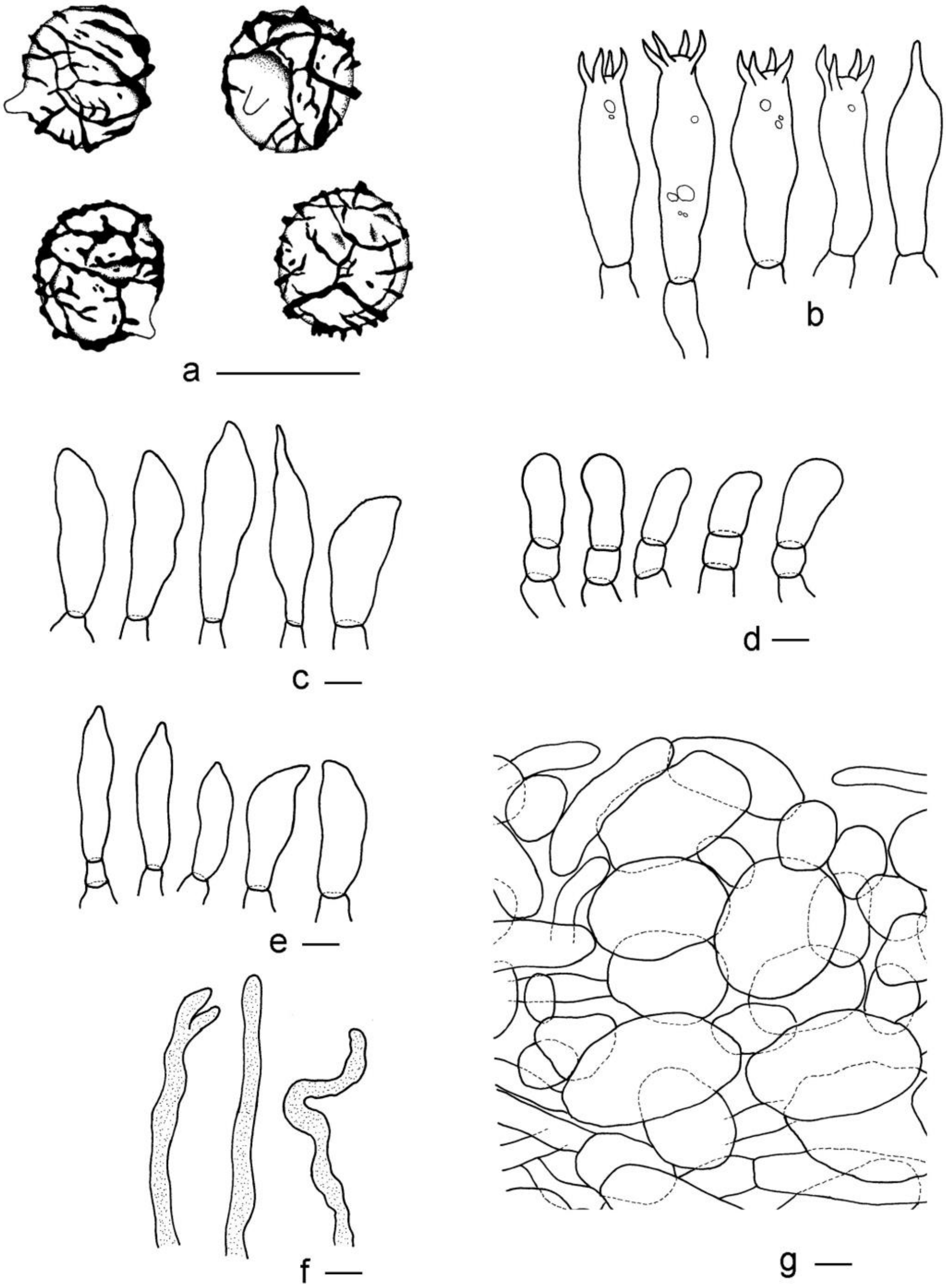

FIGURE 3.4. *Lactarius glabrigracilis* (KW093): a. basidiospore, b. basidia, c. pleuromacrocystidia, d. lamellae edge, e. cheilocystidia, f. pseudocystidia, g. pileipellis (scale bar = 10 µm).

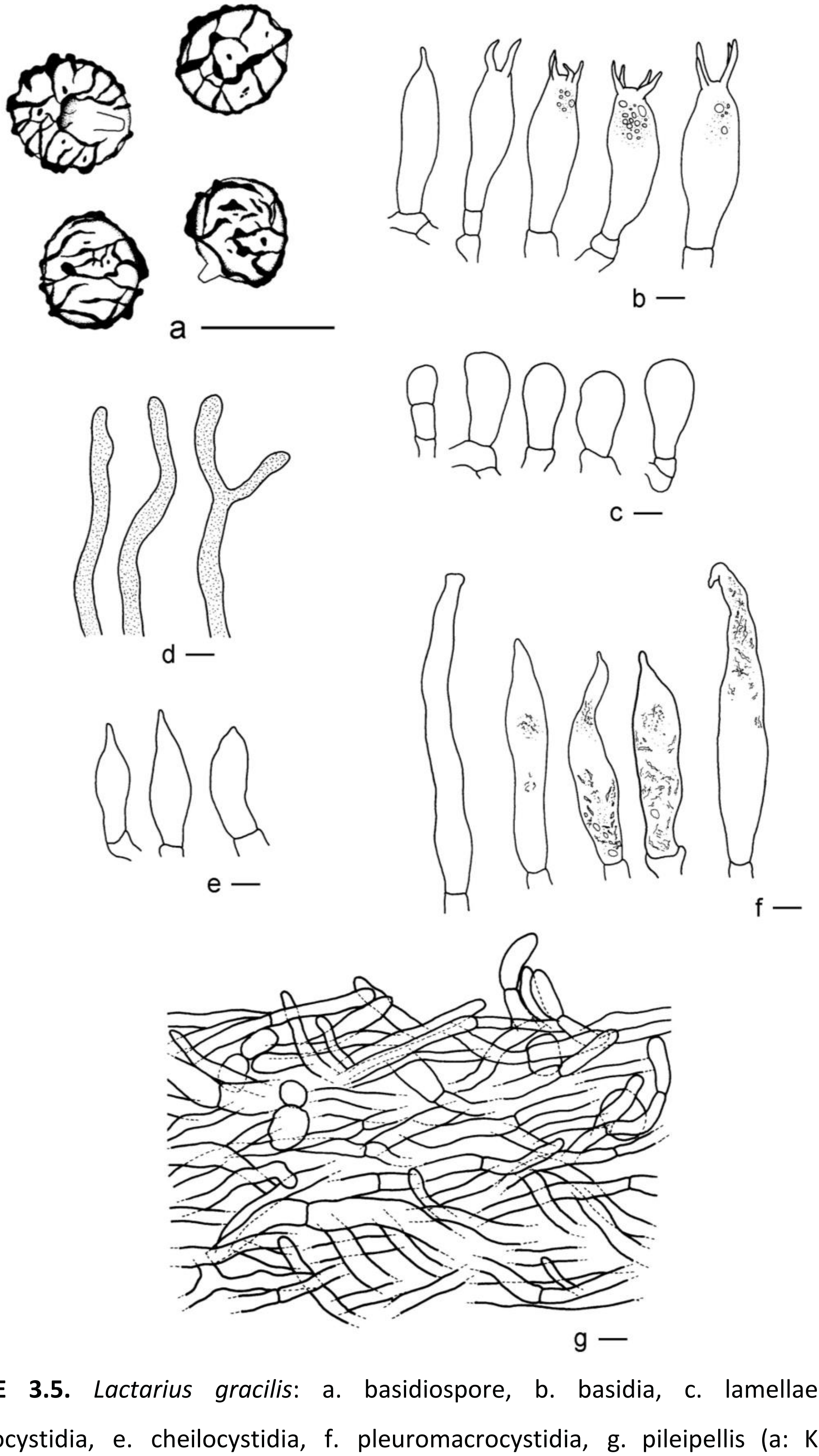

FIGURE 3.5. *Lactarius gracilis*: a. basidiospore, b. basidia, c. lamellae edge, d. pseudocystidia, e. cheilocystidia, f. pleuromacrocystidia, g. pileipellis (a: KW096, b-f: KW330, g: KW334) (scale bar = 10 µm).

center, fading with age or in dry condition; hairs at the margin of pileus orange white (5A2) to greyish yellow to greyish orange. *Lamellae* decurrent, close to crowded, with 2–3 series of lamellulae, 1–2 mm broad, orange white (5A2) in young basidiocarps and turning brownish orange (6C5–C6) with age; edge concolourous. *Stipe* 23–57 × 1–4 mm, mostly slightly tapering upwards, slender, hollow pith, orange, often a paler orange at apex, with orange white or pale orange strigose hairs at base. *Context* thin in pileus, 1 mm broad, greyish orange (5B3), unchanging with 10% KOH, greyish brown (6D5) with $FeSO_4$; smell like the European *L. quietus*; taste mild. *Latex* milky or watery white, unchanging on exposure, unchanging on white paper, unchanging with 10%KOH; taste mild.

Basidiospore subglobose to ellipsoid; 7.0–*8.1–8.4*–9.4 × 5.9–*7.0–7.3*–8.3 µm, Q = 1.07–*1.15–1.16*–1.27 (n = 60); ornamentation amyloid up to 1.0 µm high, comprising of irregularly elongated ridges or warts interconnected by fine lines, forming an incomplete reticulum to subreticulum; isolated warts numerous; plage inamyloid to slightly distally amyloid. *Basidia* 48–57 × 14–19 µm, subclavate, mostly 4-spored, 1-or 2-spored present, consisting of granular or guttate contents; sterigmata 6–16 × 1.5–3 µm. *Pleuromacrocystidia* numerous, 69–107 × 10–20 µm, emergent, generally subfusiform to subclavate with mucronate or tortuous moniliform apex, occasionally subcylindrical with obtuse apex; with needle-liked or granular contents. *Pleuropseudocystidia* fairly abundant, 5–7 µm diam., aseptate, not emergent or slightly emergent, cylindrical with rounded apex, sometimes branched near apex. *Lamellae edge* sterile, consisting of marginal cells and cheilocystidia; marginal cells abundant, 12–39 × 10–16 µm, slightly emergent, mostly subclavate, with rounded apex; cheilocystidia scarce, 37–45 × 11–13 µm, not emergent, subfusiform with mucronate apex. *Hymenophoral trama* consisting of abundant rosettes of sphaerocytes and lactifers. *Pileipellis* a cutis, 100–130 µm thick, consisting of a dense layer of repent e, with a few erect hyphae and rare globose cells. *Stipitipellis* a cutis, 40–60 µm thick, consisting of parallel hyphae, underlying layer composed of globose cells and hyphae, 4–7 µm diam., septate, thin-walled.

Habitat: gregarious on soil between leaf litter, in montane broad-leaved forest dominated by the member of Fagaceae.

Studied material: Thailand, Chiang Mai province, Muang district, Suthep sub-district, Doi Suthep-Pui National Park, Sangasabhasri Lane to Huai Kok Ma village, 1145 m elev., N18°48.62 E98°54.60, 27/4/2011, KW006 (MFLU!, GENT!);–ibid., 26/5/2011, KW020 (MFLU!, GENT!);–ibid., 2/7/2011, KW094 (MFLU!, GENT!);–ibid., 2/7/2011, KW096 (MFLU!, GENT!);–ibid., 2/7/2011, KW102 (MFLU!, GENT!);–ibid., 5/7/2012, KW334 (MFLU!, GENT!), Thailand, Chiang Rai province, Mae Fah Luang district, Mae Salong Nok sub-district, Doi Mae Salong, 1269 m elev., N20°16.90 E99°62.30, 30/5/2012, KW222 (MFLU!, GENT!);–ibid., 13/7/2012, KW350 (MFLU!, GENT!), Thailand, Chiang Mai province, Mae-On district, Huaikaew sub-district, Ban Mae Kampong, 1450 m elev., N18°51.43 E99°22.09, 3/6/2012, KW250 (MFLU!, GENT!);–ibid., 15/7/2012, KW354 (MFLU!, GENT!), Thailand, Chiang Mai province, Mae-On district, Mae Takraii national park, Thep Saded waterfall, 1150 m elev., N18°55.32 E99°21.31, 1150 m elev., 5/7/2012, KW340 (MFLU!, GENT!), Thailand, Lampang province, Muangparn district, Chaesorn sub-district, along highway No. 1252, 1420 m elev., N18°55.50 E99°23.30, 14/6/2013, KW422 (MFLU!, GENT!).

Comment: *Lactarius gracilis* has been described from Japan and China. The type collection was reported under *Shiia* from Japan (Hongo 1957). We generally found the species in rainforest dominated with the members of Fagaceae e.g. *Castanopsis*, *Quercus* and *Lithocarpus*. Wang (2007) suggested that the pellis structure consists of a layer of rounded cells. In this study we found a cutis as pileipellis structure which is concordant to the study of Le (2007).

Discussion

Lactarius subg. *Russularia* has some species with remarkably small basidiocarps, which due to the rather limited colour range in this subgenus (dominantly orange, reddish, brown) all look rather similar and are difficult to distinguish in the field. Several small species, such as *L. obscuratus* (5–35 mm pileus diam.), *L. cyathuliformis* (12–45 mm pileus diam.), *L. omphaliformis*. (8–22 mm pileus diam.) and *L. rostratus* (8–35 mm) have been documented in Europe. These species are not conspecific with tropical taxa. One of the most commonly collected species in Asia is *L. gracilis* (Hongo 1957a). In Thailand I found a rather common look-alike differing by the lack of the characteristic tufts of hairs at the pileus margin, described here as *L. glabrigracilis*. Two other new species with small basidiocarps are *L. crenulatulus* and *L. perparvus*. Our molecular analyses show that three of the Thai dwarf species (*L. gracilis*, *L. perparvus* and *L. glabrigracilis*) are related and form a separate clade. The fourth one, *L. crenulatulus*, has a distant position and is closely related to *L. pasohensis* from Malaysia, a species with larger dimensions. While most representatives of the subgenus have pleuromacrocystidia, they are absent in clade 1, the clade with *L. crenulatulus* and *L. pasohensis*. Apparently the presence of macrocystidia is a plesiomorphic state in the group that was lost more than once during the evolution in this subgenus, because I also observed the absence of macrocystidia in *L. laccarioides*, *L. pasohensis L. perconicus*, *L. condimentus* Verbeken & Horak and *L. corrugatus* Verbeken & Horak.

Since *L. perparvus* and *L. glabrigracilis* are phylogenetically related with *L. gracilis*, we here find concordance between morphology and phylogenetic data among these taxa. They share their small, slender habit and the presence of macrocystidia. In point of view of spore ornamentation, there is a difference in the degree of reticulum forming. *Lactarius glabrigracilis* has an incomplete reticulum which is similar to the ornamentation in *L.gracilis.* The clear difference between *L. glabrigracilis* and *L. gracilis* is in the pileipellis structure. The pileipellis of *L. glabrigracilis* is an epithelium with cylindrical to inflated hyphae in the underlying layer, while *L. gracilis* has a cutis. The spore ornamentation of *L. perparvus* is composed of rather abundant, isolated warts and main ridges are typically disconnected. A small and slender habit arose independently several times within *L.* subg. *Russularia*. *Lactarius crenulatulus*, with reticulate spores is recognized by its brighter colours and differs from representatives in clade 8 by lacking true cystidia. The spore ornamentation is dense and forms a complete reticulum. Pellis structure also plays an important role in this subgenus for species delimitation. In comparison with the other two subgenera, *L.* subg. *Russularia* shows a very large variation in microscopical pileipellis structures (presence or absence cellular elements, erected hyphae or repent hyphae) reflected in the macroscopical aspect ranging from smooth and non-velvety to pruinose or even cracked. Some species show uncommon pileipellis structure in the subgenus. *Lactarius liliputianus* and *L. inamyloideus* have a palisade (trichoderm) consisting of cylindric to subclavate erected terminal elements. Cutis structure is presented in *L. perparvus* and an epithelium is observed in the collections of *L. crenulatulus* and *L. glabrigracilis*. So far, there are some species with small basidiocarps documented from both temperate and tropical regions. These three newly described small species undoubtedly differ from extant European representatives of the subgenus. Four Papuan species have been described with very small habit: *L. liliputianus* (10 mm pileus diam.), *L. inamyloideus* (14 mm pileus diam.), *L. perconicus* (8 mm pileus diam.) and *L. pennulatus* (20 mm pileus diam.). Although we lack of the molecular evidence of the Papuan species in the present study, none of them are conspecific to these newly proposed species. *Lactarius liliputianus* and *L. inamyloideus* are unique in their spore ornamentation and pileipellis structure, while *L. pennulatus* has reddish brown fruiting bodies and watery latex.

It is obvious that several species remain to be described in this study (in clade 2, 5, 7, 11, 13, 15 and 16). Full illustrations, descriptions and discussion of these undescribed species will be provided in a later series of publications (in preparation).

FIGURE 3.6: Basidiocarps; a-b: *L. crenulatulus* (Photo a: Todd Elliott), c: *L. perparvus* (Photo: Todd Elliott), d: *L. glabrigracilis* (Photo: Felix Hampe), e: *L. gracilis* (Photo: Felix Hampe).

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CHAPTER 4

NEW DIVERSITY FROM THAILAND AND VIETNAM

Abstract

Lactarius subgenus *Russularia* is a dominant group of milkcaps in Southeast Asia. This paper reveals the large diversity of the subgenus, with eight new species and one known species being described from montane evergreen and coniferous forests. All new species are supported by both morphological and molecular data, the latter using Maximum likelihood and Bayesian analysis based on ITS sequence data. Complete macro- and micro-morphological descriptions and illustrations are given. A key to all described taxa is provided. *Lactarius chichuensis* is reported for the first time from Thailand.

Keywords: Russulales, indentification, ectomycorrhizal fungi, biodiversity.

Modified from: Wisitrassameewong, K., Nuytinck, J., Le, H.T., de Crop, E., Hampe, F., Hyde, K.D. & Verbeken, A. (2015). *Lactarius* subgenus *Russularia* (Russulaceae) in South-East Asia: 3. new diversity in Thailand and Vietnam. *Phytotaxa* 207(3): 215−241.

Introduction

Tropical Southeast Asia is recognized as one of the world's biodiversity hotspots and contains a high concentration of endemic species (Myers et al. 2000). The high number of endemic plants includes several families of ectomycorrhizal trees (e.g. Dipterocarpaceae, Fagaceae, Betulaceae, and Pinaceae). However, biodiversity is strongly declining in Southeast Asia due to deforestation for urbanization, logging, and agricultural expansion by local people and agricultural companies, who clear vast areas for crop cultivation (Sodhi et al. 2010). Since ectomycorrhizal (ECM) fungi are obligate symbionts of ECM trees and shrubs, an inevitable consequence of forest logging is the loss of ECM species, including those belonging to the genus *Lactarius* Pers. Issues regarding forest logging have therefore become of great concern in many countries (Mortimer et al. 2012).

Mycorrhizal trees have been used for reforestation programs in many regions such as tropical Africa, South America and Southeast Asia. Thus many attempts used mycorrhizal symbionts to improve the reforestation performance of transplanted mycorrhizal trees (Bâ et al. 2009, Ergiles et al. 2009, Sanon et al. 2010, Aggangan et al. 2012). Since ECM fungi facilitate water and nutrient uptake for their host plants, they are considered as microorganisms that can promote plant growth in forests. There is little information regarding the use of *Lactarius* species in tree seedling production. *Lactarius deliciosus* (L.: Fr.) Gray appears to be the most used *Lactarius* species applied to improve seedling establishment of *Pinus* trees (Guerin-Laguette et al. 2003, Parladé et al. 2004, Diaz et al. 2009). Our research aims at exploring the biodiversity of *Lactarius* subgenus *Russularia* (Fr.) Kauffman in Southeast Asia, as in local ecosystems it is one of the dominant groups in terms of numbers of basidiocarps covering the forest floor (Le 2007).

Lactarius subgenus *Russularia* is one of the three major subgenera of *Lactarius*. Species traditionally placed in this subgenus are recognized by basidiomata which are typically dry and vary in colour from orange to warm brown (Heilmann-Clausen et al. 1998). As mentioned in Wisitrassameewong et al. (2014b), it is remarkable that most taxa in this group have unchanging latex and that the group as a whole is characterized by few colour changes in the latex as compared to the other subgenera of *Lactarius* and *Lactifluus* (which together form the large group of milkcaps). Some species are described as having white to pale yellow latex e.g. *L. quietus* (Fr.: Fr.) Fr., and *L. decipiens* Quél. *Lactarius tabidus* Fr. has white latex which slowly turns straw yellow, while *L. duplicatus* A.H. Sm. has white latex that becomes sulphur yellow (Heilmann-Clausen et al. 1998). In the Northern hemisphere, this group is well-studied in Europe (Heilmann-Clausen et al. 1998, Basso 1999), North America (Hesler & Smith 1979) and Japan (Hongo 1957a, b, 1971).

Many mycological excursions have focused on milkcap diversity (both *Lactarius* and *Lactifluus*) in Southeast Asia in the last decade (Le et al. 2007a, 2007b, 2007c, Stubbe et al., 2007, 2008, Van de Putte et al. 2010, Verbeken et al. 2014, Wisitrassameewong et al. 2014a, 2014b). Compared to the exploration rate between *L.* subg. *Russularia* and other groups of milkcaps, the exploration rate in *Russularia*-group is quite low. So far about 13 described species belonging to *L.* subg. *Russularia* have been reported. However, many species are only morphological supported but still lack of molecular data, e.g. three species from Indonesia and *L. subzonarius*. We need a comprehensive contribution to show the diversity of *L.* subg. *Russularia* for this subcontinent. This paper contributes to the knowledge of the biodiversity of the subgenus in Thailand and Vietnam. Only one significant contribution to milkcap diversity in Vietnam is given by Morozova et al. (2013) but that was on two new species of *Lactifluus*, therefore this will be the first report of *Lactarius* subgenus *Russularia* for the country. We collected specimens mainly from montane evergreen forests dominated by Fagaceae and coniferous forests dominated by *Pinus kesiya* Royle ex Gordon from Northern Thailand and Southern Vietnam. The aim was to use both morphological and molecular tools to reveal the diversity of this ectomycorrhizal group in order to stress their importance when it comes to conservation of the whole ecosystem.

Materials and Methods

Taxon sampling

Fresh basidiomata were gathered in montane rainforests and planted coniferous forests in Northern Thailand and the regions around Dalat in Vietnam by the first author. Some collections were made by Huyen Thanh Le and Eske De Crop. The studied materials are deposited in the herbarium Universitatis Gandavensis (GENT), Belgium; Mae Fah Luang University herbarium (MFLU) and Chiang Mai University (CMU), Thailand; and/or San Francisco State University (SFSU), USA.

Morphological study

Macromorphological characters were observed on fresh material. Specimens were described and photographed in fresh condition during daylight hours. Colour coding is according to Kornerup and Wanscher (1978). Macro-morphological features of the basidiocarp were documented in term of size, shape and features of pileus, lamellae and stipe. Latex features were tested by recording colour when the latex was immediately exposed to the air, colour change after exposing to the air for a certain time, colour change when a drop of latex tested with 10% KOH and colour change when a drop of latex touched on white tissue paper and a white cotton handkerchief. For morphological terminology, we refer to Vellinga (1988) and to Verbeken (1996) and Heilmann-Clausen et al. (1998) particularly for pileipellis structures. Microscopic features were studied from dried material, mainly in Congo Red in L4 (Clémençon 1973). Basidiospores were observed in Melzer's reagent. Basidia were measured excluding sterigmata length. The spore measurement included at least 20 spores from each collection, and excludes the ornamentation. Basidiospore 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 collection, AVb = highest mean value for the measured collection and SD = standard deviation. Q corresponds to spore "length/width ratio" and is given as (MINQa) Qa–Qb (MAXQb), where Qa and Qb are the lowest and the highest mean ratio for a measured specimen, respectively. All line drawings were made by Komsit Wisitrassameewong (KW).

DNA extraction, PCR amplification and DNA sequencing

Genomic DNA was extracted from fresh material stored in 2×CTAB buffer using the protocol described by Nuytinck & Verbeken (2003) with the modifications described in Van de Putte et al. (2010). The internal transcribed spacer of the nuclear ribosomal DNA (ITS) was amplified and sequenced using the ITS1-F and ITS4 primers (White et al. 1990, Gardes & Bruns 1993). Sequencing was conducted with an ABI 3730XL or ABI 3700 by MACROGEN (Amsterdam, the Netherlands). Obtained sequences were assembled and edited with the software Sequencer$^{\text{TM}}$ v5.0 (Gene Code corporation, Ann Arbor, Michigan, U.S.A.).

TABLE 4.1. List of specimens and GenBank accession number of DNA sequences used in this study. Newly described species in this study are in boldface.

Species	Voucher collection	Origin	ITS accession no.
Lactarius subg. _Russularia_			
L. rubrobrunneus sp. nov.	AV12-044 (GENT, MFLU) (Type)	Thailand	KF432985
L. rubrobrunneus sp. nov.	LTH334 (GENT, CMU, SFSU)	Thailand	KR025598
L. rubrobrunneus sp. nov.	LTH149 (GENT, CMU, SFSU)	Thailand	KR025599
L. fuscomaculatus sp. nov.	KW111 (GENT, MFLU)	Thailand	KF433021
L. fuscomaculatus sp. nov.	KW112 (GENT, MFLU)	Thailand	KR025602
L. fuscomaculatus sp. nov.	KW128 (GENT, MFLU)	Thailand	KR025601
L. fuscomaculatus sp. nov.	KW370 (GENT, MFLU)	Thailand	KR025600
L. fuscomaculatus sp. nov.	KW373 (GENT, MFLU) (Type)	Thailand	KR025603
L. austrorostratus sp. nov.	KW108 (GENT, MFLU) (Type)	Thailand	KF433012
L. austrorostratus sp. nov.	KW109 (GENT, MFLU)	Thailand	KF433013
L. austrorostratus sp. nov.	KW110 (GENT, MFLU)	Thailand	KF433014
L. rubrocorrugatus sp. nov.	KW453 (GENT, MFLU)	Thailand	KR025592
L. rubrocorrugatus sp. nov.	KW042 (GENT, MFLU)	Vietnam	KF433010
L. rubrocorrugatus sp. nov.	KW043 (GENT, MFLU)	Vietnam	KR025589
L. rubrocorrugatus sp. nov.	KW045 (GENT, MFLU)	Vietnam	KR025591
L. rubrocorrugatus sp. nov.	KW123 (GENT, MFLU)	Thailand	KR025588
L. rubrocorrugatus sp. nov.	KW294 (GENT, MFLU)	Thailand	KF433011
L. rubrocorrugatus sp. nov.	KW384 (GENT, MFLU) (Type)	Thailand	KR025590
L. rubrocorrugatus sp. nov.	EDC14-505 (GENT, MFLU)	Thailand	KR025587
L. aquosus sp. nov.	LTH262 (GENT, CMU, SFSU)	Thailand	KR025622
L. aquosus sp. nov.	KW231 (GENT, MFLU) (Type)	Thailand	KF432984
L. tangerinus sp. nov.	KW091 (GENT, MFLU)	Thailand	KR025626
L. tangerinus sp. nov.	LTH203 (GENT, CMU, SFSU) (Type)	Thailand	KR025627
L. tangerinus sp. nov.	EDC14-475 (GENT)	Thailand	KR025625
L. inconspicuus sp. nov.	KW339 (GENT, MFLU) (Type)	Thailand	KR025584
L. inconspicuus sp. nov.	KW100 (GENT, MFLU)	Thailand	KF433001
L. inconspicuus sp. nov.	KW003 (GENT, MFLU)	Thailand	KR025583
L. inconspicuus sp. nov.	LTH256 (GENT, CMU, SFSU)	Thailand	KF433003
L. inconspicuus sp. nov.	LTH098 (GENT, CMU, SFSU)	Thailand	KF433004
L. kesiyae sp. nov.	KW034 (GENT, MFLU)	Vietnam	KF432995
L. kesiyae sp. nov.	AV12-022 (GENT, MFLU)	Thailand	KR025618
L. kesiyae sp. nov.	KW427 (GENT, MFLU)	Thailand	KR025614
L. kesiyae sp. nov.	KW346 (GENT, MFLU)	Thailand	KF432993
L. kesiyae sp. nov.	KW033 (GENT, MFLU)	Vietnam	KF432994
L. kesiyae sp. nov.	KW219 (GENT, MFLU)	Thailand	KR025616
L. kesiyae sp. nov.	KW210 (GENT, MFLU)	Thailand	KR025617
L. kesiyae sp. nov.	KW036 (GENT, MFLU)	Vietnam	KR025620
L. kesiyae sp. nov.	KW224 (GENT, MFLU)	Thailand	KR025615
L. kesiyae sp. nov.	KW032 (GENT, MFLU) (Type)	Vietnam	KR025619
L. sublaccarioides	KW300 (GENT, MFLU) (Type)	Thailand	KF432996
L. sublaccarioides	KW323 (GENT, MFLU)	Thailand	KF432997
L. sublaccarioides	KW332 (GENT, MFLU)	Thailand	KF432998
L. camphoratus	AV10-40 (GENT)	Norway	KF432971
L. camphoratus	JV2006-20 (GENT)	Belgium	KR025610
L. subumbonatus	JKLAC110902 (GENT)	Germany	KR025596
L. subumbonatus	RC-KVP10-002 (GENT)	Belgium	KF432981
L. subumbonatus	EDC11-237 (GENT)	Belgium	KR025595
L. serifluus	JV2006-028 (GENT)	Belgium	KR025597

TABLE 4.1. Continued.

Species	Voucher collection	Origin	ITS accession no.
L. crenulatulus	KW125 (GENT, MFLU)	Thailand	KR025605
L. crenulatulus	KW368 (GENT, MFLU)	Thailand	KJ458979
L. crenulatulus	KW383 (GENT, MFLU) (Type)	Thailand	KR025604
L. pasohensis	KW355 (GENT, MFLU)	Thailand	KF432988
L. pasohensis	DS06-231 (GENT, KEP)	Malaysia	KF432987
L. pasohensis	DS06-245 (GENT, KEP) (Type)	Malaysia	KF432986
L. perparvus	KW320 (GENT, MFLU) (Type)	Thailand	KJ458981
L. perparvus	KW337 (GENT, MFLU)	Thailand	KJ458982
L. glabrigracilis	KW093 (GENT, MFLU) (Type)	Thailand	KR025606
L. glabrigracilis	KW335 (GENT, MFLU)	Thailand	KJ458985
L. glabrigraclis	KW321 (GENT, MFLU)	Thailand	KR025607
L. rubidus	M.Kuo 01131106 (NY)	USA	KC691205
L. laccarioides	KW336 (GENT, MFLU) (Type)	Thailand	KF432991
L. laccarioides	KW360 (GENT, MFLU)	Thailand	KF432992
L. atlanticus	LAC11121201 (GENT)	Spain	KF432976
L. atlanticus	JKLAC13122801 (GENT)	Portugal	KR025611
L. atlanticus	AV13-047 (GENT)	Italy	KR025612
L. subserifluus	JMP0046	USA	EU819486
L. strigosipes		Mexico	JN003629
L. gracilis	KW096 (GENT, MFLU)	Thailand	KR025609
L. gracilis	KW354 (GENT, MFLU)	Thailand	KR025608
L. gracilis	KW334 (GENT, MFLU)	Thailand	KF433017
L. hirtipes	XHW1243 (HKAS)	China	KF433007
L. chichuensis	XHW1236 (HKAS)	China	KF475766
L. chichuensis	KW271 (GENT, MFLU)	Thailand	KR025593
L. chichuensis	KW012 (GENT, MFLU)	Thailand	KF433008
L. chichuensis	KW359 (GENT, MFLU)	Thailand	KF433009
L. chichuensis	KW421 (GENT, MFLU)	Thailand	KR025594
L. rostratus	691(MUVE)	Italy	JF908276
L. falcatus	KVP08-038 (GENT) (Type)	Thailand	KF133262
L. quietus	JN2012-040 (GENT)	Germany	KR025623
L. quietus	KW131 (GENT)	Belgium	KF432972
L. quietus	KW133 (GENT)	Belgium	KR025624
L. omphaliformis	PAM08083009	France	HQ714719
L. lacunarum	JKLAC11092901 (GENT)	Germany	KF432982
L. lacunarum	EDC11-231 (GENT)	Belgium	KR025570
L. lacunarum	JKLAC13122201 (GENT)	Portugal	KR025569
L. tabidus	KW130 (GENT)	Belgium	KR025582
L. tabidus	IMN98142	France	KR025581
L. aurantiacus	JN11-089 (GENT)	Greece	KR025580
L. aurantiacus	JN2001-60 (GENT)	Slovakia	KF432974
L. lanceolatus	IA-F20	Norway	UDB002454
L. brunneohepaticus	PAM08090315	France	HQ714726
L. obscuratus	ED2008-15 (GENT)	USA	KR025579
L. obscuratus	LVL02-006 (GENT)	Belgium	KF432978
L. cyathuliformis	UE04.09.2004-2 (UPS)	Sweden	KF133266
L. hepaticus	JN02-049 (GENT)	Belgium	KF432980
L. hepaticus	JV2006-025(GENT)	Belgium	KR025574
L. hepaticus	JV2006-021 (GENT)	Belgium	KR025573
L. hispanicus	MA-Fungi 53339	Spain	AJ555567
L. fulvissimus	JN2012-025 (GENT)	Germany	KR025576

TABLE 4.1. Continued.

Species	Voucher collection	Origin	ITS accession no.
L. fulvissimus	JV2006-006 (GENT)	Belgium	KR025577
L. borzianus		Switzerland	AF373599
L. rubrocinctus	EDC12-210 (GENT)	Germany	KF432977
L. rubrocinctus	JKLAC10082201 (GENT)	Germany	KR025575
L. sphagneti	JKLAC11091502 (GENT)	Germany	KF432975
L. badiosanguineus	AV04-235 (GENT)	France	KF432983
L. badiosanguineus	AV10-44 (GENT)	Norway	KR025578
L. decipiens	VDKO882 (GENT)	Belgium	KR025586
L. decipiens	AV2000-137 (GENT)	Italy	KF432973
L. decipiens	AV13-044 (GENT)	Italy	KR025585
L. subdulcis	ED2008-27 (GENT)	Belgium	KR025572
L.subdulcis	JN2012-020 (GENT)	Germany	KR025571
L. subg. Lactarius			
L. austrozonarius	FH12-007 (GENT, MFLU)	Thailand	KF432965
L. purpureus	FH12-008 (GENT, MFLU)	Thailand	KF432966
L. scrobiculatus	JN01-058 (GENT)	Slovakia	KF432968
L. hatsudake	JN2011-065 (GENT)	Vietnam	KF432967
L. tornimosus	JN11-086 (GENT)	Greece	KR025613
L. subg. Plinthogalus			
L. fuliginosus	MTB97-24 (GENT)	Sweden	JQ446111
L. pterosporus	DS09-614 (GENT)	Italy	KR025628
L. friabilis	FH12-103 (GENT, MFLU)	Thailand	KF432961

Nucleotide alignment and phylogenetic reconstruction

This study comprises the DNA sequence data of most European and Southeast Asian representatives of *L.* subg. *Russularia*, most of which were generated from GENT. Additional sequences of European species were obtained from GenBank and UNITE. We included all European representatives except *L. duplicatus* A.H. Sm. The sequences of North American taxa, *L. rubidus* (Hesler & A.H. Sm.) Methven, *L. subserifluus* Longyear and *L. strigosipes* Montoya & Bandala were retrieved from GenBank. Representatives belonging to the other subgenera of *Lactarius* were also included in the analysis. Three species of *L.* subgenus *Plinthogalus* (Berk.) Hesler & A.H. Sm., *L. pterosporus* Romagn., *L. fuliginosus* Romagn. and *L. friabilis* H.T. Le & Stubbe, were used as the outgroup in the phylogeny. Table 4.1 shows an overview of all sequences used in the phylogenetic analysis. Nucleotide sequence alignment was made using MAFFT v7 (Katoh & Standley 2013) and later manually edited in MEGA6 (Tamura et al. 2013). The program Gblocks v0.91b (Castresana 2000) was used to eliminate poorly aligned positions in the alignment, with settings allowing gaps within selected blocks, smaller blocks (minimum 5 bp) and bigger segments with contiguous non-conserved positions (maximum 10 bp). The Alignment Transformation Environment (ALTER) was used to convert sequence alignment formats (Glez-Peña et al. 2010). RAxML v7.0.3 (Stamatakis 2006) was used to infer the maximum likelihood (ML) topology, applying the Rapid Bootstrapping algorithm for 1000 replicates using the GTRGAMMA model. To determine the model of character evolution of each gene partition, we used MrModeltest v2.3 (Nylander 2004) and the suggested parameters were applied for Bayesian inference (BI) analysis. Four separate runs in parallel with 10 million Markov chain Monte Carlo (MCMC) generations were executed using MrBayes v3.1.2 (Ronquist & Huelsenbeck 2003). Sample frequency was set at 100. To recognize that the number of generations was sufficient, we looked for a stationary likelihood graph and the effective sample size (ESS) value (should exceed 200) from independent runs using the graphing function in Tracer v1.6 (Drummond & Rambaut 2007). The proper burn-in value for the dataset was observed using this program. All phylograms were displayed using FigTree v1.3.1 (Rambaut 2009).

Result

Phylogeny

The ITS sequence alignment consists of 119 sequences and 1099 bases (including gaps). Gblocks retained 76% of the original sequence alignment; the excluded regions comprising about 257 bases, which mostly are at the beginning and the end of the multiple sequence alignment. The excluded regions of this study are largely due to the length variability of sequences available in GenBank. Twenty three, ten and three known *Russularia* species from Europe, Asia and North America respectively are included in the study. Figure 4.1 shows the ML topology based on the ITS sequence alignment. The bootstrap values (BS) and posterior probabilities (PP) are indicated in the phylogram when BS and PP exceed 50 and 0.85, respectively. All new species are indicated in boldface. Taxa in blue are obtained from public databases. Based on ITS sequence data, taxa from *L.* subg. *Russularia* form a monophyletic group apart from *L.* subg. *Lactarius*, but with low bootstrap support (48%). Except for Indonesian and Papuan representatives from Verbeken et al. (2001) and Verbeken & Horak (2000), we gathered all known Southeast Asian representatives here and the molecular evidence shows that all newly proposed ones are new species.

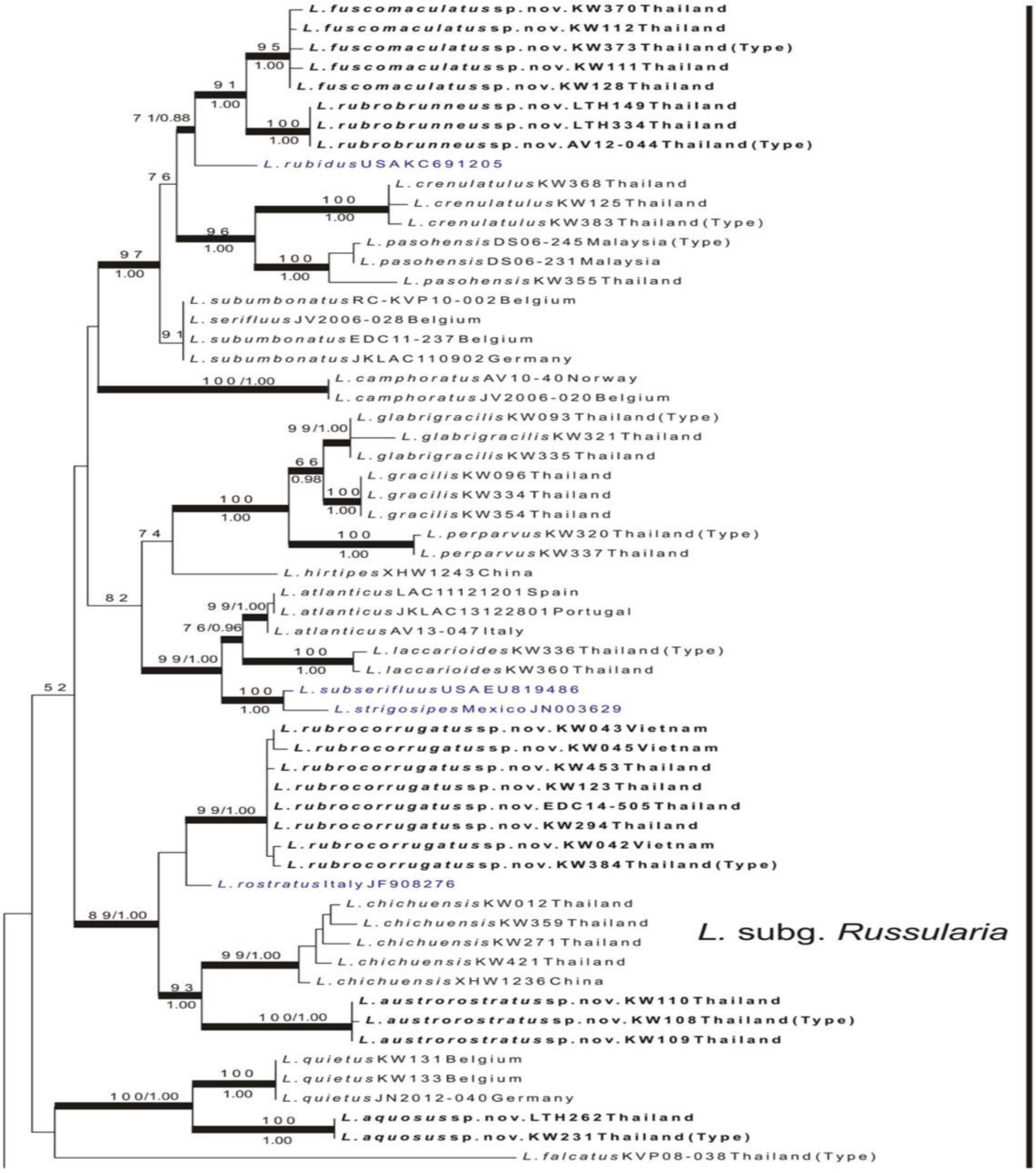

L. fuscomaculatus sp. nov. KW370 Thailand
L. fuscomaculatus sp. nov. KW112 Thailand
95
L. fuscomaculatus sp. nov. KW373 Thailand (Type)
1.00
L. fuscomaculatus sp. nov. KW111 Thailand
91
L. fuscomaculatus sp. nov. KW128 Thailand
1.00
L. rubrobrunneus sp. nov. LTH149 Thailand
71/0.88
100
L. rubrobrunneus sp. nov. LTH334 Thailand
1.00
L. rubrobrunneus sp. nov. AV12-044 Thailand (Type)
L. rubidus USA KC691205
76
L. crenulatulus KW368 Thailand
100
L. crenulatulus KW125 Thailand
1.00
L. crenulatulus KW383 Thailand (Type)
96
L. pasohensis DS06-245 Malaysia (Type)
1.00
97
100
L. pasohensis DS06-231 Malaysia
1.00
1.00
L. pasohensis KW355 Thailand
L. subumbonatus RC-KVP10-002 Belgium
L. serifluus JV2006-028 Belgium
91
L. subumbonatus EDC11-237 Belgium
L. subumbonatus JKLAC110902 Germany
100/1.00
L. camphoratus AV10-40 Norway
L. camphoratus JV2006-020 Belgium
L. glabrigracilis KW093 Thailand (Type)
99/1.00
L. glabrigracilis KW321 Thailand
L. glabrigracilis KW335 Thailand
66
L. gracilis KW096 Thailand
0.98
100
L. gracilis KW334 Thailand
100
1.00
L. gracilis KW354 Thailand
1.00
74
100
L. perparvus KW320 Thailand (Type)
1.00
L. perparvus KW337 Thailand
L. hirtipes XHW1243 China
82
L. atlanticus LAC11121201 Spain
99/1.00
L. atlanticus JKLAC13122801 Portugal
76/0.96
L. atlanticus AV13-047 Italy
100
L. laccarioides KW336 Thailand (Type)
99/1.00
1.00
L. laccarioides KW360 Thailand
52
100
L. subserifluus USA EU819486
1.00
L. strigosipes Mexico JN003629
L. rubrocorrugatus sp. nov. KW043 Vietnam
L. rubrocorrugatus sp. nov. KW045 Vietnam
L. rubrocorrugatus sp. nov. KW453 Thailand
L. rubrocorrugatus sp. nov. KW123 Thailand
99/1.00
L. rubrocorrugatus sp. nov. EDC14-505 Thailand
L. rubrocorrugatus sp. nov. KW294 Thailand
L. rubrocorrugatus sp. nov. KW042 Vietnam
L. rubrocorrugatus sp. nov. KW384 Thailand (Type)
L. rostratus Italy JF908276
L. chichuensis KW012 Thailand
89/1.00
L. chichuensis KW359 Thailand
L. subg. Russularia
L. chichuensis KW271 Thailand
99/1.00
L. chichuensis KW421 Thailand
L. chichuensis XHW1236 China
93
L. austrorostratus sp. nov. KW110 Thailand
1.00
100/1.00
L. austrorostratus sp. nov. KW108 Thailand (Type)
L. austrorostratus sp. nov. KW109 Thailand
L. quietus KW131 Belgium
100
L. quietus KW133 Belgium
100/1.00
1.00
L. quietus JN2012-040 Germany
100
L. aquosus sp. nov. LTH262 Thailand
1.00
L. aquosus sp. nov. KW231 Thailand (Type)
L. falcatus KVP08-038 Thailand (Type)

Figure 4.1 Continued.

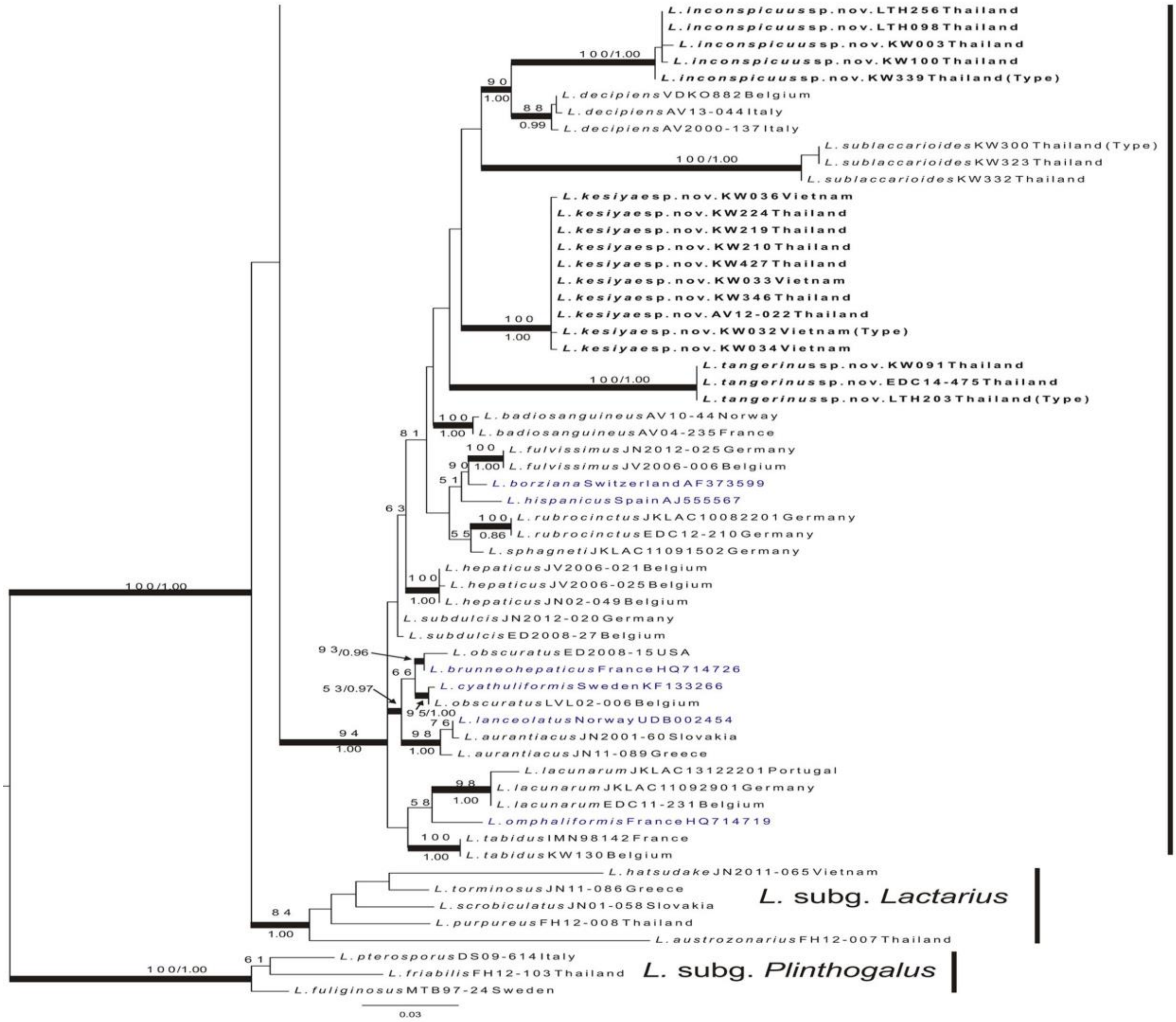


FIGURE 4.1: The obtained ML phylogeny based on ITS sequences. Names in boldface are new species. Names in blue are obtained from public databases. Bootstrap values and posterior probabilities are indicated if they exceed 50% and 0.85, respectively. The bar scale represents the expected number of nucleotide changes per site.

Taxonomy

Lactarius aquosus H.T. Le & K.D. Hyde sp. nov. (Figure 4.2 and 4.11a).

MycoBank number: MB811741; **Facesoffungi number:** FoF00634

Diagnosis*:* a medium-sized species with a smooth cap surface which is zonate, brown to orange-brown colouration and a paler margin, transparent latex, completely transparent, incompletely to almost completely reticulate basidiospores, protruding pleuromacrocystidia and a cutis as pileipellis.

Etymology: 'aquosus' is referring to the transparent latex.

Typus: Thailand, Chiang Mai Province, Mae-on district, Mae Takraii National Park, Thepsaded Waterfall, alt. 1150 m , N18º55.32 E99º21.31, 01/06/2012, KW231 (holotype, GENT!, isotype, MFLU!).

Basidiocarps medium-sized. *Pileus* 21−37 mm diam., rather plane with depressed center to infundibuliform at maturity, typically darker coloured in center and appearing more or less zonate near the margin; surface smooth, dry and glossy in moist condition, reddish brown (8E8) in center, reddish blond (5C4) to dark blond (5D4) to reddish brown (8D5), margin pale orange (5A3) to cream coloured margin with short and inconspicuous striations, slightly crenulate. *Lamellae* subdecurrent to decurrent, 1−4.5 mm broad, crowded, with 2−4 series of lamellulae, cream to yellowish white (4A1−2) to light yellow (4A4), with light brown (7D6) discolourations in older specimens, yellowish brown (5D5) when bruised. *Stipe* 25−48 ×5−15 mm, cylindrical, hollow; surface smooth, rugose when old, often with short hairs and whitish pruinose at base, brownish orange (6C6) to light brown (6−7D6), darkening to brown (7E7) near base. *Context* 2−4.5 mm broad in pileus, pale yellow (3A3) to greyish yellow (3B4) to light greyish orange (6B−C4), unchanging when cut; smell strong, raphanoid; taste mild or slightly bitter and astringent. *Latex* watery, unchanging on exposure, moderately abundant, unchanging with 10 % KOH, unchanging on white tissue paper and on white cotton handkerchief. *Macrochemical reaction* no reaction on the context with 10 % KOH and with $FeSO_4$.

Basidiospores subglobose to broadly ellipsoid, 6.1−*6.9*−*7.2*−7.9(−8.0) × 5.4−*5.9*−*6.2*−6.9 µm; Q = 1.06−*1.15*− *1.17*−1.27 (n=60); ornamentation amyloid, composed of ridges up to 1 µm high, forming an incomplete to almost complete reticulum, with short ridges connected by finer lines which are blunt to subacute; isolated warts common;

plage inamyloid. *Basidia* 48−52 × 12−16 µm, 4-spored, subclavate, sometimes bent near base, typically with needle- like to guttate contents. *Pleuromacrocystidia* 55−74 × 9−16 µm, abundant, subfusiform to subclavate with mucronate or moniliform apex, protruding up to 30 µm, with fine granules and needle-like contents. *Pleuropseudocystidia* 3−4 µm diam., not emergent, irregularly cylindrical, bent or curved near base; apex round or capitate. *Lamellar edge* heterogeneous, consisting of basidia, capitate to subclavate marginal cells 27−38 × 12−17 µm and cheilocystidia 34−58 × 8−14 µm, abundant, subfusiform, some irregularly curved, with mucronate to slightly moniliform apex, protruding up to 35 µm above the hymenium. *Lamellar trama* consisting of smaller and larger globose cells, septate hyphae and lactiferous hyphae. *Pileipellis* a cutis, 90−140 µm thick, consisting of parallel, repent and sometimes slightly erect hyphae.

Habitat: gregarious or scattered on slope, in montane tropical forest, with Fagaceae.

Studied material: Thailand, Chiang Mai Province, Mae-on district, Mae Takraii National Park, Thepsaded Waterfall, alt. 1150 m, N18º55.32 E99º21.31, 01/06/2012, KW231 (holotype, GENT!, isotype, MFLU!); Chiang Mai province, Mae Teang district, Bahn Pha Deng village, alt. 900 m., N19º17.12 E98º44.00, 01/06/2004, LTH102 (GENT!, CMU!, SFSU!);−ibid. 05/06/2005, LTH262 (GENT!, CMU!, SFSU!).

Comments: *Lactarius aquosus* is easily recognized in the field by its smooth pileus surface, zonate cap and transparent latex. Microscopically, this species has incompletely reticulate basidiospores and a cutis as a pileipellis.

Lactarius austrorostratus Wisitrassameewong & Verbeken sp. nov. (Figure 4.3).

Mycobank number: MB811742; **Facesoffungi number:** FoF00635

Diagnosis: a medium sized, reddish brown species, with incompletely reticulate basidiospore ornamentation in a zebroid pattern, rostrate pleuromacrocystidia and a hyphoepithelium as a pileipellis.

Etymology: 'austrorostratus' refers to the occurrence in South East Asia and the rostrate cystidia.

Typus: Thailand, Chiang Mai province, Jomthong district, Bahn Luang sub-district, Doi Inthanon, nature trail at highest spot, N18º35.20 E98º29.03, alt. 2565 m, 06/07/2011, KW108 (holotype, GENT!, isotype, MFLU!).

Pileus: 10−30 mm diameter, at first convex to broadly convex with incurved margin, later expanding and with a more depressed center, or slightly infundibuliform, papillate in center; surface smooth when young, then becoming rugulose, particularly in center, dark brown (9F7) initially, turning dark brown (9F6−7) to reddish brown (9E8); margin inflexed, short striation, crenulate. *Lamellae* 1−3 mm broad, crowded, with 1−2 series of lamellulae, subdecurrent, cream to dark cream; edge minutely crenulate. *Stipe* 30−43 × 4 mm, cylindrical, central to slightly eccentric, fragile, fistulose; surface wrinkled, reddish brown (8D7) to dark brown (7F6) at base in youth, turning darker brown (8F8) when mature. *Context* 0.5−2 mm broad, fragile, pale reddish brown, unchanging when cut; odor like *L. quietus* or Pentatomidae bugs, taste mild. *Latex* somewhat watery white to whey-like, sparse to moderately abundant, unchanging on exposure, unchanging with 10% KOH, unchanging on white tissue paper and on white cotton handkerchief, taste mild. *Macrochemical reaction* no reaction on the context with 10 % KOH and with $FeSO_4$.

Basidiospores subglobose to ellipsoid, (6.1−)6.4−*7.1*−7.8×(5.6−)5.7−*6.2*−6.8(−6.9) µm; Q=1.07−*1.14*−*1.15*−1.30, (n = 40); ornamentation amyloid, up to 1 µm high, consisting of blunt irregular ridges, forming an incomplete reticulum, at times with a zebroid aspect; isolated warts abundant; plage distally amyloid. *Basidia* 34−52 × 10−17 µm, mostly 4-spored, sometimes 1-or 2-spored, irregularly subclavate, some irregularly cylindrical, with guttate contents and fine granules. *Pleuromacrocystidia* abundant, 54−107 × 9−18 µm, lanceolate to subfusiform, somewhat slender, protruding up to 40 µm, rostrate apex, often tortuous particularly near the apex, mostly with refractive contents containing fine granules. *Pleuropseudocystidia* abundant, 3−6 µm diam., cylindrical, but tortuous near the base, with fine granules, needle-like contents and sometimes with crystalline contents. *Lamellar edge* sterile, with marginal cells and cheilocystidia; marginal cells 13−26 × 8−18 µm, clavate to obovoid; cheilocystidia 32−58 × 10−16 µm, scattered to abundant, subfusiform with mucronate to rounded apex, with refractive contents containing fine granules, protruding up to 15 µm. *Lamellar trama* consisting of smaller and larger globose cells, septate hyphae and lactiferous hyphae. *Pileipellis* a hyphoepithelium, suprapellis a thin layer of repent hyphae; subpellis about 60−80 µm thick, consisting of large globose cells, up to 35 µm diam.

Habitat: solitary or gregarious on ground in evergreen rainforest, under *Castanopsis*.

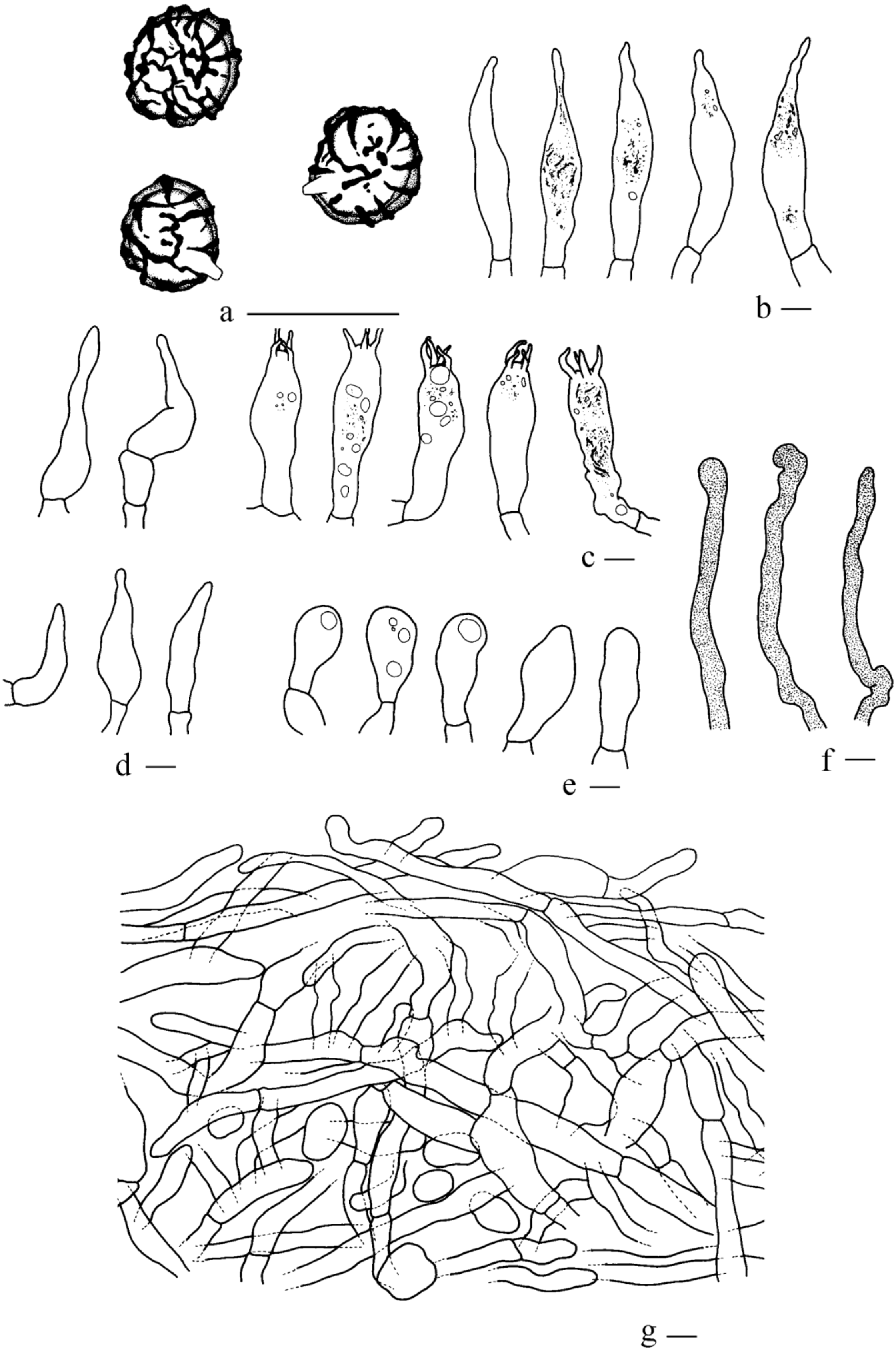


FIGURE 4.2: *Lactarius aquosus*: a. basidiospore, b. pleuromacrocystidia, c. basidia, d. cheilocystidia, e. marginal cell, f. pseudocystidia, g. pileipellis (a–g: KW231, holotype) (scale bar = 10 µm).

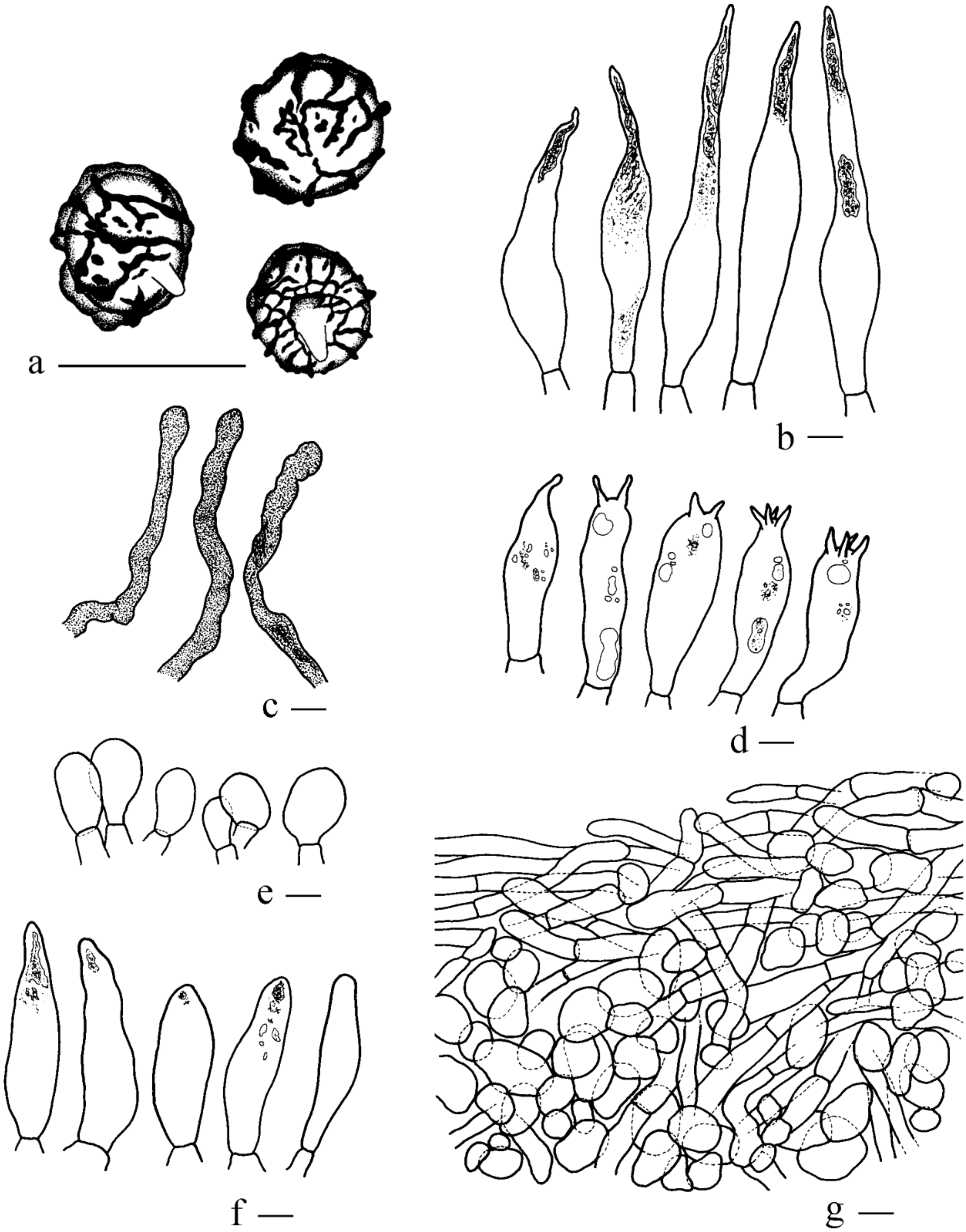


FIGURE 4.3: *Lactarius austrorostratus*: a. basidiospore, b. pleuromacrocystidia, c. pseudocystidia, d. basidia, e. marginal cell, f. cheilocystidia, g. pileipellis (a and g: KW109, b–f: KW108, holotype) (scale bar = 10 µm).

Studied material: Thailand, Chiang Mai province, Jomthong district, Bahn Luang sub-district, Doi Inthanon, nature trail at highest spot, N18º35.20 E98º29.03, alt. 2565 m, 06/07/2011, KW108 (holotype, GENT!, isotype, MFLU!);−ibid., 06/07/2011, KW109 (GENT!, MFLU!);−ibid., 06/07/2011, KW110 (GENT!, MFLU!).

Comments: This species is in some aspects similar to *L. rostratus,* e.g. in basidiocarp size, colour and latex features. Both species have rostrate pleuromacrocystidia but the Asian species has larger pleurocystidia. Both species also share basidiospore ornamentation characteristics (an incomplete reticulum with a slightly zebroid pattern) and a hyphoepithelium pileipellis. *Lactarius austrorostratus* grows under *Castanopsis* whereas *L. rostratus* is associated with *Fagus* in moss cushions. Another closely related species, *L. rubrocorrugatus*, is a small reddish brown species with completely transparent latex. The other distinguishable character between *L. austrorostratus* and *L. rubrocorrugatus* is the pleuromacrocystidia. *Lactarius austrorostratus* has conspicuous protruding cystidia (up to 40 µm) with acute apex whereas in *L. rubrocorrugatus* the cystidia are not protruding to slightly protruding up to 20 µm with a mucronate to moniliform apex.

Lactarius chichuensis W.F. Chiu, Lloydia 8(1): 38, 1945 (Figure 4.4 and 4.11j).

Basidiocarps small to medium-sized. *Pileus* 5−37 mm diam., broadly convex to convex initially with umbo, becoming infundibuliform with age; surface dry, smooth to wrinkled, colour generally varying from brown (6E4−7E6) to reddish brown (8D8, 9D−E8), with paler shade near margin, brownish orange (6C4−6) to light brown (6D7−8), in some specimens brownish (7C7) to light brown (7D4−5) to greyish brown (7E3) to brown (7E4) with cream-coloured margin, sometimes with a fine whitish powder covering the surface; margin not striate, involute initially and becoming incurved to straight in age. *Lamellae* subdecurrent to decurrent, crowded, 0.5−3 mm broad, greyish orange (5B4) to brownish orange (5C4), light brown (7D4) to brown (7E4) when older, with 2−3 series of lamellulae. *Stipe* 8−24 × 2−6.5 mm, cylindrical, central to eccentric; surface dry, smooth, light brown (7D6) to brown (6−7E6). *Context* 1−3 mm broad in pileus, hollow in stipe, pale orange (5A3) to greyish orange (5B3−B4); odor strong, like *L. quietus* or Pentatomidae bugs; taste mild. *Latex* abundant, watery white to white, unchanging on exposure, unchanging with 10 % KOH, pale yellow on white tissue paper and unchanging on white cotton handkerchief. *Macrochemical* *reaction* on context: olive brown (4D6) to light brown (5D6) with 10 % KOH, brown (6E7) with $FeSO_4$.

Basidiospores globose to broadly ellipsoid, 6.1−*6.7*−*7.3*−8.1(−8.3) × 5.5−*6.0*−*6.4*−7.2(−7.3) µm; Q = 1.02−*1.10*−*1.16*−1.28; ornamentation amyloid; ridges up to 1 µm high forming a zebroid ornamentation composed of parallel irregular, short and long ridges, never reticulate; plage inamyloid. *Basidia* 50−57 × 13−18 µm, 4-spored, subclavate, with fine granules. *Pleuromacrocystidia* rare to abundant, 45−71 × 10−15 µm, protruding up to 30 µm, subfusiform, bent to straight with mucronate to moniliform apex, typically with refractive contents at apex. *Pseudocystidia* 3−6 µm diam., slightly protruding, cylindrical, tortuous to straight, with fine granulate content. *Lamellar edge* heterogeneous, consisting of basidia, marginal cells and cheilocystidia; marginal cells 12−28 × 7−15 µm, subcylindrical to clavate; cheilocystidia abundant 31−45 × 10−19 µm, protruding up to 15 µm, subfusiform to subclavate, rarely clavate, with refractive contents at apex. *Lamellar trama* consisting of globose cells, septate hyphae and lactiferous hyphae. *Pileipellis* a hyphoepithelium, with a dense upper layer of repent hyphae and an underlying layer of globose cells, suprapellis composed of cylindrical hyphae about 20−40 µm thick; subpellis consisting of globose cells about up to 20 µm diam.

Habitat: gregarious on ground in montane mixed forest with Fagaceae and *Pinus kesiya*.

Distribution: reported from China (Chiu 1945, Wang & Liu 2002), Thailand.

Studied material: Thailand, Chiang Rai province, Mae Fah Luang district, Doi Mae Salong Nok sub-district, Doi Mae Salong, alt. 1269 m., N20º16.90 E99º62.30, 13/07/2012, KW352 (GENT!, MFLU!);−ibid., 27/07/2012, KW372 (GENT!, MFLU!);−ibid., 08/08/2012, KW388 (GENT!, MFLU!);−ibid., 16/09/2012, KW403 (GENT!, MFLU!);−ibid., 28/08/2013, KW465 (GENT!, MFLU!);−ibid., 10/09/2013, KW467 (GENT!, MFLU!); Chiang Mai province, Mae Teang district, Pa Pae sub-district, Bahn Pha Deng village, mushroom research center, N19º17.12 E98º44.00, alt. 900 m, 13/05/2011, KW012 (GENT!, MFLU!); Chiang Mai province, Mae Teang district, Pa Pae sub-district, Bahn Pha Deng village,Pathummikaram Temple, forest trail, alt. 1050 m, 07/06/2012, KW271 (GENT!, MFLU!);−ibid., 07/06/2012, KW272 (GENT!, MFLU!).

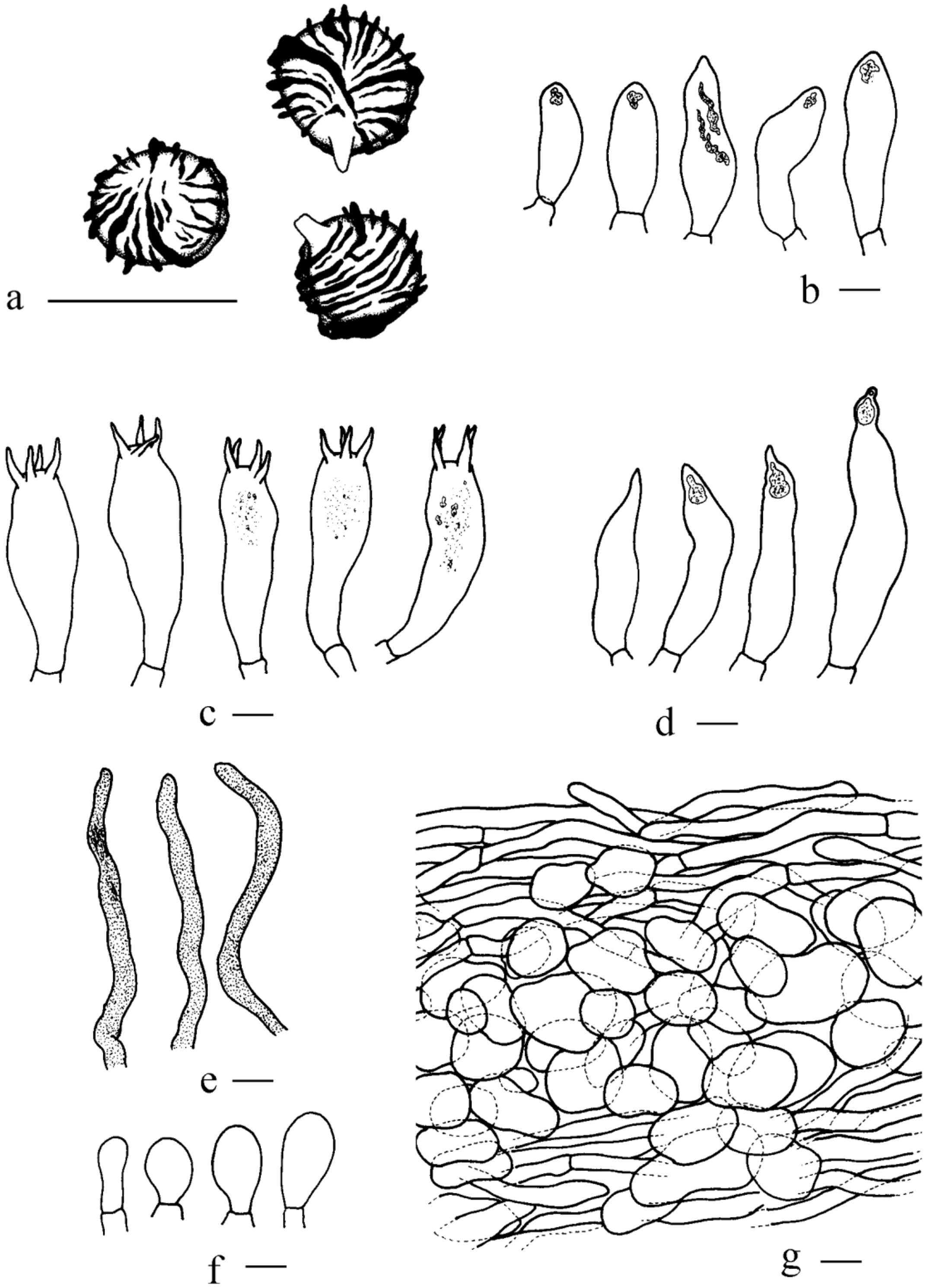


FIGURE 4.4: *Lactarius chichuensis*: a. basidiospore, b. cheilocystidia, c. basidia, d. pleuromacrocystidia, e. pseudocystidia, f. marginal cells, g. pileipellis (a: KW271, b−g: KW421) (scale bar = 10 µm).

Comments: *Lactarius chichuensis* is a distinctive species in the subgenus because of its zebroid basidiospore ornamentation. The species has small, reddish brown basidiocarps and a strong *L. quietus*-like or Pentatomidae bug odor. The species was discovered by Chiu (1945). The holotype is in poor condition and only the spore ornamentation was studied by Chiu. Later the species was described in more detail and distinguished from a look-alike species, *L. hirtipes* J.Z. Ying by Wang & Liu (2002) on account of its different basidiospore ornamentation. Morphological characters of our specimens are consistent with the documentation of Wang & Liu (2002). In our collections we sometimes observed the pileus to be whitish pruinose. Wang & Liu (2002) stated that the species is found in forests with Fagaceae, whereas we found the species in mixed forest of Fagaceae and *Pinus kesiya*.

Lactarius fuscomaculatus Wisitrassameewong & Verbeken sp. nov. (Figure 4.5 and 4.11b).

Mycobank number: MB811743; **Facesoffungi:** FoF00636

Diagnosis: a medium sized species with a brown to orange-brown pileus and remarkably dark brown spots or tinges, white latex, a strong odor of Pentatomidae bugs, incompletely reticulate basidiospore ornamentation, pleuromacrocystidia rare and pileipellis a hyphoepithelium.

Etymology: 'fuscomaculatus' refers to the dark, brownish spots on the pileus.

Typus: Thailand, Chiang Rai province, Muang district, Thasai sub-district, forest near Doi Pui Reverse Signal Station, Doi Pui, N19º49.26 E99º52.19, alt. 655 m, 31/07/2012, KW373 (holotype, GENT!, isotype, MFLU!).

Basidiocarps medium sized. *Pileus* 25−62 mm diam., broadly convex initially, turning slightly infundibuliform to deeply infundibuliform in age, with a more or less distinct papilla; surface dry, more rugose in the center in age, brown (7D8 to 7E7) to dark brown (8F7) in the center, towards the margin, paler, brownish orange (5C5) to brown (6D7) to pale yellow (3A3), typically becoming uneven in colour in age, with dark brown (8F7) discolourations, varying from spotted to brushed over the whole surface except the margin; margin indistinctly striate, slightly crenulate. *Lamellae* subdecurrent to decurrent, 1−3 mm broad, crowded, with 3−4 series of lamellulae, pale yellow (4A3) to cream-coloured, discolouring light brown (6D6) to brown (7E6), spotted in age. *Stipe* 34−76 x 4−7 mm, cylindrical, central, rarely slightly eccentric; surface dry, smooth, whitish pruinose, particularly in young specimens, dull yellow (3B3) to olive brown (4D6) at the apex, yellowish brown (5E4) to dark brown (7F5) towards the base, sometimes brownish orange (7C7), with 1−2 mm long hairs at the base. *Context* 0.5−4 mm broad in the pileus, partially hollow to completely hollow in stipe, pale yellow (3A3); odor strong, reminiscent of *L. quietus* or Pentatomidae bugs; taste mild. *Latex* watery white to white, abundant, unchanging on exposure, unchanging with 10% KOH, unchanging on white tissue paper and on white cotton handkerchief; taste slightly astringent and becoming faintly peppery. *Macrochemical reaction* no reaction on the context with 10% KOH, or with $FeSO_4$.

Basidiospores globose to broadly ellipsoid, 6.0−*6.6−7.2*−7.7(−7.8) × 5.5−*6.1−6.6*.−7.0(−7.2) µm, Q = 1.02−*1.08−1.10*−1.21 (n=100); ornamentation amyloid, composed of irregular ridges up to 1 µm high, forming an incomplete reticulum, warts and ridges connected by fine lines; isolated warts present; plage inamyloid to distally amyloid. *Basidia* 42−53 × 13−17 µm, 4-spored, some 2-spored, subclavate to clavate; with guttate contents. *Pleuromacrocystidia* rare, protruding up to 10 µm, 55−68 × 13−17 µm, fusiform, with a mucronate, occasionally ramified apex. *Pleuropseudocystidia* scarce to abundant, 3−6 µm diam., not emergent to slightly emergent, cylindrical to tortuous with an obtuse apex, often broadened at the apex. *Lamellar edge* heterogeneous, composed of basidia, abundant cylindrical to subclavate, thin- walled marginal cells 13−26 × 5−13 µm; cheilocystidia absent to rare, 34−38 ×11−14 µm, not emergent, fusiform with a mucronate apex. *Lamellar trama* consisting of lactifers and sphaerocysts. *Pileipellis* a hyphoepithelium, with a thin upper layer of repent or oblique hyphae; suprapellis composed of cylindrical hyphae, 10−20 µm thick; subpellis a layer of large globose cells, up to 30 µm diam., mixed with cylindrical to inflated hyphae.

Habitat: gregarious to scattered on the ground in montane tropical forests with Fagaceae.

Studied material: Thailand, Chiang Rai province, Muang district, Thasai sub-district, forest near Doi Pui Reverse Signal Station, Doi Pui, N19º49.26 E99º52.19, alt. 655 m, 31/07/2012, KW373 (holotype, GENT!, isotype, MFLU!);−ibid., 25/07/2011, KW111 (GENT!, MFLU!);−ibid., 25/07/2011, KW112 (GENT!, MFLU!);−ibid., 01/09/2011, KW126 (GENT!, MFLU!);−ibid., 01/09/2011, KW128 (GENT!, MFLU!);−ibid., 01/09/2011, KW129 (GENT!, MFLU!);−ibid., 25/07/2012, KW370 (GENT!, MFLU!); Chiang Rai province, Mae Fah Luang district, Doi Mae Salong Nok sub-district, Doi Mae Salong, alt. 1269 m, N20°16.90

E99°62.30, 30/05/2012, KW221 (GENT!, MFLU!);–ibid., 30/05/2012, KW223 (GENT!, MFLU!); Chiang Rai province, Mae Fah Luang district, Doi Mae Salong Nok sub-district, Doi Mae Salong, N20º17.23 E99º61.69, alt. 1193 m, 22/07/2012, KW365 (GENT!, MFLU!);–ibid., 28/08/2013, KW466 (GENT!, MFLU!); Chiang Mai province, Mae-On district, Huaikaew sub-district, Bahn Pok M.1, N18º53.08 E99º21.45, alt. 1040 m, 02/06/2012, KW236 (GENT!, MFLU!); Chiang Mai province, Mae-On district, Huaikaew sub-district, Bahn Mae Kampong, N18º51.43 E99º22.09, alt. 1450 m, 03/06/2012, KW249 (GENT!, MFLU!).

Comments: *Lactarius fuscomaculatus* is widely distributed in tropical forests with *Castanopsis* and *Quercus* in Northern Thailand. The species can be confused with a closely related species, *L. rubrobrunneus* due to similarities in basidiocarps size, colour, latex features and habitat. Both species are very similar in the immature stage, however, according to our experience, brown spots on the pileus are often found in fully mature fruiting bodies of *L. fuscomaculatus*, while *L. rubrobrunneus* is more unicoloured. Under the microscope, both species have basidiospores with an incomplete reticulum and not many cystidia. A slight difference was observed in their pileipellis structures; *L. rubrobrunneus* has in part a very thin layer of repent hyphae which is like a transition between an epithelium and a hyphoepithelium, whereas *L. fuscomaculatus* has a more complete layer of repent hyphae covering the pileus. *Lactarius fuscomaculatus* may also be confused with *L. tangerinus* in the field. *Lactarius tangerinus* has smaller basidiomata, is typically paler in colour without dark brown spots and possesses transparent latex. For more details on the difference between these two species, see under *L. tangerinus*.

Lactarius inconspicuus H.T. Le & F. Hampe sp. nov. (Figure 4.6 and 4.11d).

MycoBank number: MB811744; **Facesoffungi:** FoF00637

Diagnosis: a small to medium sized species recognized by the brownish orange cap with paler margin, white latex turning pale yellow on exposure and on white tissue paper and white handkerchief, with incomplete reticulate basidiospore ornamentation, large and protruding pleuromacrocystidia and an ixotrichodermal pileipellis.

Etymology: ‘inconspicuus’ refers to the inconspicuus macromorphological characters of the species.

Typus: Thailand, Chiang Mai province, Doi Suthep-Pui national park, Sangasabhasri Lane to Huai Kok Ma Village, N18º48.62 E98º54.60, alt. 1145 m, 05/07/2012, KW339 (holotype, GENT!, isotype, MFLU!).

Basidiocarps small to medium sized. *Pileus* 10−56 mm diam., convex-umbonate in immature specimens, becoming plano-convex with depressed disc; surface minutely rugulose, dry to slightly sticky, glossy when moist, brown (7E6) to dark brown (7F8) in center or at least on umbo, brown (6E−F6) to light brown (6D7) to brownish orange (6C−D6), paler and cream-coloured at margin; margin estriate when immature, becoming striate with age, crenulate. *Lamellae* subdecurrent to decurrent, 1−3 mm broad, crowded, with 3−4 series of lamellulae, yellowish white (2A2), pale yellow (4A3) to light yellow (4A4), turning brownish orange (6B−C7, 7C−D6) to reddish brown (8E5) when bruised. *Stipe* 20−75 × 2−8 mm, cylindrical to tapering upwards; surface dry to moist, smooth, brownish yellow (6B−C6) to brownish orange (6C8−7C8) to brown (7E−F8), fistulose, whitish pruinose at base. *Context* 1−3 mm thick in pileus, pale cream to orange-white (5A2) to brownish orange (5C−D6); odor faint, like *L. quietus* or Pentatomidae bugs; taste mild. *Latex* abundant, watery white, slowly turning pale yellow on exposure, unchanging with 10 % KOH, pale yellow on white tissue paper, pale yellow on white handkerchief; taste mild. *Macrochemical reaction* on the context: light brown (5D4−D5) with 10 % KOH, greyish yellow (4B3) with $FeSO_4$.

Basidiospores typically subglobose to broadly ellipsoid, rarely globose, 6.1−*6.9*−*7.4*−8.0(−8.1) × 5.5−*5.8*−*6.3*−6.9(−7.0) µm; Q = 1.03−*1.13*−*1.16*−1.28 (n = 100); ornamentation amyloid, up to 1.3 µm high, consisting of blunt to subacute thick ridges forming an incomplete to almost complete reticulum; isolated warts common; plage inamyloid, sometimes slightly distally amyloid. *Basidia* 30−62 × 8−18 µm, mostly 4-spored, sometimes 2-spored, subcylindrical to subclavate, with fine granules and guttate contents. *Pleuromacrocystidia* abundant, 30−114 × 8−20 µm, protruding up to 30 µm, subcylindrical to subfusiform, with mucronate to moniliform apex, with needle-like contents and fine granules. *Pleuropseudocystidia* not protruding, 3−5 µm diam., tortuous to straight, cylindrical, with fine granules. *Lamellar edge* heterogeneous, consisting of basidia, cylindrical to subclavate marginal cells 15−36 × 8−17 µm and abundant cheilocystidia 30−52 × 8−18 µm, not protruding to slightly protruding up to 10 µm, subfusiform to fusiform, with mucronate to moniliform apex. *Lamellar trama* consisting of globose cells,

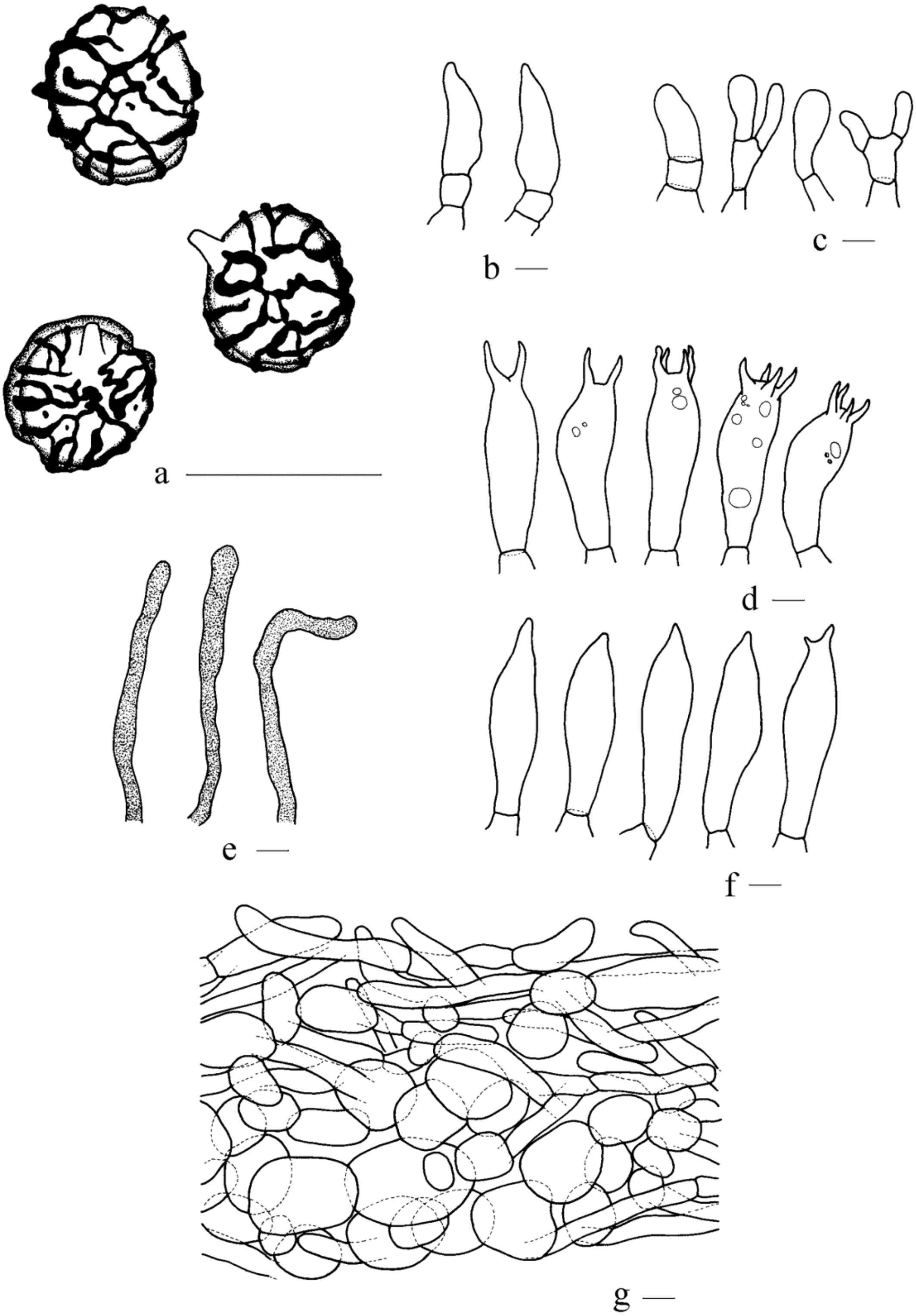


FIGURE 4.5: *Lactarius fuscomaculatus*: a. basidiospore, b. cheilocystidia, c. marginal cell, d. basidia, e. pseudocystidia, f. pleuromacrocystidia, g. pileipellis (a and c–f: KW365, b and g: KW373, holotype) (scale bar = 10 µm).

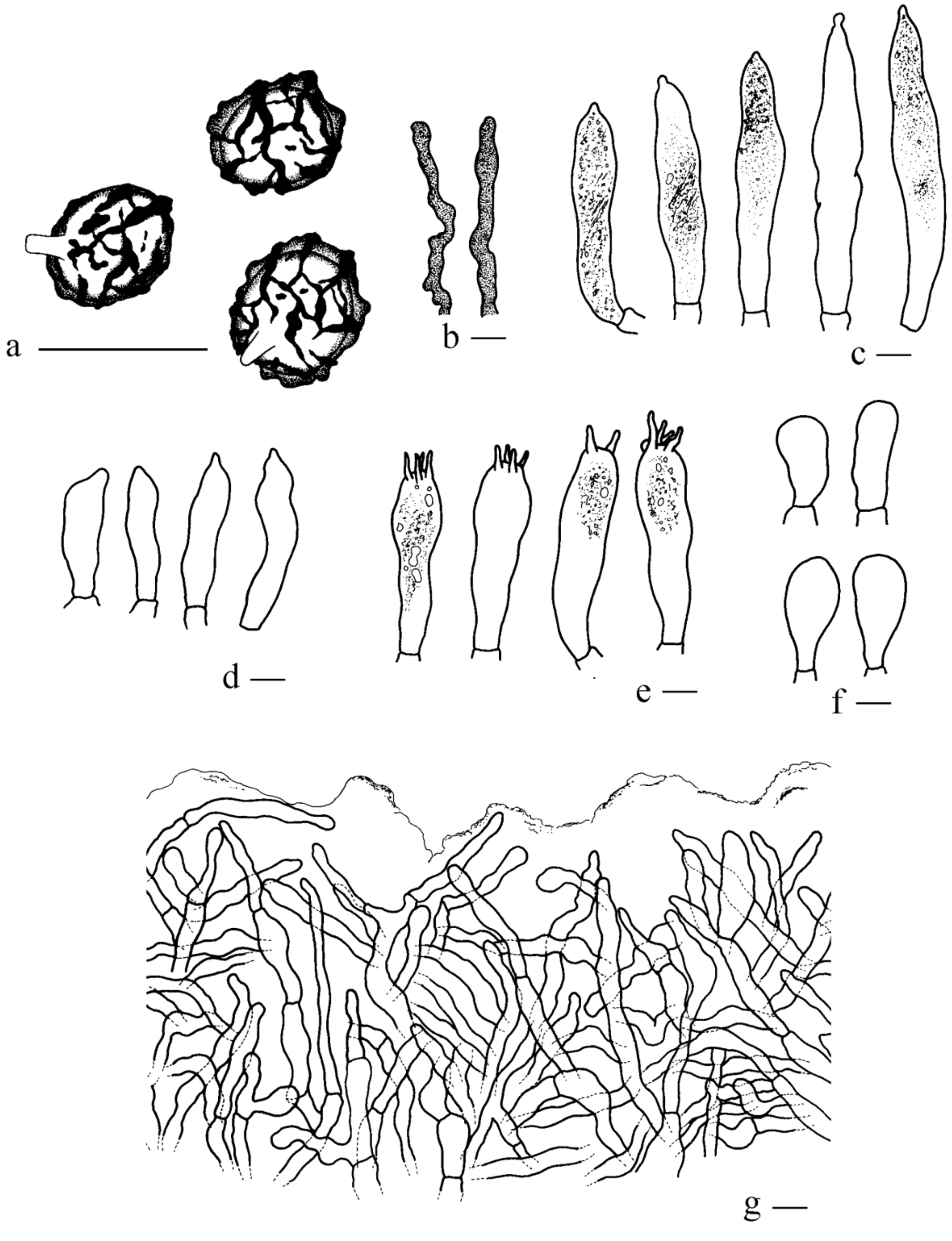


FIGURE 4.6: *Lactarius inconspicuus*: a. basidiospore, b. pseudocystidia, c. pleuromacrocystidia, d. cheilocystidia, e. basidia, f. marginal cell, g. pileipellis (a–f: LTH256, g: KW339, holotype) (scale bar = 10 µm).

septate hyphae and lactiferous hyphae. *Pileipellis* an ixotrichoderm, 100−120 µm thick,consisting of erect cylindrical hyphae, or sometimes, repent cylindrical hyphae.

Habitat: solitary to gregarious on soil in montane tropical forests, under *Castanopsis armata*.

Studied material: Thailand, Chiang Mai province, Doi Suthep-Pui national park, Sangasabhasri Lane to Huai Kok Ma Village, N18º48.62 E98º54.60, alt. 1145 m, 02/06/2005, LTH256 (GENT!, CMU!, SFSU!);−ibid., 30/05/2004, LTH098 (GENT!, CMU!, SFSU!);−ibid., 24/06/2005, LTH306 (GENT!, CMU!, SFSU!);−ibid., 24/06/2005, LTH307 (GENT!, CMU!, SFSU!);−ibid., 21/04/2011, KW003 (GENT!, MFLU!);−ibid., 05/07/2012, KW339 (holotype, GENT!, isotype, MFLU!); Chiang Mai province, Mae Taeng district, Bahn Mae Sae, 50 km marker on highway 1095, N19º14.59 E98º39.45, alt. 962 m, 03/06/2011, KW016 (GENT!, MFLU!).

Comments: This species has the general features of the subgenus but lacks any striking distinguishing characters. In the field this species is similar to *L. tangerinus*. A microscopic examination revealed that these species can be distinguished by: (1) larger pleuromacrocystidia in *L. inconspicuus*; (2) presence of a narrow mucus layer in the pileipellis in *L. inconspicuous*; and (3) higher basidiospore ornamentation in *L. tangerinus*.

Lactarius kesiyae Verbeken & K.D. Hyde sp. nov. (Figure 4.7, 4.11g and 4.11h)

MycoBank number: MB811745; **Facesoffungi number:** FoF00638

Diagnosis: a medium sized species with smooth cap and glossy surface, brownish grey to brownish orange cap with greyish green tints, latex watery white turning yellow on a white handkerchief, incompletely reticulate basidiospores, protruding pleuromacrocystidia, an ixotrichoderm and association with *P. kesiya*.

Etymology: 'kesiyae' refers to the ectomycorrhizal tree, *P. kesiya*.

Typus: Vietnam, Lam Dong province, Lac Duong district, Xa Lat, Lang Biang National park, alt. 1545 m, N12º01.57 E108º25.58, 12/06/2011, KW032 (holotype, GENT!, isotype, MFLU!).

Basidiocarps medium-sized. *Pileus* 18−49 mm diam., at first convex to broadly convex, then becoming plano-convex with a more depressed disc, finally infundibuliform; surface smooth and slightly glossy, slightly sticky when moist; colour darkest in immature specimens, dark blond (5D4) when young, greyish yellow (3−4C4), brownish grey (5C2), reddish blond (5C3−4) to brownish orange (5C5) when mature, in some collections with greyish green

(25B4−B6) tints, colour paler in dry condition, pale orange (5A3) to pale greyish orange (5B3−4); margin, paler, yellowish white (2A2), estriate when immature, later becoming striate and slightly crenulate with age. *Lamellae* subdecurrent to decurrent, 1−3 mm broad, crowded, with 2 series of lamellulae, pale cream to cream, to greyish orange (6B5) when bruised. *Stipe* 30−56 × 3−8 mm, cylindrical, centrally attached; apex becoming concolourous with lamellae, darker when moist, brownish orange (5C5), pale orange when dry. *Context* 3−5 mm broad in pileus, whitish, hollow in stipe; smell fruity; taste mild, a bit sweet after a while, then faintly acrid. *Latex* watery white, moderately abundant, unchanging on exposure, unchanging with 10% KOH, unchanging on white tissue paper, soon turning yellow on a white cotton handkerchief; taste mild to slightly astringent. *Macrochemical reaction* no reaction on the context with 10% KOH, but becoming brownish (5E7) to light brown (6D6) with $FeSO_4$ after a while.

Basidiospores globose to broadly ellipsoid, 6.3−*6.9*−*7.1*−7.8(−7.9) × 5.7−*6.1*−*6.3*−6.8(−7.0) µm; Q = 1.02−*1.10*−*1.13*−1.19 (n=60); ornamentation amyloid, up to 1.2 µm high, composed of irregular to crenulate ridges, obtuse, forming an incomplete reticulum; isolated warts common; plage distally amyloid. *Basidia* 48−54 × 12−18 µm, 4-spored, clavate, with fine granules and guttate contents. *Pleuromacrocystidia* abundant, 54−100 × 11−16 µm, protruding up to 30 µm, irregular narrowly fusiform, slender, thin-walled, partially with granular and needle-like or guttate contents; apex mucronate, occasionally ramified. *Pleuropseudocystidia* abundant, 4−6 µm diam., not protruding, cylindrical to broadened at apex, tortuous. *Lamellar edge* heterogeneous, with basidia, cylindrical to subclavate, thin-walled marginal cells 14−28 × 6−10 µm,; cheilocystidia scarce to fairly abundant, 37−58 × 10−14 µm, slightly protruding up to 10 µm, narrowly fusiform, with mucronate apex, with granules. *Lamellar trama* consisting of abundant lactifers and sphaerocysts. *Pileipellis* an ixocutis to ixotrichoderm, covered by a thin glutinous layer; upper layer 150−200 µm thick, composed of repent to erect hyphae; subpellis composed of cylindrical and inflated hyphae with a few globose cells.

Habitat: gregarious or scattered on ground, in coniferous forest with *P. kesiya*.

Studied material: Vietnam, Lam Dong province, Lac Duong district, Xa Lat, Lang Biang National park, alt. 1545 m, N12º01.57 E108º25.58 , 12/06/2011, KW032 (holotype, GENT!, isotype, MFLU!);−ibid., 12/06/2011, KW033 (GENT!, MFLU!); −ibid., 12/06/2011, KW034

(GENT!, MFLU!);–ibid., 12/06/2011, KW035 (GENT!, MFLU!);–ibid., 12/06/2011, KW036 (GENT!, MFLU!); Thailand, Chiang Rai province, Mae Fah Luang district, Doi Mae Salong Nok sub-district, Doi Mae Salong, N20º08.67 E99º40.17, alt. 1015 m, 15/05/2012, KW207 (GENT!, MFLU!);–ibid., 19/05/2012, KW210 (GENT!, MFLU!),–ibid., 24/05/2012, KW219 (GENT!, MFLU!);–ibid., 30/05/2012, KW224 (GENT!, MFLU!);–ibid., 13/07/2012, KW353 (GENT!, MFLU!);–ibid., 13/07/2012, AV12-022, (GENT!, MFLU!); Mae Hong Son province, coniferous forest along highway 1095, near Huai Nam Dang national park, alt. 1322 m, N19º16.07 E98º37.86, 08/07/2012, KW346 (GENT!, MFLU!); Lampang province, Muangparn district, Chaesorn sub-district, forest along highway 1252, N18º55.43 E99º23.40, alt. 1420 m, 15/06/2013, KW427 (GENT!, MFLU!).

Comments: *Lactarius kesiyae* grows in coniferous forests dominated by *P. kesiya*. It can be recognized by its sticky and glossy appearance in moist conditions, and its pale brownish grey to pale brownish orange colour with greenish or orange to even pinkish tints on the pileus. The latex is watery white and turns yellow on a white cotton handkerchief. Microscopically, the pileipellis is an ixotrichoderm and the pleuromacrocystidia, which are up to 100 µm long in length, protrude conspicuously from the hymenium.

Microscopically, the sticky pileus is reflected as an ixocutis and it is not a common character for this subgenus. A sticky pileus occurs in a few temperate species such as *L. decipiens*, *L. duplicatus*, and *L. badiosanguineus* Kühner & Romagn., and it seems to be less common in tropical Asia. There are two tropical Asian species recorded as having a thin slime layer in the pileipellis stucture, *L. austrotabidus* Verbeken & E. Horak, with an ixocutis or ixotrichoderm and *L. inconspicuus* with an ixotrichoderm. In the field *L. kesiyae* could be confused with representatives of *L.* subg. *Lactarius* (syn. *L.* subg. *Piperites* (Fr.) Kauffman) on account of this surface feature which is one of the dominant characters of that subgenus. However, the overall brownish orange colours of *L. kesiyae* suggest it is a member of subg. *Russularia*.

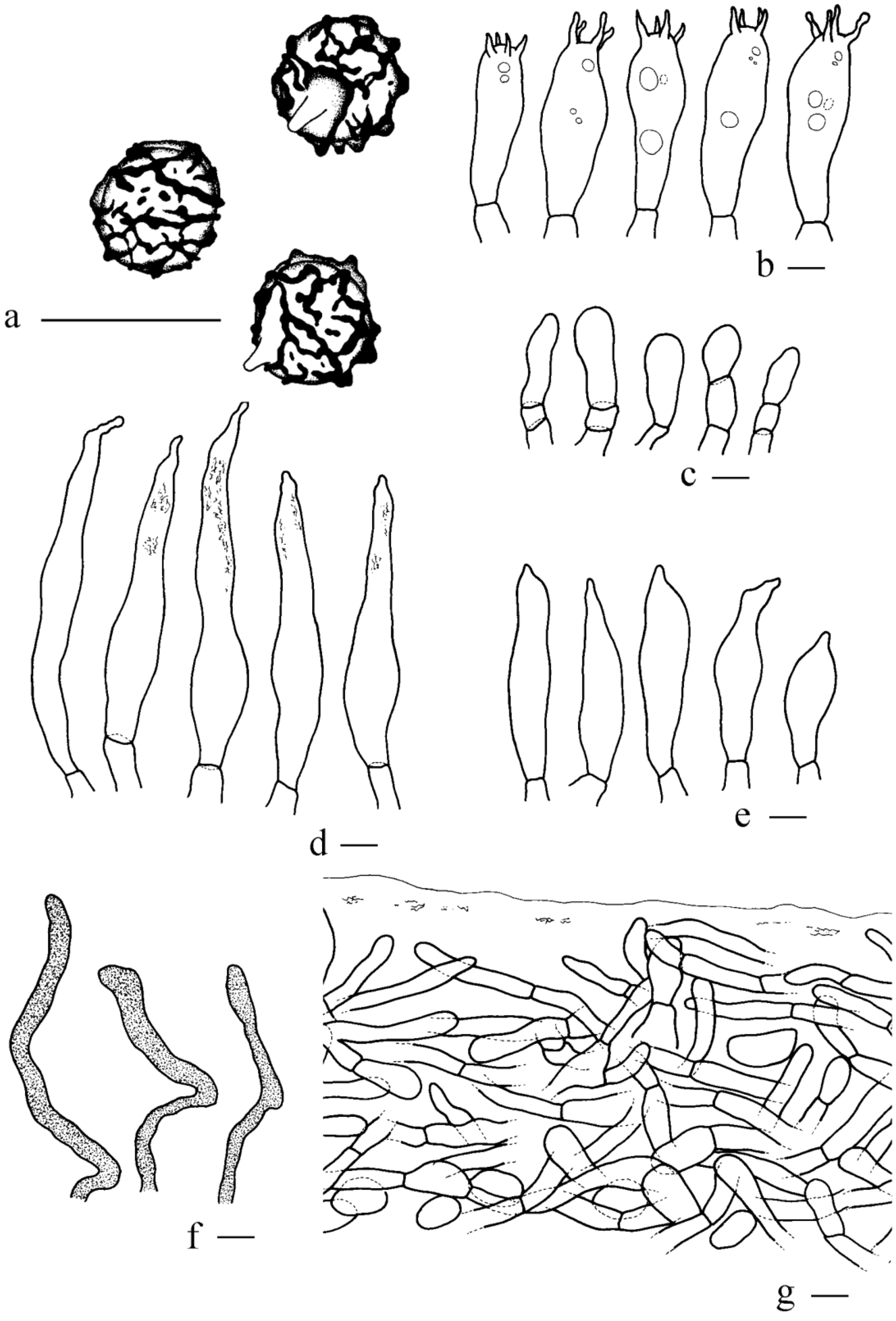


FIGURE 4.7: *Lactarius kesiyae*: a. basidiospore, b. basidia, c. marginal cell, d. pleuromacrocystidia, e. cheilocystidia, f. pseudocystidia, g. pileipellis (a–g: KW353) (scale bar = 10 µm).

Lactarius rubrobrunneus H.T. Le & Nuytinck, sp. nov. (Figure 4.8 and 4.11c)

MycoBank number: MB811746; **Facesoffungi number:** FoF00639

Diagnosis: a medium sized species with reddish brown cap, incompletely reticulate basidiospores, pleuromacrocystidia rare, pileipellis an epithelium to hyphoepithelium.

Etymology: 'rubrobrunneus' refers to the reddish brown colour of the cap.

Typus: Thailand, Chiang Mai province, Mae-On district, Huaikaew sub-district, Bahn Mae Kampong, N18º51.43 E99º22.09, alt. 1450 m, 15/07/2012, KW356 (AV12-044) (holotype, GENT!, isotype, MFLU!).

Basidiocarps medium sized. *Pileus* 15−80 mm diam., convex to widely depressed or infundibuliform, papillate; surface dry, greasy, slightly rugulose, dark brown (8F5−6) in center, reddish orange (7A6−8) to light reddish brown to yellow-brown (6C6−7) to brown (6D7) at the margin. *Lamellae* 1−4 mm broad, decurrent, crowded, with 3−4 series of lamellulae, forked, cream to greyish orange (5B4−5), sometimes paler, discolouring with reddish brown spots in age. *Stipe* 50−105 × 2−8 mm, cylindrical to tapering upwards, central, dry, smooth, whitish to pale cream pruinose at apex, light brown (6D4) to brown (6E7) to dark brown (6F6), fistulose, hairy at base. *Context* 1−3 mm thick in pileus, cream to brownish cream (5B4−5), odor strong, reminiscent of *L. quietus* or Pentatomidae bugs; taste mild. *Latex* watery white to white, abundant, unchanging on exposure, unchanging on white tissue paper and on a white cotton handkerchief and with 10 % KOH, taste mild. *Macrochemical reaction* no reaction on context with 10% KOH, or with $FeSO_4$.

Basidiospores subglobose to broadly ellipsoid, 6.1−*6.6*−*6.9*−7.4(−7.5) × 5.6−*6.0*−*6.3*−6.9(−7.0) µm, Q = 1.05−*1.10*−*1.11*−1.23 (n = 40); ornamentation amyloid, an incomplete reticulum up to 1 µm high,, consisting of blunt and irregular ridges, connected by thinner ridges, isolated warts common; plage inamyloid to distally amyloid. *Basidia* 41−57 × 12−16 µm, 4-spored, some 2-spored, subclavate, with guttate contents. *Pleuromacrocystidia* rare, 38−50 × 10−14 µm, not protruding, with guttate contents. *Pleuropseudocystidia* abundant, slightly protruding, 3−7 µm diam., with fine granules, irregular to tortuous, sometimes with broadened apex. *Lamellar edge* heterogeneous, consisting of basidia and marginal cells without cheilocystidia, marginal cells abundant, 15−36 × 5−12 µm, mostly cylindrical to subcylindrical to subclavate. *Lamellar trama* a combination of globose cells, septate hyphae and lactiferous hyphae. *Pileipellis* a transition between epithelium and hyphoepithelium, with or without a thin layer of about 5−10 µm thick, repent hyphae, subpellis layer consisting of small to large, globose cells, up to 35 µm diam., layer about 70−100 µm thick.

Habitat: gregarious in montane tropical forests with Fagaceae.

Studied material: Thailand, Chiang Mai province, Jomthong district, Bahn Luang sub-district, Doi Inthanon National Park, junction of highway 1009 and road to Mae Cham, N19º31.58 E98º29.64, alt. 1703 m, 25/06/2004, LTH149 (GENT!, CMU!, SFSU!); Mae Hong Son province, Huai Nam Dang National Park, nature trail, alt. 1538 m, N19º18.29 E98º35.88, 29/06/2005, LTH334 (GENT!, CMU!, SFSU!); Chiang Mai province, Mae-On district, Huaikaew sub-district, Bahn Mae Kampong, N18º51.43 E99º22.09, alt. 1450 m, 15/07/2012, KW356 (AV12-044) (holotype, GENT!, isotype, MFLU!).

Comments: *Lactarius rubrobrunneus* is recognized by its reddish brown to orange-brown cap with a darker brown shade in the center. *Lactarius rubrobrunneus* differs from *L. fuscomaculatus* by the lack of dark brown spots on cap. Compared to *L. fuscomaculatus*, all collections of *L. rubrobrunneus* have a thinner layer of terminal hyphae in the pileipellis, which is a transition between an epithelium and a hyphoepithelium. In addition, we have observed cheilocystidia in *L. fuscomaculatus* collections, while they are completely absent in *L. rubrobrunneus*.

Lactarius rubrocorrugatus Wisitrassameewong & Nuytinck, sp. nov. (Figure 4.9 and 4.11i)

MycoBank number: MB811747; **Facesoffungi number:** FoF00640

Diagnosis: A small to medium sized species, cap red to reddish brown with a rugulose surface, latex transparent, basidiospore ornamentation consisting of low ridges forming an incomplete reticulum, pleuromacrocystidia present and pileipellis a hyphoepithelium.

Etymology: ‘rubrocorrugatus’ refers to the red and wrinkled pileus.

Typus: Thailand, Chiang Rai province, Muang district, Thasai sub-district, forest at Doi Pui Reverse Signal Station, Doi Pui, alt. 740 m, N19º49.00 E99º52.03, 31/07/2012, KW384 (holotype, GENT!, isotype, MFLU!).

Basidiocarps small to medium sized. *Pileus* 7−44 mm diam., plane to infundibuliform, papillate initially, becoming depressed with or without a papilla; surface dry, smooth in immature specimens, later becoming wrinkled in mature specimens, hygrophanous, red

(9B7−8) to reddish brown (9D7−8−E8), typically with dark brown (7F5) shade in center; margin not striate in young specimens, becoming slightly striate in age, incurved, crenulate. *Lamellae* decurrent, 1−2 mm broad, very crowded, sometimes forked, with 1−3 series of lamellulae, yellowish white to light yellow (4A4) to cream, turning brown (6E5−E6) when

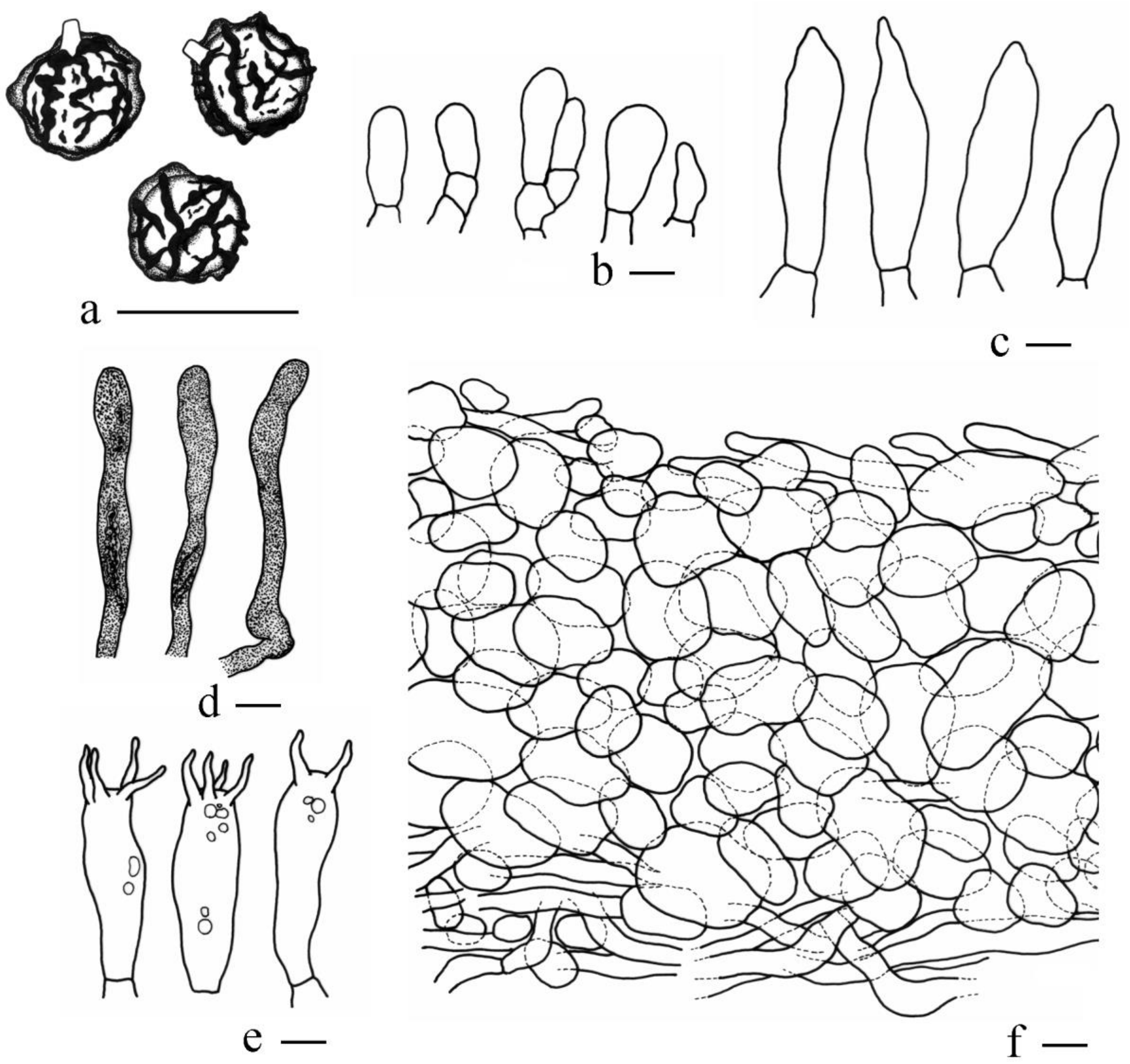


FIGURE 4.8: *Lactarius rubrobrunneus*: a. basidiospore, b. marginal cell, c. pleuromacrocystidia, d. pseudocystidia, e. basidia, f. pileipellis (a−f: AV12-044, holotype) (scale bar = 10 µm).

bruised; edge slightly crenulate to even. *Stipe* 11−42 × 2−8 mm, cylindrical, fistulose; surface dry, smooth to slightly wrinkled, brownish orange (7C7) to brown (7E7−E8), turning dark brown (7F8) when older, whitish pruinose at base. *Context* 1−3 mm broad in pileus, pale yellow (4A3) to cream, unchanging when cut; odor reminiscent of *L. quietus* or Pentatomidae bugs; taste mild, sometimes sweetish and bitter. *Latex* transparent, unchanging on exposure, unchanging on tissue paper and on a white cotton handkerchief. *Macrochemical reaction* on the context: unchanging or slowly turning pale yellow with 10 % KOH, greyish green (26E6) with $FeSO_4$.

Basidiospores globose to broadly ellipsoid, 5.8−*6.4−7.0*−7.9 × 5.2−*5.8−6.3*−6.9 µm; Q = 1.01−*1.09−1.13*−1.25 (n=120); ornamentation amyloid, composed of ridges up to 0.7 µm high, forming an incomplete reticulum; isolated warts common, sometimes clustered; plage inamyloid to distally amyloid. *Basidia* 45−73 × 9−18 µm, mostly 4-spored, sometimes 1-spored, subclavate to subcylindrical, with guttate contents. *Pleuromacrocystidia* 44−87 × 11−20 µm, abundant, not protruding to protruding up to 20 µm, subfusiform, with a mucronate to moniliform apex, with granules and guttate contents. *Pleuropseudocystidia* abundant, 3−6 µm diam., cylindrical, tortuous, with fine granules. *Lamellar edge* heterogeneous, with basidia, marginal cells and cheilocystidia; marginal cells 24−40 × 8−15 µm, subcylindrical, subclavate to clavate; cheilocystidia 25−51 × 7−13 µm, rare to abundant, subfusiform, bent or irregular, with a mucronate to moniliform apex, with granular contents. *Lamellar trama* consisting of globose cells, septate hyphae and lactiferous hyphae. *Pileipellis* a hyphoepithelium, with an upper layer of repent to oblique hyphae; suprapellis a thin layer of repent hyphae about 10−25 µm thick; subpellis 40−60 µm thick, consisting of globose cells up to 30 µm diam.

Habitat: gregarious on soil, in coniferous forest, dominated by *P. kesiya* and in tropical rain forests, dominated by *Shorea* sp., *Quercus* sp. and *Castanopsis* sp.

Studied material: Vietnam, Dalat province, Xa Xuan Truong, at km 7, left side of the main road, alt. 1499 m, N11º54.94 E108º32.01, 13/06/2012, KW042 (GENT!, MFLU!);−ibid. 13/06/2012, KW043 (GENT!, MFLU!);−ibid. 13/06/2012, KW045 (GENT!, MFLU!); Thailand, Chiang Mai province, Mae Taeng District, Pa Pae sub-district, Bahn Pha Deng village, Pathummikaram Temple, forest trail, alt. 1050 m, N19º06.28’ E98º44.47, 9/06/2012, KW294 (GENT!, MFLU!); Chiang Rai province, Muang district, Thasai sub-district, forest at

Doi Pui Reverse Signal Station, Doi Pui, alt. 740 m, N19º49.00 E99º52.03, 31/07/2012, KW381 (GENT!, MFLU!);–ibid. 31/07/2012, KW384 (holotype, GENT!, isotype, MFLU!);–ibid., 08/07/2013, KW453 (GENT!, MFLU!); Chiang Rai province, Chiang Khong district, forest near Bahn Nurnsomboon, alt. 450 m, N20º07.77 E100º26.40, 26/08/2011, KW123 (GENT!, MFLU!), Chiang Mai province, Mae Taeng District, Bahn Thapa, 22 marker on highway 1095, alt. 750 m, N19º07.41 E98º45.57, 31/07/2014, EDC14-505 (GENT!, MFLU!); Loei province, Phu Ruea district, junction from highway 203, alt. 1045 m, N17º28.09 E101º26.18, 27/06/2013, KW443 (GENT!, MFLU!).

Comments: *Lactarius rubrocorrugatus* can be easily distinguished from the other Southeast Asian species with transparent latex because of its small size, and a hygrophanous and rugulose cap. The other species with transparent latex described in the present work are *L. aquosus* and *L. tangerinus*. *Lactarius aquosus* differs from *L. rubrocorrugatus* by the zonate, smooth cap and a cutis for a pileipellis. *Lactarius tangerinus* has a reddish orange cap and a trichoderm pileipellis. *Lactarius rubrocorrugatus* is also similar to the European *L. rostratus* Heilmann-Clausen because of the characters mentioned above. The major difference can be found in the macrocystidia. *Lactarius rubrocorrugatus* has larger cystidia and the apex is not as acute as in *L. rostratus*. *Lactarius rubrocorrugatus* has been found in Vietnam and Thailand. The putative host range of the species is broad. The Vietnamese specimens were found in coniferous forest dominated by *P. kesiya* at higher altitude, around 1500 m above sea level, while the Thai specimens were found in evergreen forests dominated by members of the Fagaceae at lower altitude (alt. between 700-1000 m).

Lactarius tangerinus H.T. Le & De Crop, sp. nov. (Figure 4.10, 4.11e and 4.11f)

MycoBank number: MB811748; **Facesoffungi number:** FoF00641

Diagnosis: a small to medium sized species with reddish brown to reddish orange cap and a paler margin, watery latex, incompletely to almost completely reticulate basidiospores, pleuromacrocystidia present and pileipellis a trichoderm.

Etymology: 'tangerinus' refers to the reddish orange colour on cap.

Typus: Thailand, Chiang Mai province, Mae Teang district, Pa Pae sub-district, Bahn Pha Deng village, mushroom research center, N19º17.12 E98º44.00, alt. 900 m, 28/07/2004, LTH 203 (holotype GENT!, isotype, CMU!, isotype, SFSU!).

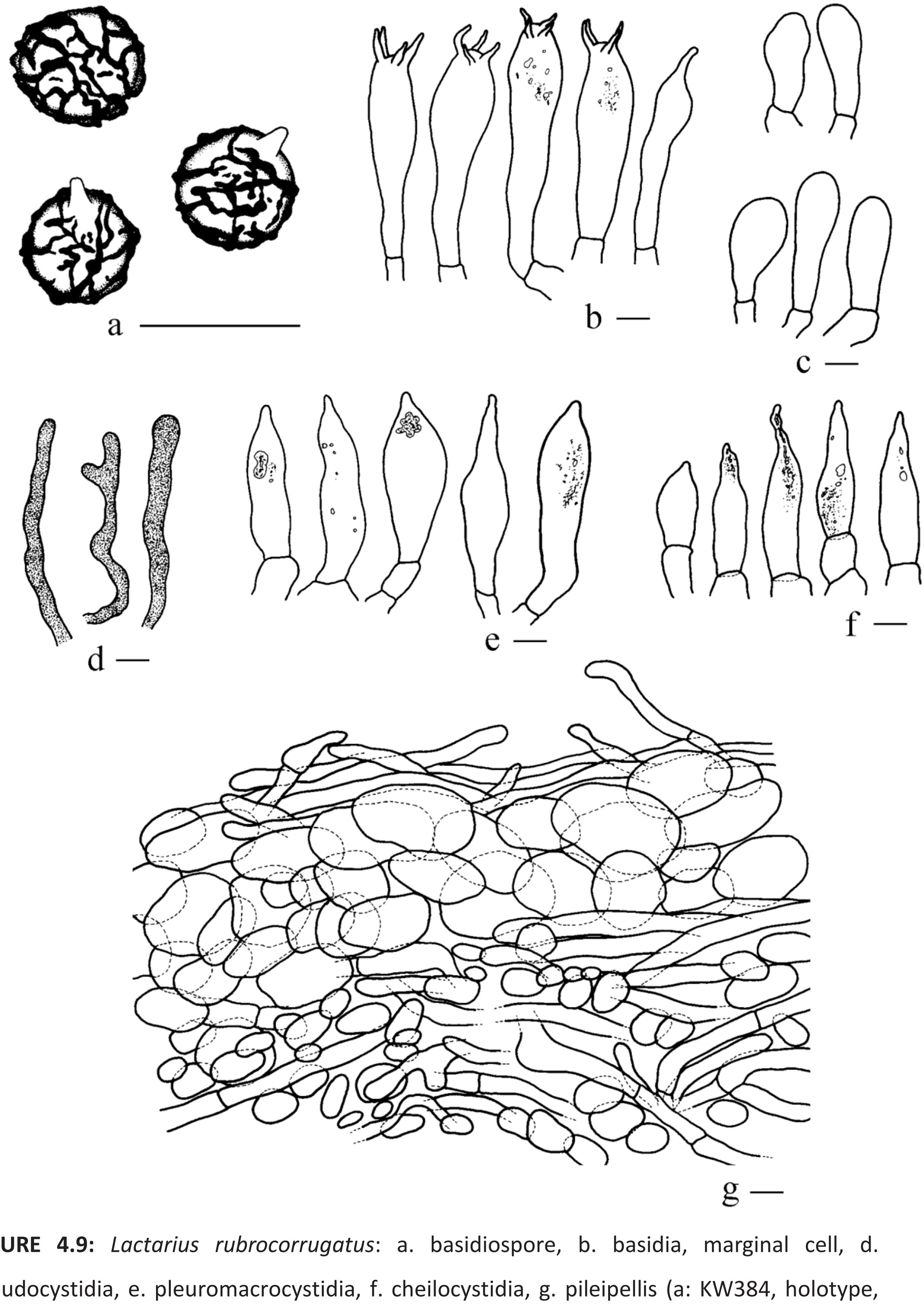
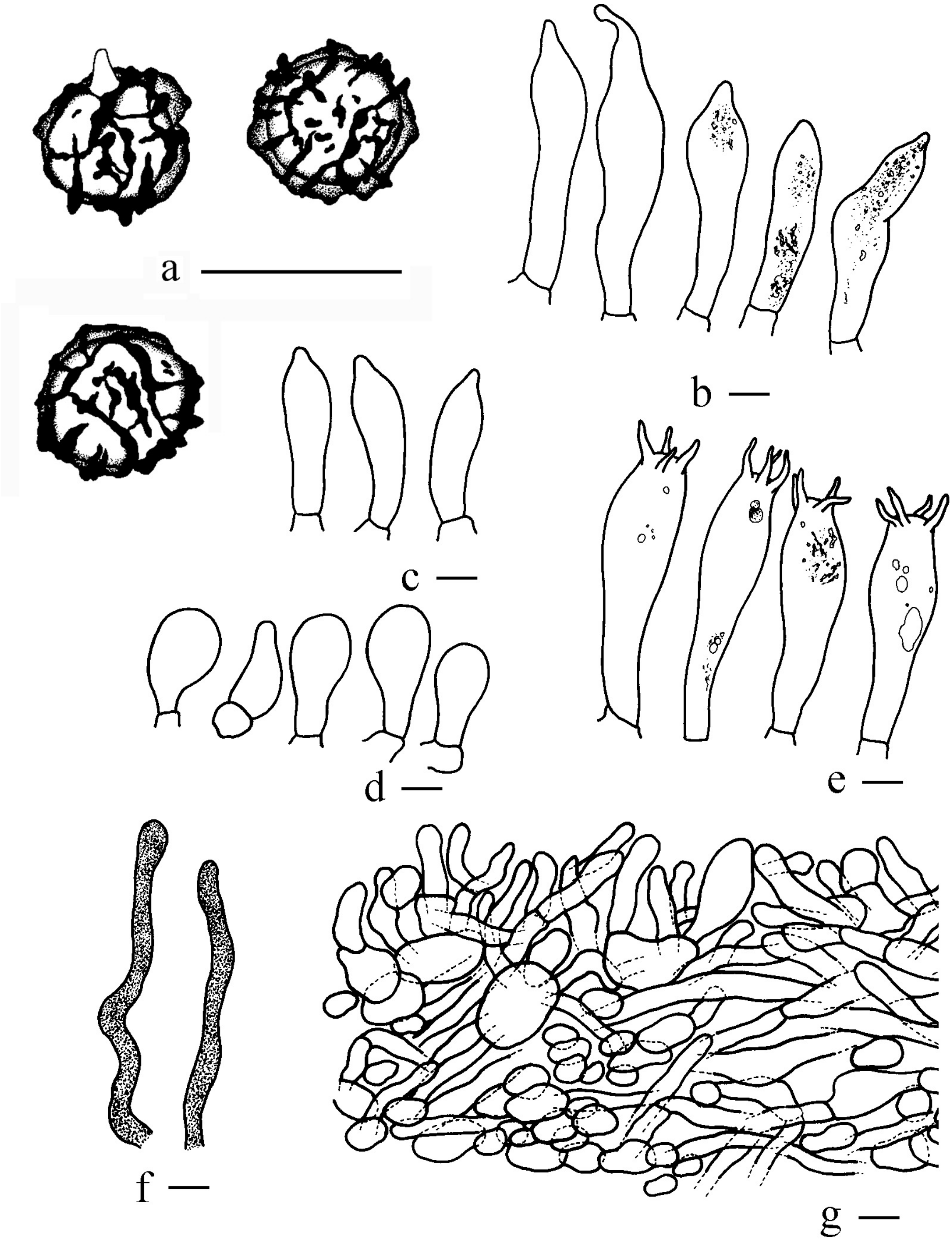


FIGURE 4.9: *Lactarius rubrocorrugatus*: a. basidiospore, b. basidia, marginal cell, d. pseudocystidia, e. pleuromacrocystidia, f. cheilocystidia, g. pileipellis (a: KW384, holotype, b–g: KW294) (scale bar = 10 µm).



FIGRUE 4.10: *Lactarius tangerinus*: a. basidiospore, b. pleuromacrocystidia, c. cheilocystidia, d. marginal cell, e. basidia, f. pseudocystidia, g. pileipellis (a–g: LTH203, holotype) (scale bar = 10 µm).

Basidiocarps small to medium sized. *Pileus* 8−36 mm diam., plane to slightly infundibuliform with a central depression; surface rugulose, typically darker in center, reddish brown (8E8−F8) to dark brown (7F8), reddish blond (6C6), to beige, paler towards the margin, orange-yellow (4A3−5), light yellow (4A5) at the margin; margin striate at maturity. *Lamellae* 1.5−4 mm broad, decurrent with tooth, crowded, with 3−4 series of lamellulae, yellowish white (4A1−2) to light yellow (4A4) to greyish orange (5B5). *Stipe* 10−36 × 2−6 mm, cylindrical to tapering downwards, central to eccentric; surface dry, smooth, rugose when old, with paler colour at apex, greyish orange (6B4) to pale orange (5A3) at apex, brownish orange (6C4), light brown (6D4), light brown (7D7−8), whitish pruinose at the base solid to hollow. *Context* 1−2 mm broad in the pileus, pale pinkish cream (5A3) to grey cream (5A−B3); odor like *L. quietus* or Pentatomidae bugs; taste mild. *Latex* transparent, abundant, unchanging on exposure, unchanging with 10 % KOH; taste mild. *Macrochemical reaction* on context: becoming pale greyish green with 10 % KOH, light greyish blue with $FeSO_4$.

Basidiospores subglobose to broadly ellipsoid, (5.8−)6.1−*6.6−7.2*−7.9 × 5.4−*6.1−6.3*−7.0 (−7.2) µm; Q = 1.02−*1.09−1.13*−1.25 (n=40); ornamentation amyloid, up to 1.8 µm high, composed of blunt to subacute, thick irregular ridges, forming an incomplete to almost complete reticulum; short ridges interconnected by fine lines; plage inamyloid to slightly amyloid. *Basidia* 30−65 × 9−18 µm, 4-spored, mostly subclavate, rarely subcylindrical, with fine granules and guttate contents. *Pleuromacrocystidia* not abundant, 35−77 × 10−18 µm, subcylindrical to subclavate, with mucronate apex, protruding up to 10 µm. *Pleuropseudocystidia* 2−4 µm broad, not protruding, tortuous to straight, cylindrical, with fine granules. *Lamellar edge* heterogeneous, consisting of basidia, subcylindrical to subclavate to obovoid marginal cells 15−30 × 4−15 µm, and a few cheilocystidia 39−49 × 11−15 µm, not protruding, subclavate, with mucronate apex. *Lamellar trama* composed of cylindrical hyphae, lactiferous hyphae and sphaerocysts. *Pileipellis* a trichoderm, about 35−50 µm thick, consisting of erect cylindrical hyphae, subpellis mainly with small globose cells, around 10−20 µm diam., intermixed with hyphae.

Habitat: solitary or gregarious on soil among leaf litter, near *Lithocarpus thomsonii* and *L. elegon* stands.

FIGURE 4.11: Basidiocarps: a. *L. aquosus* (KW231), b. *L. fuscomaculatus* (KW373), c. *L. rubrobrunneus* (AV12-044, photo by Annemieke Verbeken), d. *L. inconspicuus* (KW339, photo by Felix Hampe), e-f. *L. tangerinus* (EDC14-475, photo by Eske De Crop), g-h. *L. kesiyae* (g: KW032, h: KW353), i. *L. rubrocorrugatus* (KW384), j. *L. chichuensis* (KW271).

Studied material: THAILAND, Chiang Mai province, Mae Teang district, Pa Pae sub-district, Bahn Pha Deng village, mushroom research center, N19º17.12 E98º44.00, alt. 900 m, 28/07/2004, LTH 203 (holotype GENT!, isotype, CMU!, isotype, SFSU!);–ibid., 13/08/2004, LTH217 (GENT!, CMU!, SFSU!);–ibid., 01/07/2011, KW091 (GENT!, MFLU!); – ibid., 28/07/2014, EDC14-475 (GENT!, MFLU!).

Comments: Immature basidiocarps of this species might be mistaken for *L. fuscomaculatus* or *L. inconspicuus*. However, *L. fuscomaculatus* has watery white latex and develops dark brown spots on the cap in mature specimens. The latex also separates *L. tangerinus* from *L. inconspicuus*. The latter species has watery white latex that slowly turns yellowish white or pale yellow. For the microscopic differences between both species, see under *L. inconspicuus*.

Identification key to the Southeast Asian *Lactarius* subg. *Russularia* species

1. Pileus rather small sized, an average size around 20 mm……………………………………………(2)

1. Pileus medium sized ……………………………………………………………………………(5)

2. Pileus margin typically with triangle tufts……………………………………*Lactarius gracilis*

2. Pileus margin without tufts……………………………………………………………………(3)

3. Basidiospores complete reticulum; pleuromacrocystidia absent; stipe mostly with whitish pruinose at base……………………………………………………………*Lactarius crenulatulus*

3. Basidiospores incomplete reticulum; pleuromacrocystidia present; stipe mostly with strigose at base……………………………………………………………………………(4)

4. Pileus smaller dimension (5−7 mm diam.), with or without an inconspicuous papilla; pileipellis a cutis ……………………………………………………………*Lactarius perparvus*

4. Pileus larger dimension (5−40 mm diam.), with an acute papilla; pileipellis an epithelium ……………………………………………………………*Lactarius glabrigracilis*

5. Distant lamellae; pileus with sulcate striations……………………………………………(6)

5. Crowded lamellae; pileus without sulcate striations………………………………………(9)

6. Pleuromacrocystidia present…………………………………………………………………(7)

6. Pleuromacrocystidia absent …………………………………………………………………(8)

7. Pleuromacrocystidia strikingly protruding from the hymenium; basidiospores complete reticulum………………………………………………………*Lactarius sublaccarioides*

7. Pleuromacrocystidia not protruding from the hymenium; basidiospores incomplete reticulum……………………………………………………………*Lactarius stubbei*

8. Pileus surface non-velutinous; stipe long (26−71 mm in length) and often very hispid ……………………………………………………………*Lactarius laccarioides*

8. Pileus surface minutely velutinous; stipe shorter (9−22 mm in length) and hairs only at the base...*Lactarius pasohensis*

9. Latex transparent, completely watery and unchanging on exposure...(10)

9. Latex watery white to white...(13)

10. Pileus with inconspicuous zonation, reddish brown to brown, paler towards margin; lamellae discolouring brown or with brown spots; pileipellis a cutis...***Lactarius aquosus*** **sp. nov.**

10. Pileus without zonation..(11)

11. Pileipellis a trichoderm...***Lactarius tangerinus*** **sp. nov.**

11. Pileipellis a hyphoepithelium...(12)

12. Pleuromacrocystidia with a rostrate apex, remarkably protruding up to 40 µm...***Lactarius austrorostratus*** **sp. nov.**

12. Pleuromacrocystidia different; basidiospores with low ornamentation (less than 1 µm high), an incomplete reticulum; pileus rugulose...***Lactarius rubrocorrugatus*** **sp. nov.**

13. Pileipellis covered with a thin slime layer..(14)

13. Pileipellis without a thin slime layer..(15)

14. Latex watery white, unchanging on exposure, turning yellow on a white handkerchief, associated with conifers (*Pinus*)..***Lactarius kesiyae*** **sp. nov.**

14. Latex watery white, slowly turning to pale yellow on exposure; growing with *Castanopsis*...***Lactarius inconspicuus*** **sp.nov.**

15. Basidiospore ornamentation zebroid, ridges up to 1 µm high..*Lactarius chichuensis*

15. Basidiospore ornamentation incomplete reticulum, ridges up to 1 µm high...(16)

16. Pileipellis a hyphoepithelium; pileus reddish brown to orange-brown with distinct dark brown discolourations and spots...***Lactarius fuscomaculatus*** **sp. nov.**

16. Pileipellis a transition between hyphoepithelium and epithelium; pileus more unicolourous, reddish orange to reddish brown...***Lactarius rubrobrunneus*** **sp. nov.**

Discussion

This contribution is the third in a series of publications reporting the diversity of *L.* subg. *Russularia* in Southeast Asia. According to the species concept stated in de Queiroz (2007), speciation occurs when a lineage acquires genotypic divergence and different recognizable characters. Thus, species delimitation is ideally based on the concordance of morphological characters and molecular evidence. Including the previous studies of Wisitrassameewong et al. (2014a, 2014b), we have described 15 new species of *L.* subg. *Russularia* from mycological expeditions in Thailand, Malaysia and Vietnam. Most of them grow in broadleaf evergreen forests with members of the Fagaceae, except for the conifer associated taxon, *L. kesiyae*.

Relatively few distinct field characters could be defined and applied for all described species because of subtle macromorphological differences among species on this subcontinent. *Lactarius fuscomaculatus* and *L. rubrobrunneus* seem to form one of the most complex groups. These two species can be easily confused in the field and are also closely related in our phylogenetic analysis. *Lactarius fuscomaculatus* mainly differs from *L. rubrobrunneus* by the dark brown spots on its pileus and the subtle difference in the terminal layer of the pileipellis. Although most Southeast Asian taxa are superficially similar in their basidiocarp colour and latex features, which could lead to confusion in field identification, we consider none of the Southeast Asian species to be morphologically cryptic (as described in the part of identification keys). All 15 described species are well-delimited using ITS sequence data and the molecular evidence is consistent with their morphological differences. Distant gills, sulcate pileus striation and sticky pileus surface can be used as field characters in some species. We notice that representatives with a sticky surface seem to be rare, at least in this region. Considering the low variety in latex features, this character is not particularly useful to differentiate among species. Basidiospore ornamentation, presence or absence of true cystidia and pileipellis structure appear to be more reliable for species delimitation. From our experience, many representatives possess basidiospores with an incomplete to almost complete reticulum while a complete reticulum, isolated warts and zebroid ornamentation sometimes occur. The absence of true cystidia arose during a speciation event in several taxa. The presence of a thin slime layer and presence or absence of terminal hyphal elements in the pileipellis are considered important characters.

The molecular analysis involves specimens from Asia, North America and Europe. So far all Southeast Asian taxa are endemic to the subcontinent and no intercontinental conspecificity with temperate representatives has been found (Figure 4.1). All new species are well-supported in molecular phylogram and we found concordance between field characters, microscopic characters and phylogenetic positions. The closest relative of *L. austrorostratus* is *L. chichuensis* and the newly proposed species is sister to the clade that includes *L. rostratus* and *L. rubrocorrugatus*. *Lactarius rubrocorrugatus*, *L. kesiyae* and *L. tangerinus* form distinct groups in the phylogram. *Lactarius aquosus* and the European *L. quietus* split from the same ancestor. Although both species are phylogenetically related and have an inconspicuous pileus zonation, other features are different. *Lactarius quietus* is easy to recognize due to its pinkish buff cap and strong Pentatomidae bug odour (Heilmann-Clausen et al., 1998). Microscopically, *L. aquosus* has a cutis and *L. quietus* has a trichopalisade as a pileipellis. *Lactarius inconspicuus* falls within a clade with *L. decipiens*. Both species have similar characters, such as yellowing milk, large and acute pleurocystidia and a thin glutinous layer with erect terminal hyphae in the pileipellis. *Lactarius decipiens* mainly differs from the Asian taxon by the pinkish fruitbody, the odour of *Pelargonium* and reticulate basidiospore ornamentation.

The results in this stdy and recent studies suggest that the diversity of *L.* subg. *Russularia* in Southeast Asia is higher than we previously thought. We emphasize that tropical rainforests in this continent comprise many ectomycorrhizal trees and a large diversity of *Lactarius* species. However, many regions in this continent are undersampled. DNA sequence data of several known Southeast Asian taxa are lacking. Further expeditions are necessary to explore undescribed indigenous species in undersampled areas and to obtain more sample collections and molecular data of known Asian species. Additional gene markers are necessary in order to better resolve the evolutionary relationship between species.

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CHAPTER 5

TWO NEW SPECIES FROM THAILAND

Abstract

This paper is a part of a large paper on the diversity of 110 fungal taxa, including one new family, 10 new genera, and 76 new species, representing a wide taxonomic and geographic range. Two new species of *L.* subg. *Russularia* are also introduced: *L. atrobrunneus* and *L. politus*. A full description of each species is provided with light micrographs (or drawings). Molecular data is provided for 90 taxa and used to generate phylogenetic trees to establish a natural classification for species.

Keywords: Ascomycota, Basidiomycota, Global Taxonomy Initiative, Phylogeny, Taxonomy.

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Introduction

The estimated number of described fungi is 100000 (including 17500 lichens), but only approximately 1000 new species are described annually (Kirk et al. 2008). The total number of fungi is, however, estimated at between 700000 to 1.5 million species (Hawksworth 1991, 2001; Schmit & Mueller 2007) or even 5.1 million species (Blackwell 2011). The majority of the undescribed fungal species are expected to be discovered in poorly studied areas (Hyde et al. 2001), such as in tropical forests or in under-explored habitats, for example living on or in insects, plants or lichens (Hawksworth & Rossman 1997; Fröhlich & Hyde 1999; Taylor et al. 2000; Sipman & Aptroot 2001; Lawreya & Diederich 2003; Arnold & Lutzoni 2007). Phylogenetic studies are revealing numerous new genera and species (Alves et al. 2004, 2006; Crous et al. 2006, 2009a,b; Phillips & Alves 2009; Phillips et al. 2008, 2013; de-Gruyter et al. 2010; Liu et al. 2011, 2012; Maharachchikumbura et al. 2012). Before molecular phylogenetics, most fungal species have been described based on their morphology and host associations and thus the number of species is understandably underestimated. Phylogenetic studies have indicated that numerous morphologically similar taxa may be represented as distinct lineages in different families, e.g. Botryosphaeriaceae (Liu et al. 2012; Phaeosphaeriaceae (Phookamsak et al. 2014), Didymosphaeriaceae (Ariyawansa et al. 2014). Cryptic species of plant pathogens have been shown to comprise several taxa, e.g. *Colletotrichum* (Hyde et al. 2009, Sharma et al. 2014), *Pestalotiopsis* (Maharachchikumbura et al. 2011, 2012, 2014), *Diaporthe* (Udayanga et al. 2011, 2012). Traditionally, systematic studies, papers and monographic revisions have just one or a few authors, but the aforementioned collaborative initiatives have initiated a mind-shift with a multi-authored, community-wide classification for the fungal kingdom (Hibbett et al. 2007). In addition, the mycological community has several, regularly updated fundamental resources for species and higher level classification and nomenclature available, such as Myconet (http://www.fieldmuseum.org/myconet), Index Fungorum (http://www.indexfungorum.org) and other CABI Bioscience databases, and the Dictionary of the Fungi (Kirk et al. 2008).

Materials and methods

The phylogenetic analyses were performed based on up to date ex-type, ex-epitype or otherwise authentic sequence data available in GenBank as a concerted effort of multiple contributors listed in the authors section. New and reference species were sequenced based on the genomic DNA which was extracted from the fresh mycelium except for lichenised and lichenicolous fungi and fungi not readily cultivatable, specimens in this case were used for direct extraction of DNA.

Gene sequences and genetic markers used for each genus were selected based on the current publications and have commonly been used for each of the genera. The single gene sequence alignments were initially aligned with ClustalX2 and improved in MAFFT V. 7.017 (Katoh et al. 2002) and BioEdit 7.0 (Hall 2004). Individual alignments were then concatenated and used to construct the backbone trees of each group listed.

The phylogenetic analyses were performed for maximum parsimony in PAUP v. 4.0b10 (Swofford 2002), maximum likelihood in RAxML 7.4.2 Black Box or RAxMl GUI (Stamatakis 2006; Stamatakis et al. 2008), PhyML 3.0 (Guindon et al. 2010) or Bayesian inference in MrBayes v.3.1.2 (Huelsenbeck and Ronquist 2001) as specified in the legend of each phylogenetic tree. The trees used to represent each order; family and genus were analyzed by multiple contributors based on the selection of genes in given publications under each description.

Material and methods for *Lactarius*

Morphological study

For macromorphological characters, specimens were described and photographed in fresh condition during daylight hours. The terminology of morphological characters of Vellinga (1988) and of Verbeken (1996) and Heilmann-Clausen *et al*. (1998) were used in this study. Colour coding is according to Kornerup and Wanscher (1978). Macro-morphological features of the basidiocarp were documented in term of size, shape and features of pileus, lamellae and stipe. Latex features were tested by recording colour when the latex was immediately exposed to the air, colour change after exposing to the air for a certain time, colour change when a drop of latex tested with 10% KOH and colour change when a drop of latex touched on white tissue paper and a white cotton handkerchief.

Microscopic features were studied from dried material, Most microscopic characters were observed in congo red solution in L4 (Clémençon 1973), except the basidiospore ornamentation. The spore measurement included at least 20 spores from each collection, and excludes the ornamentation. Basidiospore 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 collection, AVb = highest mean value for the measured collection and SD = standard deviation. Q corresponds to spore "length/width ratio" and is given as (MINQa) Qa–Qb (MAXQb), where Qa and Qb are the lowest and the highest mean ratio for a measured specimen, respectively.

Molecular study

Genomic DNA was extracted from fresh material stored in 2×CTAB buffer using the protocol described by Nuytinck & Verbeken (2003) with the modifications described in Van de Putte *et al*. (2010). The internal transcribed spacer of the nuclear ribosomal DNA (ITS) region was amplified using ITS1 and ITS4 primers (White *et al*. 1990, Gardes & Bruns 1993). Later DNA sequencing was done with an ABI 3730XL or ABI 3700 by MACROGEN (Amsterdam, the Netherlands).

Obtained ITS sequences were manually edited and assembled using the software Sequencer$^{\text{TM}}$ v5.0 (Gene Code corporation, Ann Arbor, Michigan, U.S.A.). Except the sequences of our newly proposed species, 18, 16 and 15 representatives for *L.* subg. *Russularia*, subg. *Lactarius* and subg. *Plinthogalus* of *Lactarius* are included in this study, respectively (Table 5.1). Three *Multifurca* spp. were used as the outgroups in this study. Nucleotide sequence alignment was made using MAFFT v7 (Katoh & Standley 2013) and later manually edited in MEGA6 (Tamura *et al*. 2013). Maximum likelihood (ML) algorithm was executed using RAxML v7.0.3, applying the Rapid Bootstrapping algorithm for 1000 replicates using the GTRGAMMA model (Stamatakis et al. 2008).

Results and discussion

The new species are described below in alphabetical order. They represent a total of 67 genera in 33 families, 17 orders and three classes in the Ascomycota. The individual taxon entries are standardized as far as possible, but reflect in style and content the diversity of participating authors and different groups of fungi.

Table 5.1. Specimens and GenBank accession numbers of DNA sequences used in the molecular analyses. Newly described species in this study are in boldface.

Species	Voucher collection	Origin	ITS accession no.
Lactarius* subg. *Russularia			
***L. atrobrunneus* sp. nov.**	KW270 (GENT, MFLU)	Thailand	KP744443
***L. atrobrunneus* sp. nov.**	KW347 (GENT, MFLU, Type)	Thailand	KP744442
***L. politus* sp. nov.**	KW107 (GENT, MFLU)	Thailand	KP744444
***L. politus* sp. nov.**	KW331 (GENT, MFLU)	Thailand	KF433020
***L. politus* sp. nov.**	KW351 (GENT, MFLU, Type)	Thailand	KF433019
***L. politus* sp. nov.**	KW396 (GENT, MFLU)	Thailand	KF433018
***L. politus* sp. nov.**	KW468 (GENT, MFLU)	Thailand	KP744445
L. subdulcis	KW134 (GENT)	Belgium	KF432969
L. hepaticus	JN02-049 (GENT)	Belgium	KF432980
L. fulvissimus	JKLAC10082001 (GENT)	Germany	KF432970
L. sphagneti	JKLAC11091502 (GENT)	Germany	KF432975
L. rubrocinctus	EDC12-210 (GENT)	Germany	KF432977
L. obscuratus	LVL02-006 (GENT)	Belgium	KF432978
L. badiosanguineus	AV04-235 (GENT)	France	KF432983
L. aurantiacus	JN2001-60 (GENT)	Slovakia	KF432974
L. lacunarum	JKLAC11092901 (GENT)	Germany	KF432982
L. tabidus	KW136 (GENT)	Belgium	KF432979
L. decipiens	AV2000-137 (GENT)	Italy	KF432973
L. camphoratus	AV10-40 (GENT)	Norway	KF432971
L. pasohensis	DS06-231 (GENT, KEP)	Malaysia	KF432987
L. hirtipes	Wang1243 (HKAS)	China	KF433007
L. gracilis	KW006 (GENT, MFLU)	Thailand	KF433015
L. laccarioides	KW336 (GENT, MFLU, Type)	Thailand	KF432991
L. sublaccarioides	KW323 (GENT, MFLU)	Thailand	KF432997
L. chichuensis	Wang1236 (HKAS)	China	KF475766
Lactarius* subg. *Lactarius			
L. purpureus	FH12-008 (GENT, MFLU)	Thailand	KF432966
L. scrobiculatus	JN01-058 (GENT)	Slovakia	KF432968
L. hatsudake	JN2011-065 (GENT)	Vietnam	KF432967
L. austrozonarius	FH12-007 (GENT, MFLU)	Thailand	KF432965
L. torminosus	RW3183 (GENT)	Czech Republic	KF133281
L. pubescens	AV96-931 (GENT)	Norway	AY336958
L. formosus	LTH382 (type, CMU, SFSU, GENT)	Thailand	EF141549

Table 5.1. Continued.

Species	Voucher collection	Origin	ITS accession no.
L. deliciosus	JN2001-046 (GENT)	Slovakia	KF133272
L. citriolens	UE20.09.2004-03 (UPS)	Sweden	DQ422003
L. controversus	AV00-117 (GENT)	Italy	KF241544
L. akahatsu	AV2004-141 (GENT)	Thailand	KF133269
L. albocarneus	AV98-080 (GENT)	France	KF241545
L. alboscrobiculatus	LTH175 (CMU, SFSU, GENT)	Thailand	EF141538
L. mairei	AV00-118 (GENT)	Italy	AY336950
L. quieticolor	UE10.09.2004-1 (UPS)	Sweden	DQ422002
L. necator	AV04-231 (GENT)	France	KF133276
Lactarius **subg.**			
Plinthogalus			
L. acris	BG2011-31 (GENT)	Belgium	KF432962
L. pterosporus	JN2012-037 (GENT)	Germany	KF432963
L. romagnesii	UE29.09.2002-6 (UPS)	France	DQ421989
L. friabilis	FH12-103 (GENT, MFLU)	Thailand	KF432961
L. azonites	AV00-124 (GENT)	Belgium	KF241540
L. crassiusculus	LTH369 (GENT)	Thailand	EF560684
L. cyanescens	DS06-058 (GENT)	Malaysia	GU258278
L. fuliginosus	MTB97-24 (GENT)	Sweden	JQ446111
L. lignyotus	UE06.09.2003-5 (UPS)	Sweden	DQ421993
L. montoyae	KD1065 (type, BSHC)	India	EF560673
L. subplinthogalus	AV04-219 (GENT)	USA	KF241539
L. spinosulus	AT2003068 (UPS)	Sweden	KF133262
L. baliophaeus	AV05-155 (GENT)	Malawi	GU258277
L. picinus	SAV.F-2223	Slovakia	JQ446130
L. ruginosus	KVP08-082	Austria	JQ446144
Multifurca			
M. furcatus	RH7804 (NY)	Costa Rica	DQ421994
M. ochricompacta	BB02.107 (PC)	USA	DQ421984
M. zonaria	DED7442 (PC)	Thailand	DQ421990

In the study of *Lactarius*, a dataset of ITS sequence alignment consists of 59 sequences. Figure 5.1 shows the Maximum likelihood (ML) topology based on the ITS sequence data. Two clades within *L.* subg. *Russularia* are well-supported (with 100% bootstrap support) and belong to *L.* subg. *Russularia*. The molecular evidence is in accordance with the morphology (see under the taxonomy part).

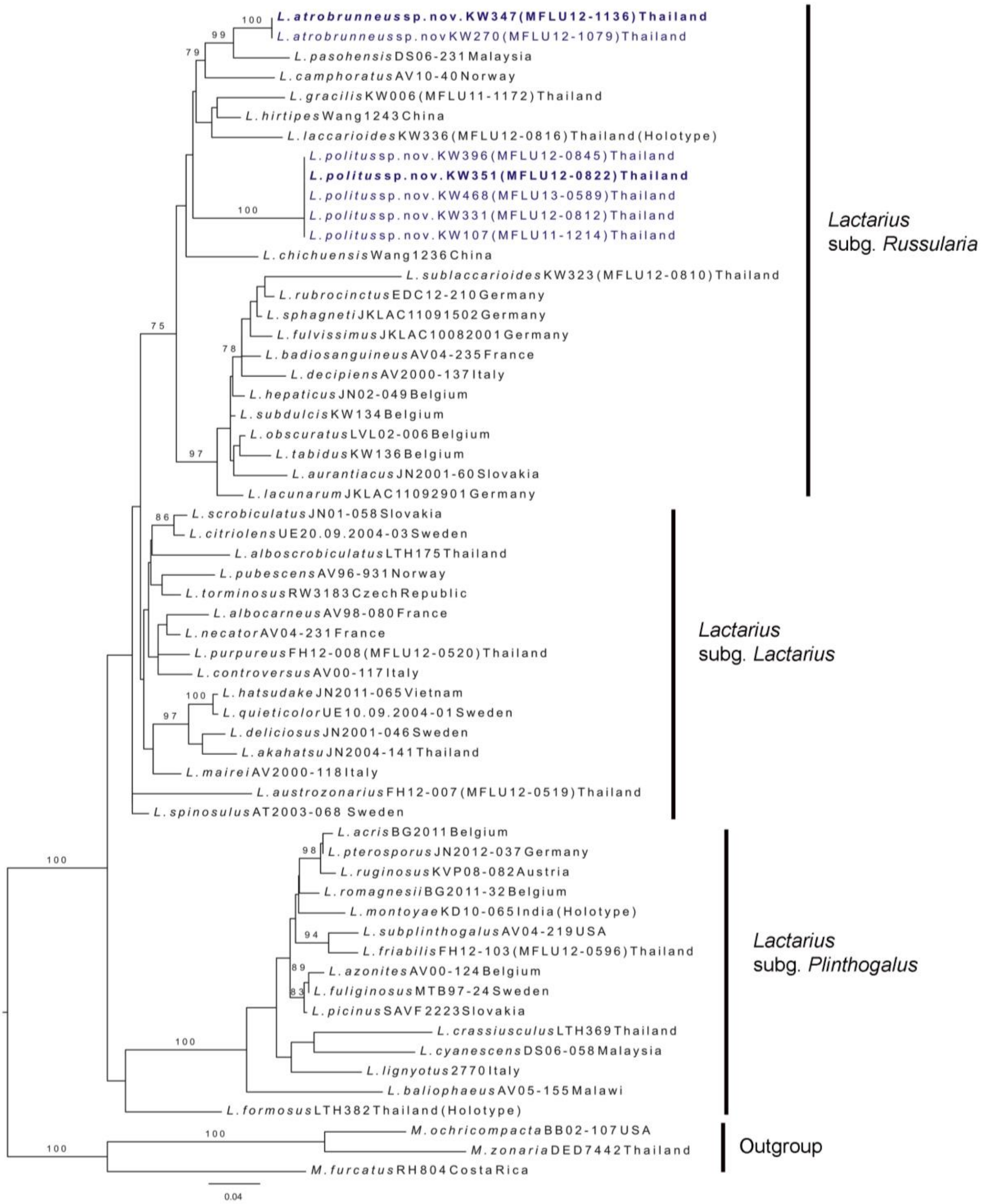

FIGURE 5.1: The obtained ML phylogeny showing phylogenentic position of *L. atrobrunneus* and *L. politus* based on ITS sequence data. ML bootstrap support values greater than 75% are indicated above or below the nodes, species for which obtained sequences are based on type material names in boldface.

Taxonomy

Lactarius atrobrunneus Wisitrassameewong & K. D. Hyde sp. nov. (Figure 5.2, 5.4a)

Index Fungorum number: IF551005, **Facesoffungi number:** FoF: FoF00483

Diagnosis: a small sized species with distant gills, reddish to dark brown cap, completely transparent latex and an epithelium as a pileipellis.

Etymology: 'atrobrunneus' meaning dark brown colour.

Holotype: Thailand, Chiang Mai province, Mae Taeng district, Pa Pae sub-district Mushroom Research Center, N19º17.12 E98º44.00, elev. ca 900 msl., KW347 (MFLU!, GENT!).

Basidiocarps small sized. *Pileus* 6−31 mm diam., broadly convex in young specimens, with or without a small papilla, becoming plane to infundibuliform at maturity; surface dry, radially rugulose, particularly in inner half of pileus, strongly and irregularly wrinkled in the outer half of pileus, with grooves that show the lamellae in transparency; margin striate, occasionally fissured, incurved; surface blackish to dark brown when young, turning less unicolourous and between dark brown (9F8), reddish-brown (9E8) and blackish when older. *Lamellae* subdecurrent to decurrent, 1−2 mm broad, distant, with 1−2 series of lamellulae, beige to greyish beige (4C2−4C3) to fuliginous. *Stipe* 14−37 × 1−4 mm, fragile, cylindrical, more or less concolourous to pileus, with darker brown tints when old. *Context* thin-fleshed, 0.5−1 mm broad in pileus, pinkish to clay buff, darker in cortex, unchanging when cut, but changing yellow with 10%KOH within seconds; smell like *L. quietus*, like Pentatomidae bugs; taste mild. *Latex* rather abundant, pure watery, unchanging when exposure, unchanging with 10% KOH, unchanging on white tissue or paper; taste faintly bitter.

Basidiospores subglobose to ellipsoid; 6.9−*8.1*−*8.2*−9.6(−10.7) × 6.0−*7.0*−*7.2*−8.3(−8.5) µm, Q = 1.06−*1.15*−*1.16*−1.29; ornamentation amyloid, subreticulate, composed of ridges up to 1.5 µm high; ridges often spiny and irregular, rather broad, interconnected by finer lines, forming an incomplete to subcomplete reticulum; isolated warts infrequent; plage distally amyloid. *Basidia* 55−72 × 18−20 µm, mostly 4−spored, with small or large guttate contents. *Pleuromacrocystidia* absent. *Pseudocystidia* 4−6 µm diam., cylindrical, often curved, slightly emergent to emergent. *Lamellae edge* heterogeneous, with some basidia present; marginal cells 11−44 × 5−12 µm, subcylindrical to subclavate; cheilocystidia absent. *Lamellar trama* mixed, composed of small and large globose cells, septate hyphae and lactiferous hyphae. *Pileipellis* an epithelium, about 40−70 µm thick, composed of globose cells of 5−25 µm. diam., with some subclavate elements on top; underlying layer composed of smaller globose cells and cylindrical hyphae.

Habitat: gregarious on naked soil, in montane deciduous forest with Fagaceae trees.

Studied material: Thailand, Chiang Mai province, Mae Taeng district, Pa Pae sub-district, Mushroom Research Center, N19º17.123 E 98º44. 009', elev. ca 900 msl., KW270, 07/06/2012 (MFLU!, GENT!); ibid., KW324, 01/07/2012 (MFLU!, GENT!); ibid., KW347, 09/07/2012 (MFLU!, GENT!).

Comment: *Lactarius atrobrunneus* is rather unique and well-recognisable in the subgenus by its small size and striking dark or blackish brown colour. Furthermore the strongly rugose cap, the pure transparent latex and distant gills are striking field characters. Microscopically, the rather spiny and heavy spore ornamentation and the well-developed epithelium as a pileipellis structure are striking. Other species of the subgenus with remarkably distant lamellae are *L. laccarioides* Wisitrassameewong & Verbeken, *L. sublaccarioides* Wisitrassameewong & Verbeken, *L. stubbei* Wisitrassameewong & Verbeken and *L. pasohensis* Wisitrassameewong & Stubbei (Wisitrassameewong et al. 2014a). All of them are larger and have more orange brown colours and white latex.

Lactarius politus Wisitrassameewong & K.D. Hyde sp. nov. (Figure 5.3, 5.4b)

Index Fungorum number: IF551004, **Facesoffungi number:** FoF00484

Diagnosis: a medium sized species with polished surface, reddish brown or dark brown with darker zonation at the center, completey transparent latex, heavy spore ornamentation, high ridges up to 2.5 µm forming a complete reticulum and a hyphoepithelium as a pileipellis.

Etymology: 'politus' meaning polished surface.

Holotype: Thailand, Chiang Rai province, Mae Fah Luang district, Doi Mae Salong Nok sub-district, Doi Mae Salong, elev. ca 1,269 msl., N20º16.90 E99º62.30, 13/7/2012, KW351 (MFLU!, GENT!).

Basidiocarps medium sized. *Pileus* 25−66 mm diam., at first broadly convex, later turning infundibuliform, inconspicuously mucronate in center; surface dry and polished, somewhat smooth when young, becoming minutely wrinkled; margin incurved, translucently striate up to almost halfway the radius, crenulate when mature; surface reddish brown or dark brown

(8F8), with darker zonation at least in inner half of pileus, palest at pileus margin, in outer half ranging from dark brown (7F8−8F8) to reddish brown (7E8) to brownish orange (5C4−C5) to brown (5E8) to olive brown (4E8) to greyish orange (5B4). *Lamellae* decurrent, 3 mm broad, medium crowded, sometimes forked, with 1−2 series of lamellulae, light yellow (4A4) to greyish yellow (4B4) to olive brown (4D5−E3), with greyish brown (5E3) tinge. Stipe 30−62 × 5−8 mm, cylindrical; surface dry, longitudinally wrinkled, brown (5E5) to greyish orange (6B3) to dark brown (9F8). *Context* 2 mm broad in pileus, medium firm, hollow in stipe, greyish pink; smell strong, sweetish and spicy, maggi-like in dried specimens; unchanging with 10% KOH and unchanging with $FeSO_4$. *Latex* completely transparent, unchanging on exposure, unchanging with 10% KOH, unchanging on white tissue or paper; taste mild.

Basidiospores globose to subglose, sometimes ellipsoid; 7.7−*8.6−9.0*−10.2(−10.6) × 6.7−*7.6*−7.8−8.7 µm; Q = 1.03−*1.11−1.17*−1.33; ornamentation amyloid, reticulate, ornamented with very heavy, dense and high ridges up to 2.5 µm; ridges irregular and thick interconnected with finer lines, forming a dense reticulum, sometimes with a slight tendency of zebroid ornamentation; plage sometimes distally amyloid. *Basidia* 55−84 × 18−23 µm, 4−spored, subclavate, with guttate contents. *Pleuromacrocystidia* very scarce, 55−77 × 16−20 µm, not emergent, subfusiform to subclavate, mucronate to rounded, with small granules and guttate contents. *Pseudocystidia* 3−6 µm diam., not emergent, tortuous cylindrical, often curved, sometimes ramified. *Lamellae edge* heterogenous, with some basidia present; marginal cells subcylindrical to subclavate to obovoid to capitate, 25−43 × 11−22 µm; cheilocystidia scarce, 30−55 × 8−14 µm, not emergent, subcylindrical to subclavate, with mucronate apex. *Lamellar trama* consisting of small and large globose cells, septate hyphae and lactiferous hyphae. *Pileipellis* a hyphoepithelium; upper layer 20−30 µm thick, consisting of repent cylindrical hyphae; subpellis consisting of globose cells of 10−40 µm diam.

Habitat: solitary or scattered on soil in montane deciduous forest near *Lithocarpus sootepensis* stand.

Studied material: Thailand, Chiang Rai province, Mae Fah Luang district, Doi Mae Salong Nok sub-district, Doi Mae Salong, elev. ca 1,269 msl., N20°16.90 E99°62.30, 13/7/2012, KW351 (MFLU!, GENT!), ibid., 22/8/2012, KW396 (MFLU!, GENT!), ibid., 22/8/2012, KW468

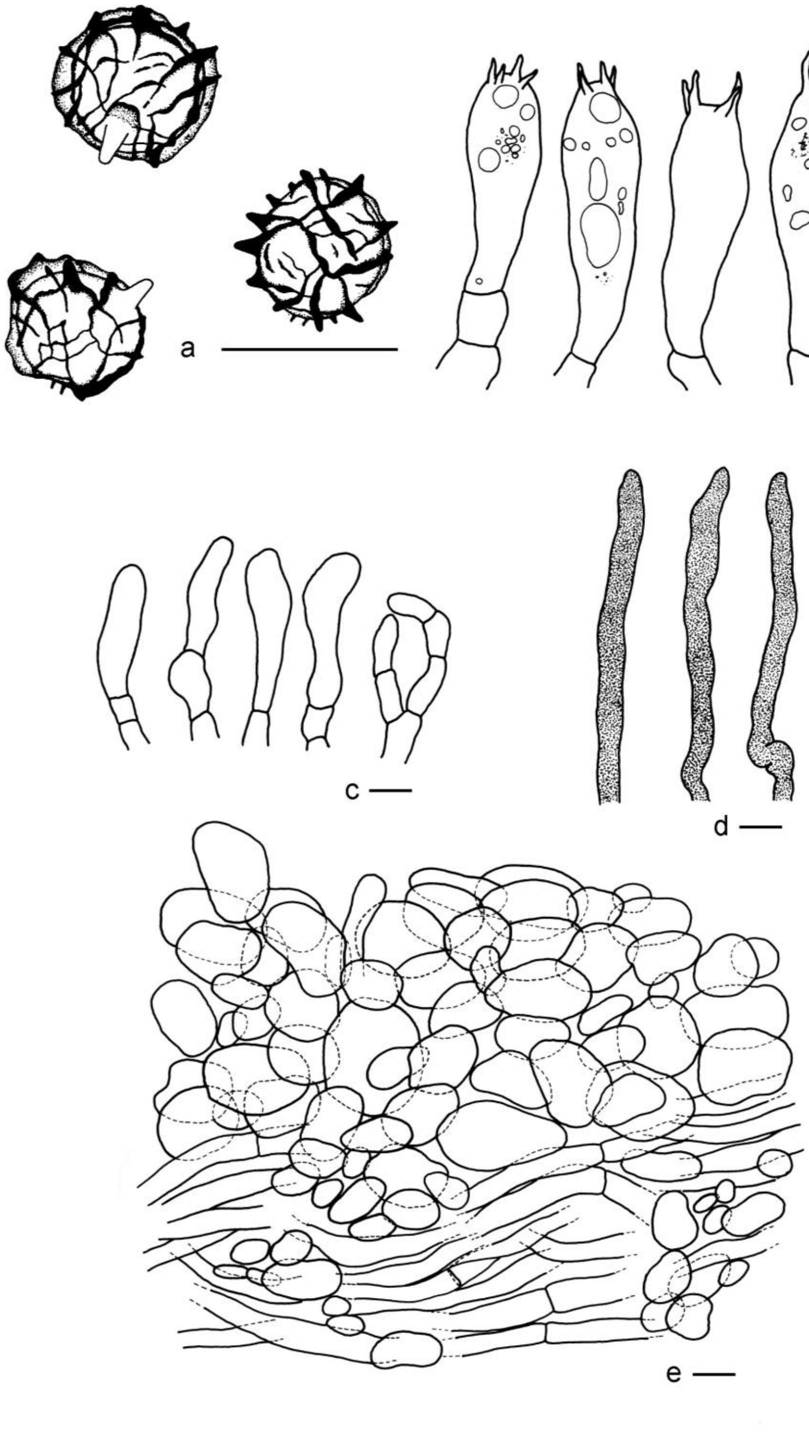

Figure 5.2: *Lactarius atrobrunneus*: a. basidiospore, b. basidia, c. marginal cells, d. pseudocystidia, e. pileipellis (a−e: KW347, holotype) (scale bar = 10 µm).

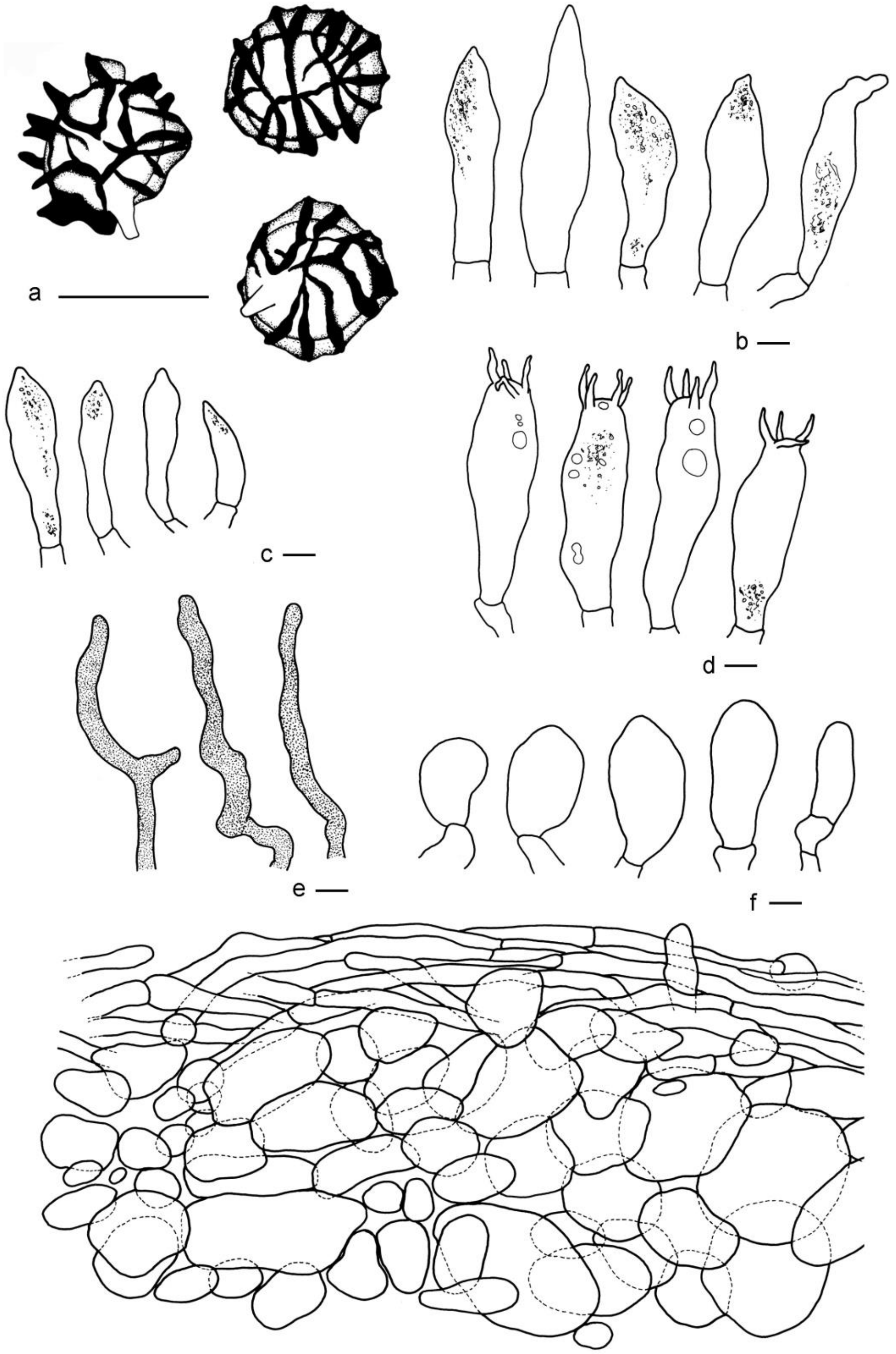

FIGURE 5.3: *Lactarius politus*: a. basidiospore, b. pleuromacrocystidia, c. cheilocystidia, d. basidia, e. pseudocystidia, f. marginal cells, g. pileipellis (a−g: KW351) (scale bar = 10 µm).

(MFLU!, GENT!), Thailand, Chiang Mai province, Mae Taeng district, Pa Pae sub-district, Mushroom Research Center, elev. ca 900 msl., N19°07.20 E98°44.04, 3/7/2012, KW331(MFLU!, GENT!), Thailand, Chiang Mai province, Mae Taeng district, Bahn Mae Sae village, on Highway no.1095 near 50 km marker, elev. ca 962 msl., N19º14.59 E098º39.45, 4/7/2011, KW107 (MFLU!, GENT!).

Comment: *Lactarius politus* is recognized by the large, ellipsoid spores and the firm ornamentation forming complete reticulum. The other important characters are transparent latex, the strong smell and distinct dark brown colour at the center or inner half of the pileus and paler margin. The pileipellis structure is hyphoepithelium which is common feature in subgenus *Russularia*.

FIGURE 5.4. Basidiocarps: a. *L. atrobrunneus* (KW347), b. *L. politus* (KW351).

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CHAPTER 6

BIODIVERSITY, MOLECULAR PHYLOGENY AND EVOLUTIONARY RELATIONSHIPS OF *LACTARIUS* SUBGENUS *RUSSULARIA*

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 and divergence time and origin of the subgenus. Six conifer symbionts are new to science: *L. atrii*, *L. aurantionitidus*, *L. dombangensis*, *L. flavigalactus*, *L. lachungensis* and *L. sikkimensis*. Species delimitation is accessed based on the concordance between morphological characteristics and ITS phylogeny. Infrageneric relationships were studied using a phylogeny constructed from concatenated ITS-*rpb2* data using Maximum Likelihood and Bayesian inference methods. Species in this subgenus do not cluster together according to their geographic origin. Intercontinental sister relationships between Europe/Asia/North America are common but true conspecificity is rare. This result suggests that allopatric speciation played an important role within this subgenus. Few morphological characteristics tend to be phylogenetically informative: the presence or absence of true cystidia and the pileipellis structure. A fossil-calibrated phylogeny of *L.* subg. *Russularia* using ITS and *rpb2* sequence data is generated. The proposed origin of *L.* subg. *Russularia* is estimated to be in the Mid Miocene period.

Keywords: molecular evolution, milkcaps, molecular dating

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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 earth is unknown (Hawksworth 1991, Hawksworth & Rossman 1997, O'Brien et al. 2005, Kirk et al 2008). Even for striking macrofungi the biodiversity is often underexplored (Verbeken & Buyck 2002, Mueller et al. 2007). Within the genus *Lactarius* Pers., more than 400 species have been published so far (Verbeken 2001, Das et al. 2004, Le et al. 2007a, 2007b, Verbeken et al. 2014, Das et al. 2015). Many endemic species are recognized by local people but remain undescribed, particularly in tropical forest ecosystems (Wisitrassameewong et al. 2015, Wang et al. 2015). Many species are hidden under species names that are described and known from a different continent. Molecular phylogenetic analyses and detailed morphological studies are needed to test intercontinental conspecificity (Nuytinck et al. 2007). Some 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 *L.* subg. *Russularia* (Fr.) Kauffman being one of them (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 et al. 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, 2014b, 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 appendix 6), we followed Hesler & Smith (1979) for North American representatives, and Heilmann-Clausen et al. (1998) and Basso (1999), Verbeken & Vesterholt (2008) for the European mycoflora. These contributions are the most recent and complete monographs for the 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 accepted as members of *L.* subg. *Russularia* (see Table 6.1) up to present. Most species are agaricoid, except for three sequestrate species: *L. hispanicus* Calonge & Pegler, *L. borzianus* Cavara and *L. 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 is shown in Table 6.1.

Species traditionally placed in the subgenus are characterized by small to medium sized fruiting bodies which have a dry surface and orange, warm brown to reddish brown colours. Among the three subgenera of *Lactarius*, latex features of *L.* subg. *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 in 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 ITS sequences are adequate to delimit species in *L.* subgenus *Russularia* but cannot tackle evolutionary relationships of ancestral nodes in the phylogeny (Wisitrassameewong et al. 2014a, 2014b, 2015, Das et al. 2015). Multi-locus phylogeny can resolve species complexes, higher taxonomic evolutionary relationships, and (sub)generic classification. Since our previous studies dealing with *L.* subg. *Russularia* have been relying on ITS only, we are lacking phylogenetic insights this group. In this study we use two locus analyses (ITS and *rpb2*) to answer evolutionary questions.

In this study we aim to: 1) propose six new *Russularia* species from India and China, 2) explore the delimitation of the subgenus by including borderline taxa (*L. rufus* (Scop.: Fr.) Fr., *L. helvus* (Fr.: Fr.) Fr., *L. chrysorrheus* Fr. and *L. vinaceorufescens* A.H. Sm.), 3) carry out two locus analyses (ITS−*rpb2*) and investigate whether we can find concordance between morphology and phylogeny, 4) discuss intercontinental conspecificity and 5) estimate divergence time of *Lactarius* subg. *Russularia*.

Table 6.1: The current number of accepted species of *Lactarius* subg. *Russularia* according to geographic region and references up to present. The total numbers of species per regions include morphologically described species but exclude synonyms and variations of species.

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

Material and Methods

Examined specimens

Indian samples were collected in Sikkim Province (Himalaya region) in 2009. Chinese samples were collected in Yunnan from 2012 to 2013. Indian specimens were deposited in the Herbarium Universitatis Gandavensis (GENT), Belgium and Central National Herbarium (CAL), India. Chinese specimens were 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). The internal transcribed spacer of the nuclear ribosomal DNA (ITS) was 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^{TM} v5.0 (Genecode Corporation, Ann Arbor, Michigan, U.S.A.).

Sequence sampling and alignment

Apart from newly generated sequences, published DNA sequences of *L. herrarae* Montoya, Bandala & Garay (Montoya et al. 2014), *L. indochrysorrheus* K. Das & Verbeken (Das et al. 2015), *L. politus* Wisitrassameewong & K. D. Hyde and *L. atrobrunneus* Wisitrassameewong & K. D. Hyde (Liu et al. 2015) were included in this study, supplied with some other ITS sequences from the GenBank and UNITE databases. An overview of sequences included in this study is given in the appendix 7. 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) and bigger segments with contiguous non-conserved positions (maximum 10 bp).

Phylogenetic reconstruction

Two datasets of DNA sequences of *L.* 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 5.8 S 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. Representatives of *L.* 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 Bootrapping 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 (see the appendix 5). We investigated the compatibility of both dataset 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. on 1983). 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 *L.* 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 dataset. Two datasets were generated: dataset 1 and 2. An overview of sequences used in both dating phylogenies is given in the appendix 7.

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. The aim of dataset 1 is to estimate the mean ages of genera in Russulaceae. The dataset 1 was analzed 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* 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* 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 summarised 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) and Verbeken (1996) and Heilmann-Clausen et al. (1998) for the pileipellis structure. Colour coding is according to Kornerup and Wanscher (1978). The observation of latex features is done immediately when the latex is exposed to the air, colour reaction is noted after 15 to 30 minutes. 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 Figure 6.2): 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 215 ITS sequences that comprises 78 species of *L.* 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 form strong-supported clades in the ITS phylogeny (Figure 6.1). The final *rpb2* dataset contains 61 sequences. Due to the low success rate of amplification when using dried collections, several temperate taxa remain missing. Additional *rpb2* sequences (from domain 6 to 7) of European taxa were downloaded from GenBank. The dataset of rpb2 sequence was analysed by the ML algorithm. The rpb2 phylogram is shown in the appendix 5.

The phylogeny generated by the concatenated dataset comprises 51 *L.* subg. *Russularia* species in total (Figure 6.2). Before combining both datasets, 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 (Figure 6.2). 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 thirteen 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: *L. fuscomaculatus*, *L. rubrobrunneus*, *L. subumbonatus*, *L. crenulatulus*, *L. pasohensis* and *L. atrobrunneus*. This

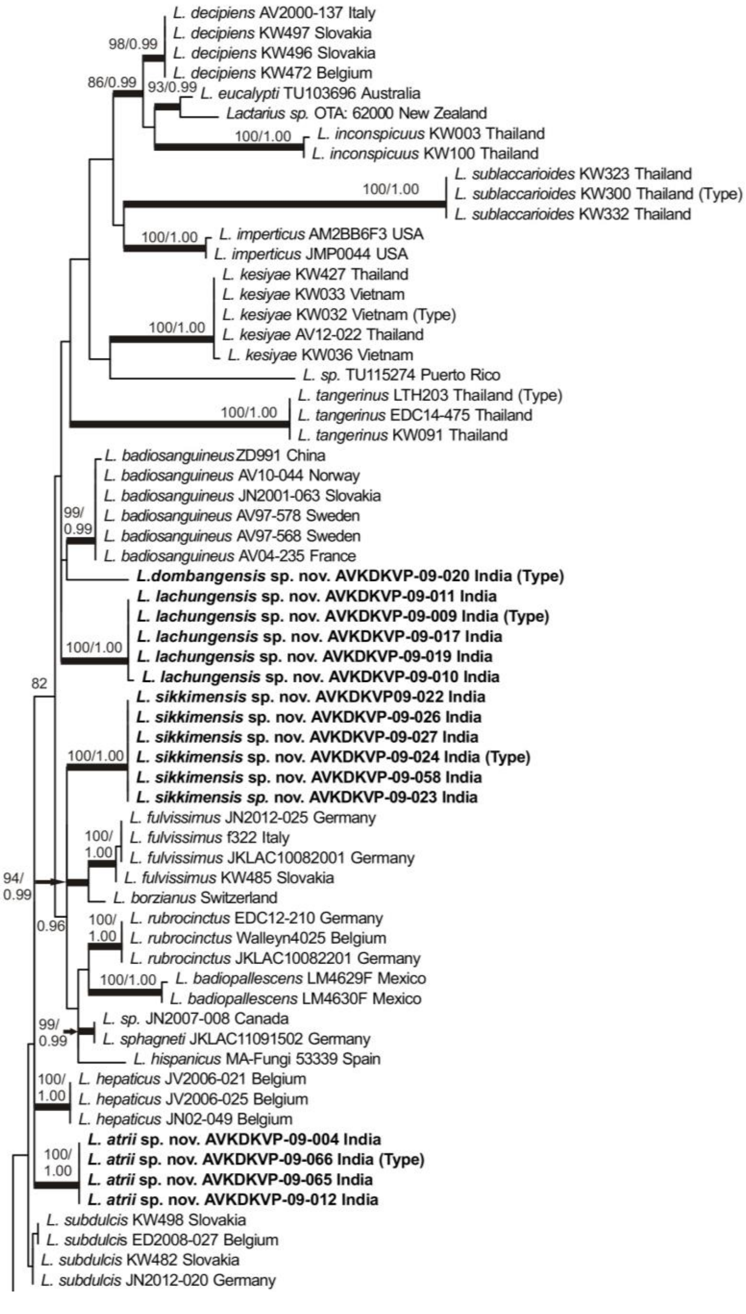

Figure 6.1 Continued.

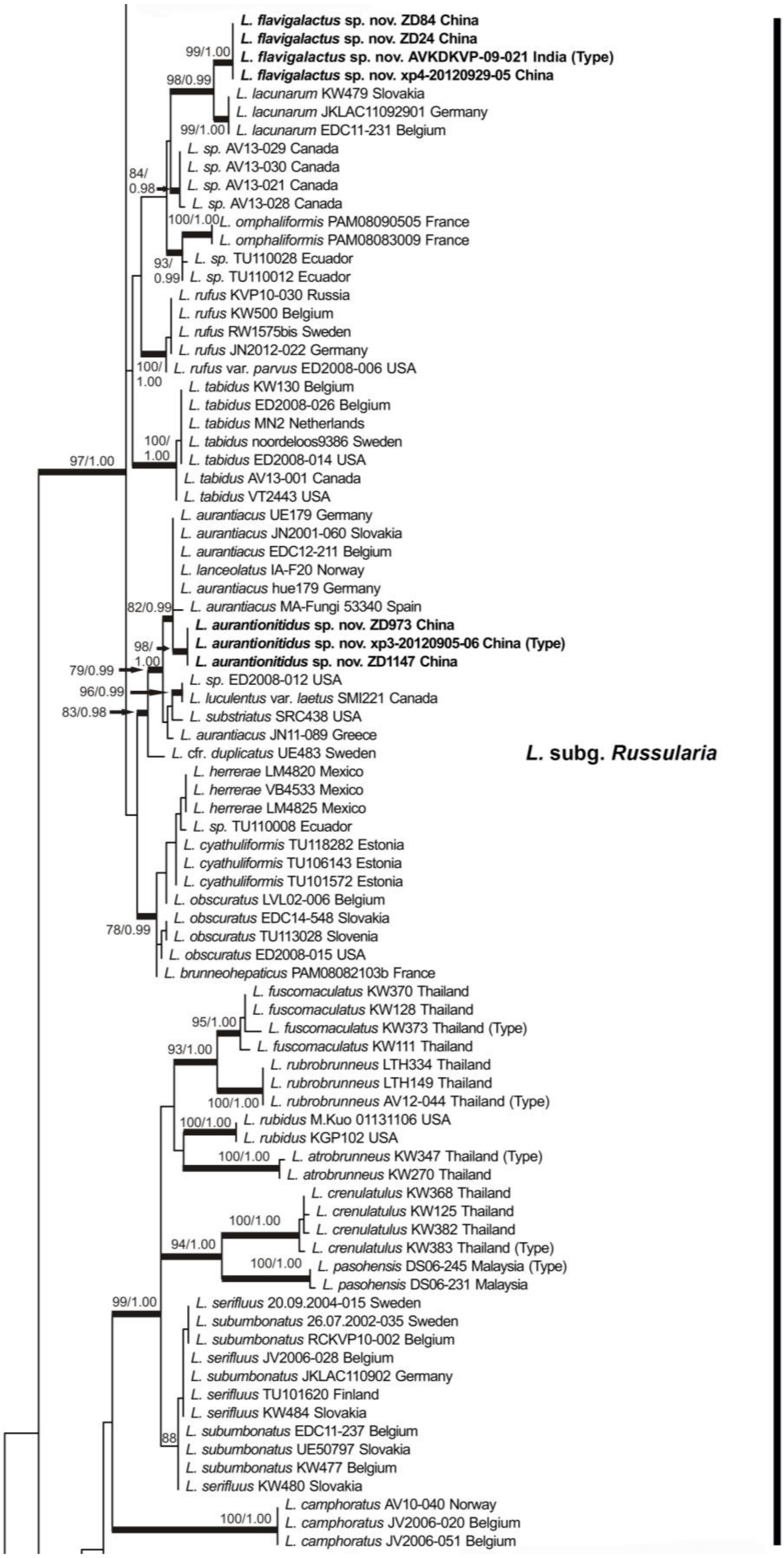

Figure 6.1 Continued.

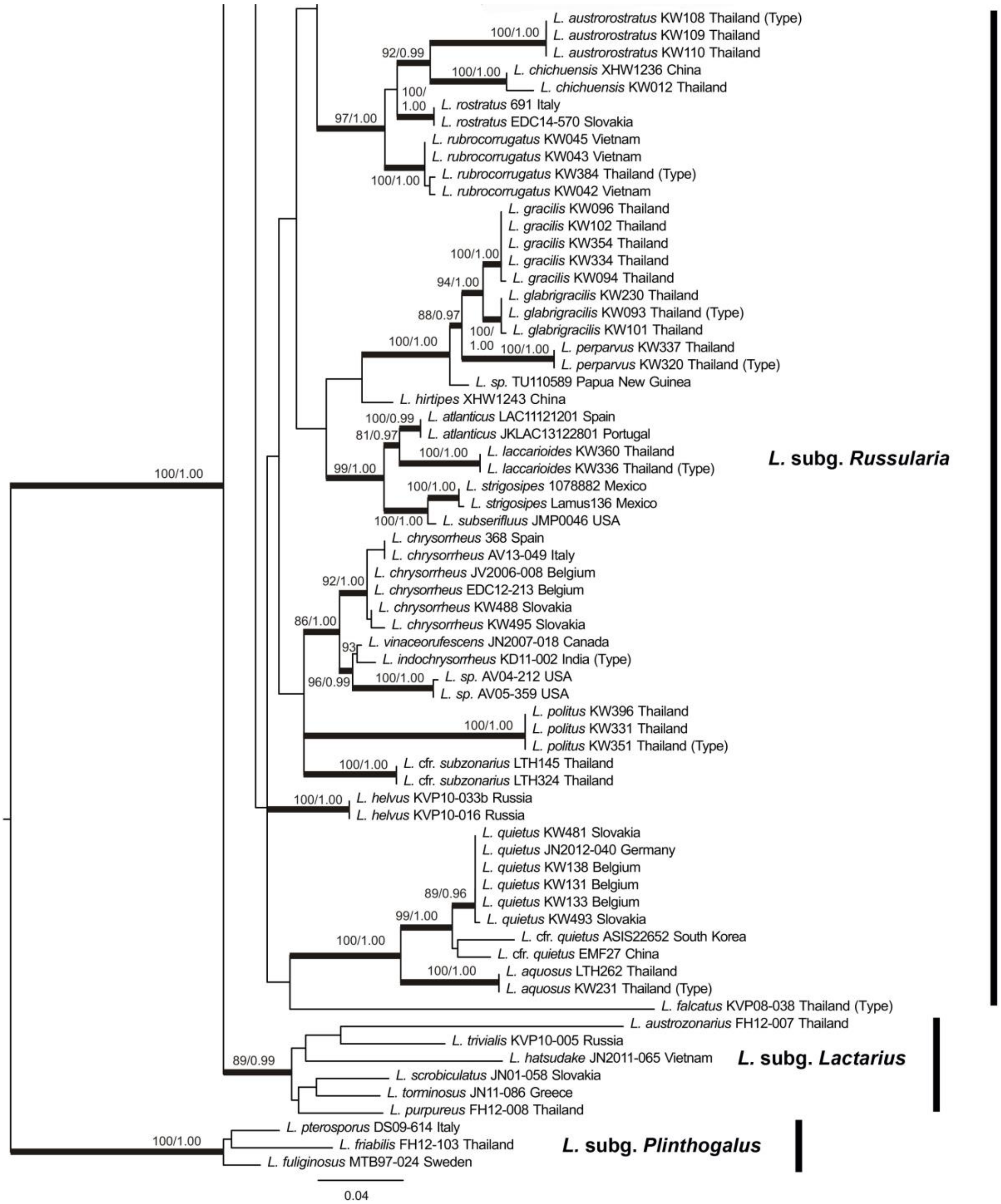

Figure 6.1: A global ITS tree of *L.* subg. *Russularia*. The thick branches indicated supported clades. Bootstrap support and posterior probabilities are indicated when they exceed 75% and 0.95. New species in this study are in boldface.

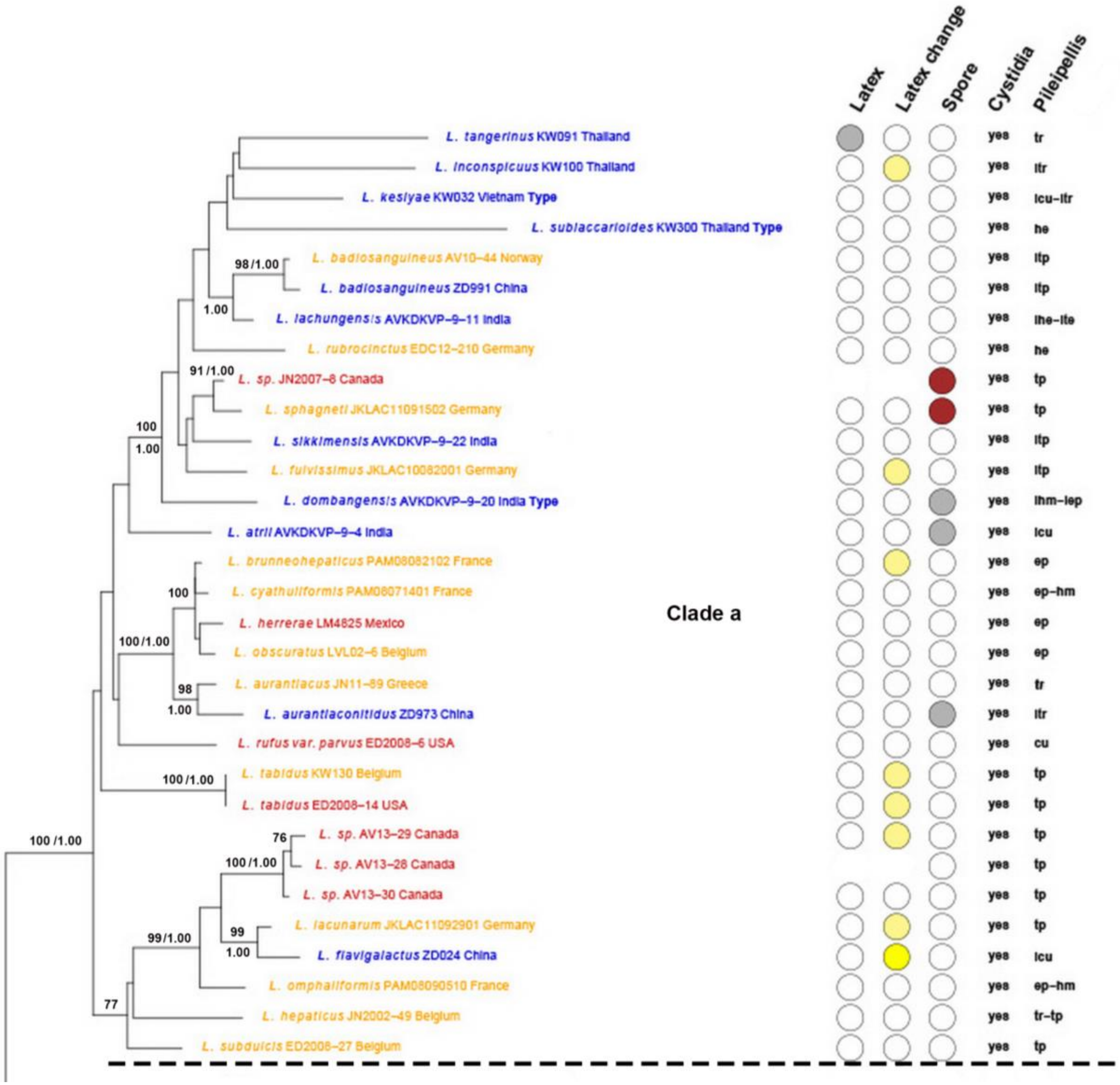
Latex
Latex change
Spore
Cystidia
Pileipellis
L. tangerinus KW091 Thailand
yes
tr
L. inconspicuus KW100 Thailand
yes
itr
L. kesiyae KW032 Vietnam Type
yes
icu–itr
L. sublaccarioides KW300 Thailand Type
yes
he
98 / 1.00
L. badiosanguineus AV10–44 Norway
yes
itp
L. badiosanguineus ZD991 China
yes
itp
1.00
L. lachungensis AVKDKVP–9–11 India
yes
ihe–ite
L. rubrocinctus EDC12–210 Germany
yes
he
91 / 1.00
L. sp. JN2007–6 Canada
yes
tp
L. sphagneti JKLAC11091502 Germany
yes
tp
100
1.00
L. sikkimensis AVKDKVP–9–22 India
yes
itp
L. fulvissimus JKLAC10082001 Germany
yes
itp
L. dombangensis AVKDKVP–9–20 India Type
yes
ihm–iep
L. atrii AVKDKVP–9–4 India
yes
icu
L. brunneohepaticus PAM08082102 France
yes
ep
100
L. cyathuliformis PAM08071401 France
yes
ep–hm
L. herrerae LM4825 Mexico
yes
ep
100 / 1.00
L. obscuratus LVL02–6 Belgium
yes
ep
98
L. aurantiacus JN11–89 Greece
yes
tr
1.00
L. aurantiaconitidus ZD973 China
yes
itr
L. rufus var. parvus ED2008–6 USA
yes
cu
100 / 1.00
L. tabidus KW130 Belgium
yes
tp
L. tabidus ED2008–14 USA
yes
tp
100 / 1.00
76
L. sp. AV13–29 Canada
yes
tp
100 / 1.00
L. sp. AV13–28 Canada
yes
tp
L. sp. AV13–30 Canada
yes
tp
99
L. lacunarum JKLAC11092901 Germany
yes
tp
99 / 1.00
1.00
L. flavigalactus ZD024 China
yes
icu
L. omphaliformis PAM08090510 France
yes
ep–hm
77
L. hepaticus JN2002–49 Belgium
yes
tr–tp
L. subdulcis ED2008–27 Belgium
yes
tp
Clade a

Figure 6.2 Continued.

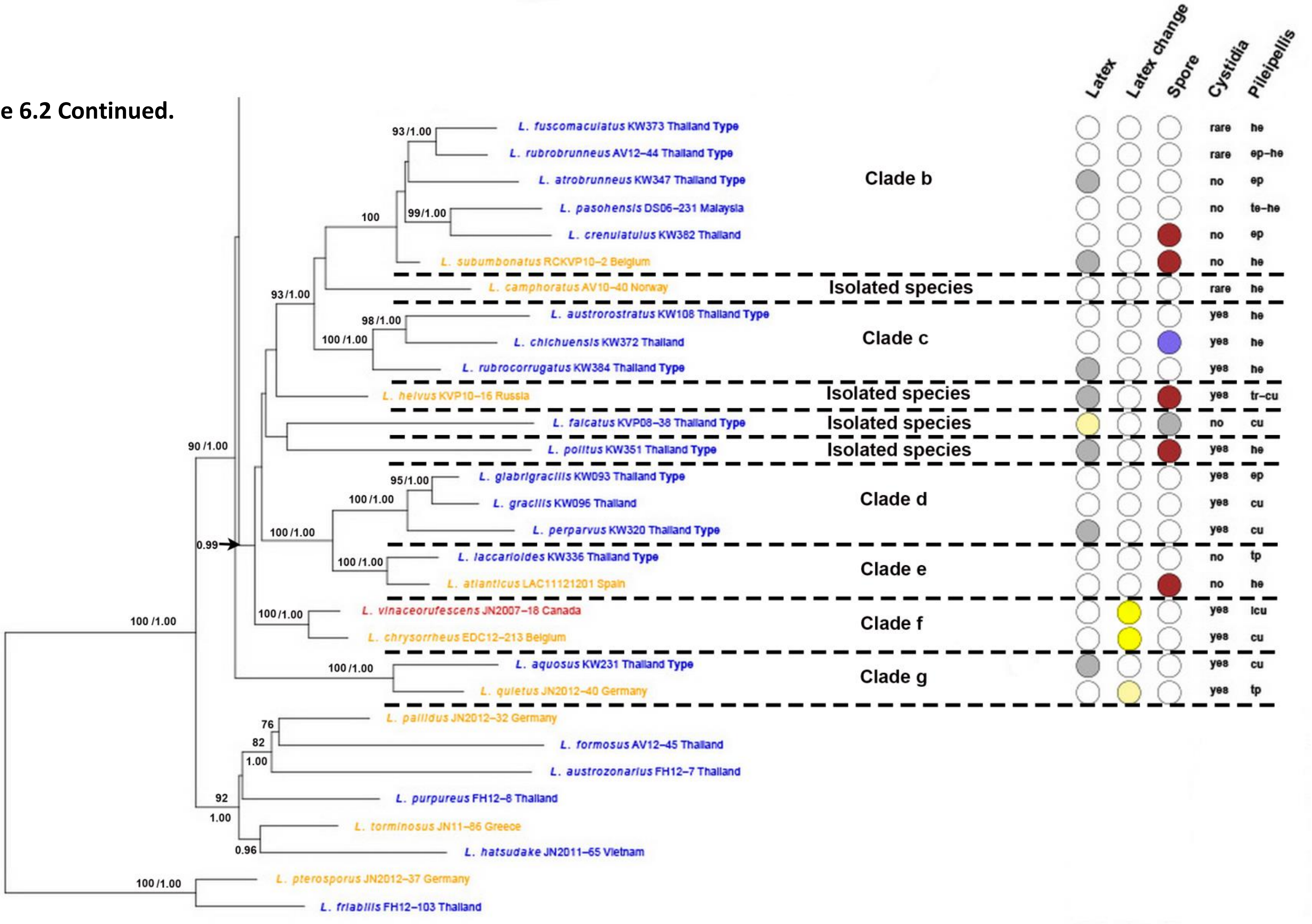

Figure 6.2: Tree of concatenated ITS-*rpb2* dataset of *L.* subg. *Russularia*. Species names are coloured by continent; blue: Asia, orange: Europe, red: North America. Bootstrap values above 75 and posterior probabilities exceeding 0.95 are indicated. Five morphological characters are plotted at the 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 reaction, 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.

clade has a sister relationship with clade c where *L. chichuensis*, *L. austrorostratus, L. rubrocorrugatus* and *L. camphoratus* are grouped together. *Lactarius politus*, *L. helvus* and *L. falcatus* have isolated positions in the phylogeny. Three small-sized species are grouped in clade d: *L. gracilis*, *L. glabrigracilis* and *L. perparvus*. Clade e consists of two species: *L. atlanticus* and *L. laccarioides* and clade f consists of two resembling species: *L. chrysorrheus* and *L. vinaceorufescens*. Clade g comprises two species: *L. quietus* and *L. aquosus*.

Divergence time estimation

The chronogram was obtained using two calibration points for estimating the origin of Russulaceae (dataset 1) is shown in Figure 6.3. The estimated times for major clades are summarised in Table 6.2.

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 diversification of the genus *Lactarius* might have taken place during the Early Miocene (28.17 mya, 16.94−41.62 mya; 95% HPD) (Figure 6.3). The analyses of dataset 2 showed that diversification of the major clades within *L.* subg. *Russularia* might have occurred during the Miocene (18.67 mya, 15.85−28.28 mya; 95% HPD) (Figure 6.4).

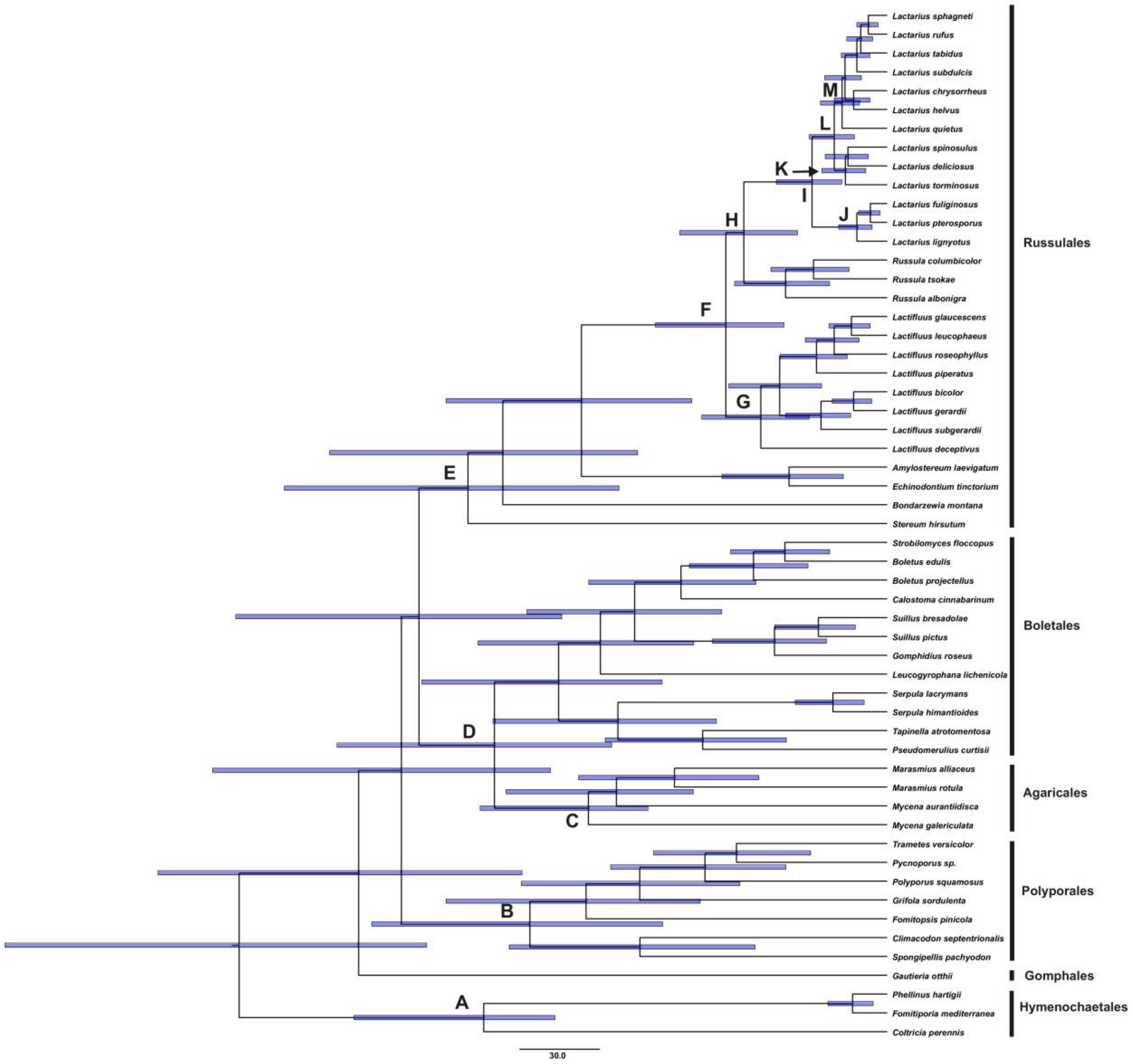

Figure 6.3: Fossil-calibrated maximum-clade-credibility tree of the BEAST analysis of dataset 1 (based on LSU and *rpb2*), including representatives in Russulaceae. Two calibration points were used: *Quatsinoporites cranhamii* (125 mya) for the minimum age of Hymenochaetales and *Archaeomarasmius leggetti* (90 mya) for the minimum age of the split between *Mycena* and *Marasmius* (Agaricales). The letters A–M refer to Table 6.2.

Table 6.2: 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

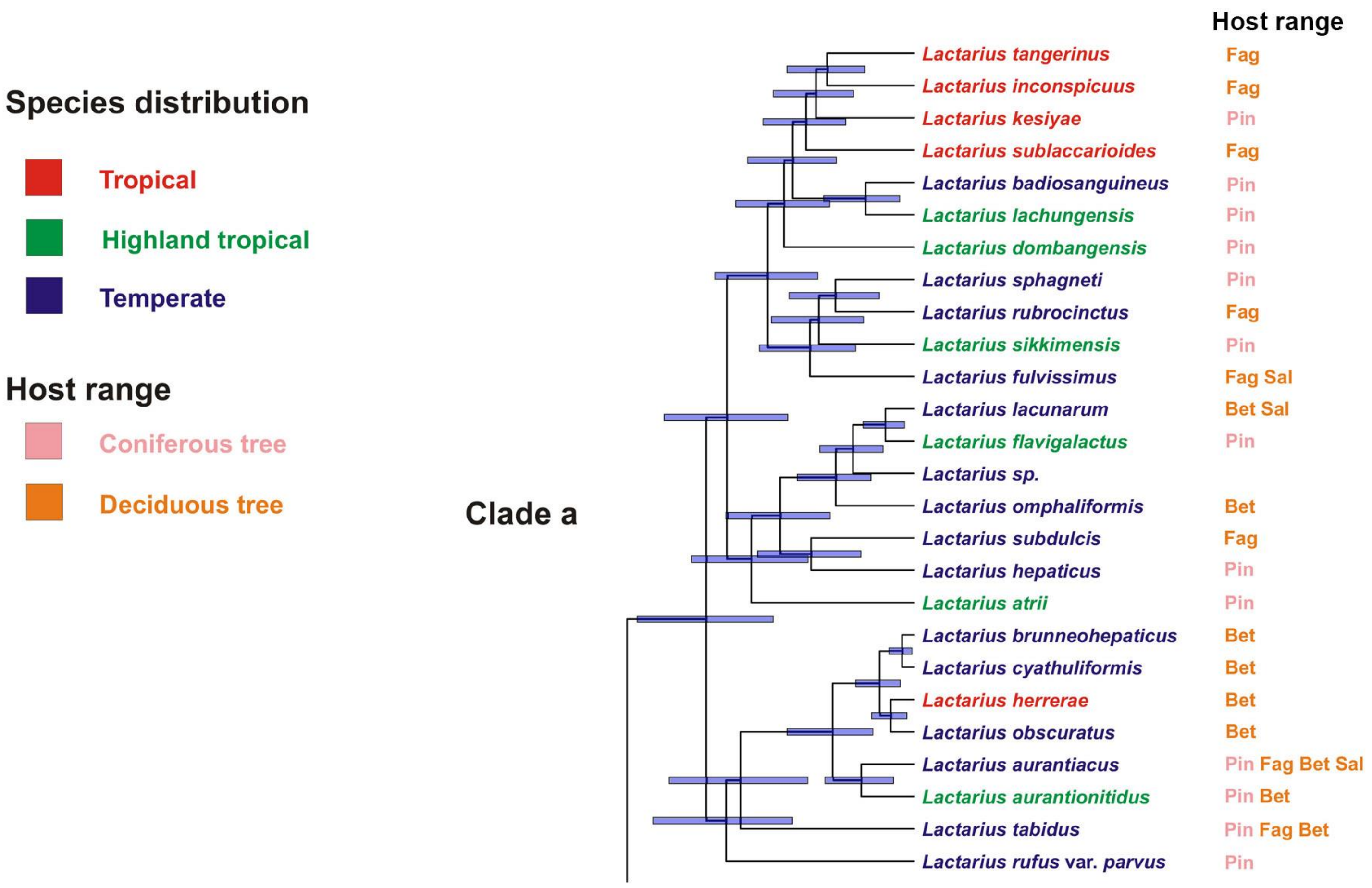
Species distribution
Tropical
Highland tropical
Temperate
Host range
Coniferous tree
Deciduous tree
Clade a
Host range
Lactarius tangerinus Fag
Lactarius inconspicuus Fag
Lactarius kesiyae Pin
Lactarius sublaccarioides Fag
Lactarius badiosanguineus Pin
Lactarius lachungensis Pin
Lactarius dombangensis Pin
Lactarius sphagneti Pin
Lactarius rubrocinctus Fag
Lactarius sikkimensis Pin
Lactarius fulvissimus Fag Sal
Lactarius lacunarum Bet Sal
Lactarius flavigalactus Pin
Lactarius sp.
Lactarius omphaliformis Bet
Lactarius subdulcis Fag
Lactarius hepaticus Pin
Lactarius atrii Pin
Lactarius brunneohepaticus Bet
Lactarius cyathuliformis Bet
Lactarius herrerae Bet
Lactarius obscuratus Bet
Lactarius aurantiacus Pin Fag Bet Sal
Lactarius aurantionitidus Pin Bet
Lactarius tabidus Pin Fag Bet
Lactarius rufus var. parvus Pin

Figure 6.4. Continued.

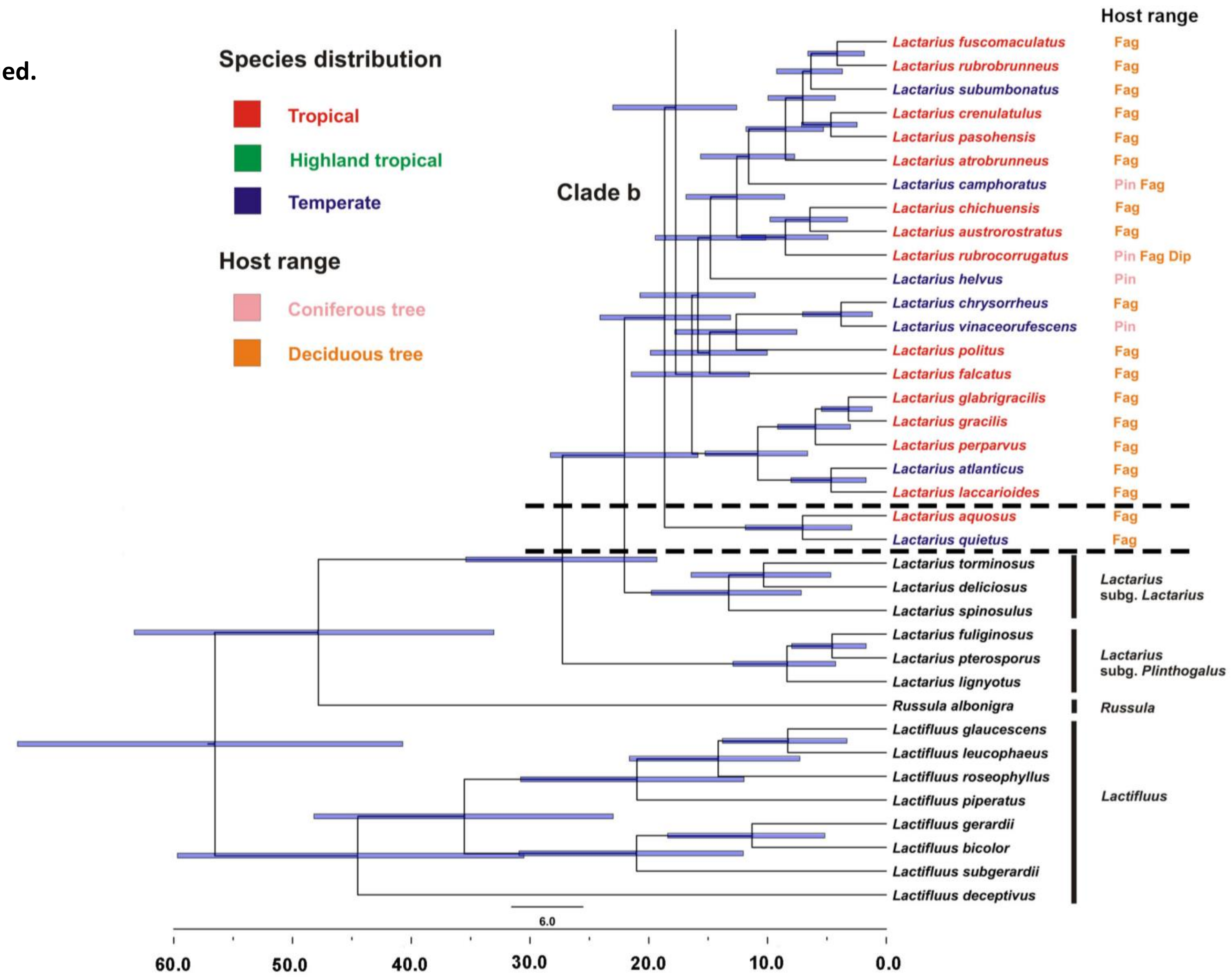

Figure 6.4: Fossil-calibrated maximum-clade-credibility tree of the BEAST analysis using dataset 2 (based on ITS and *rpb2*), including representatives of *L.* subg. *Russularia* and Russulaceae. Species distribution are colour as follow; red = tropical, green = highland tropical, blue = temperate. Families of host tree are annotated and coored at the right of the tip labels; pink = coniferous tree and orange = deciduous tree. Abbreviations are used for families of tree: Bet = Betulaceae, Dip = Dipterocarpaceae, Fag = Fagaceae, Pin = Pinaceae and Sal = Salicaceae.

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 forest in India and China. The description of morphology, diagnostic characters of species and illustration are given as follows.

Lactarius atrii Van de Putte & K. Das sp. nov. (Figure 6.5)

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 for 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 (*Picea*, *Tsuga*, *Juniperus*) with few *Rhododendrons* sp., 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,

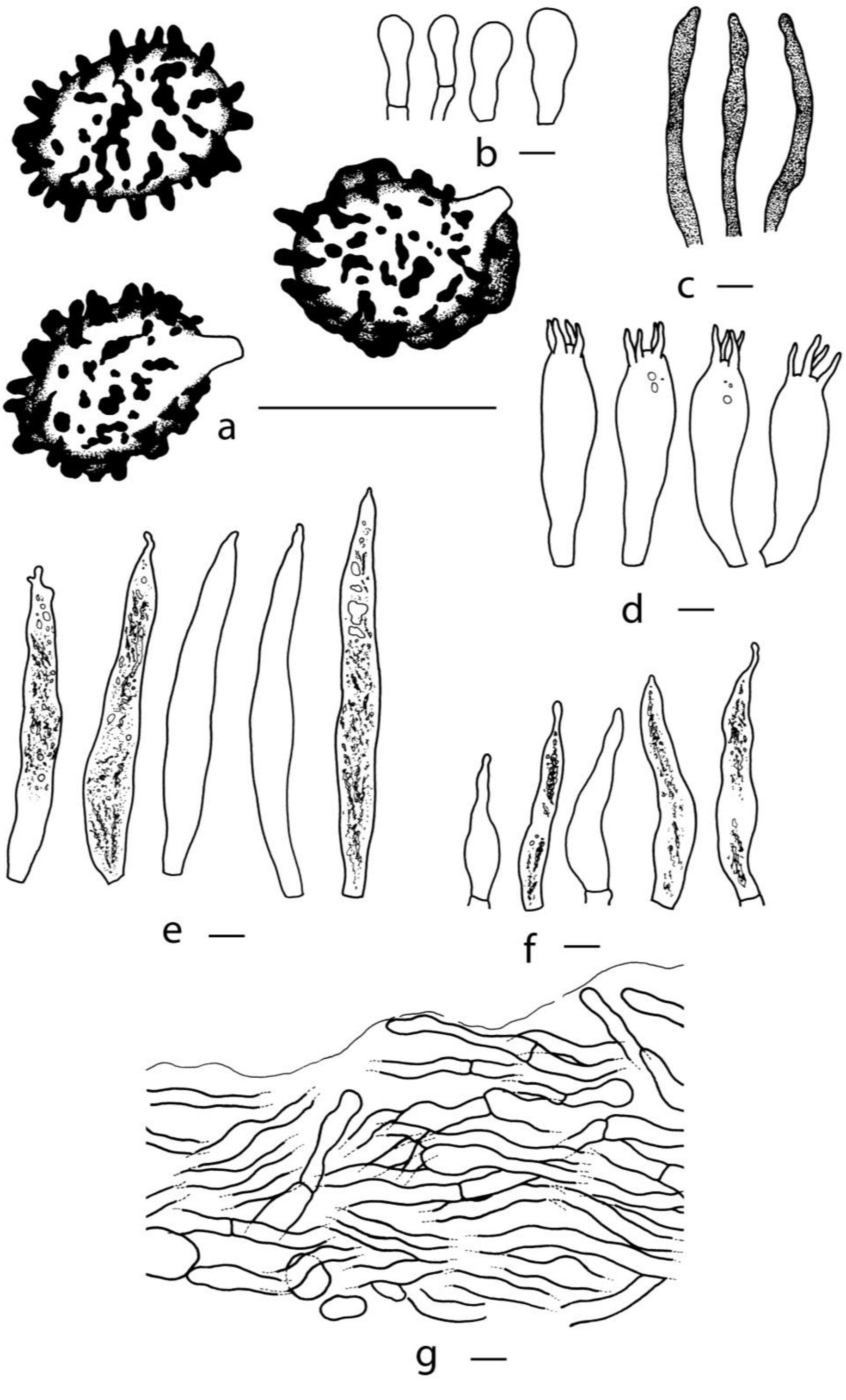

Figure 6.5: *Lactarius atrii* (a−g: AVKDKVP09-066, holotype): a. basidiospores, b. marginal cells, c. pseudocystidia, d. basidia, e. pleuromacrocystidia, f. cheilocystidia, g. pileipellis (scale bar = 10 µm).

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 x 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 to 1.7 µm high, composed of distinctly isolated or aligned warts, which are rather obtuse and rarely connected by lower ridges and; 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, 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, *Rhododendron* sp., *Abies densa*, *Tsuga*, *Picea*,, 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 (*Taxus*, *Tsuga*, *Juniperus*) with few *Rhododendrons*, 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 the species is characterized by the pale brown colour, without any orange or reddish tinge. Microscopically, the distinct characters are the large and emergent pleuromacrocystidia, the basidiospore ornamentation composed of distinctly isolated or aligned warts and the ixocutis for pileipellis.

Lactarius aurantionitidus Wisitrassameewong & G. Jiayu sp. nov. (Figure 6.6, 6.11a and 6.11b)

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, composed of ridges up to 1 µm high, forming isolated warts and short and low ridges connected by thin lines, obtuse, 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* sp., 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 (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* by the viscid cap, isolate warts on the basidiospores, the large macrocystidia and the ixotrichoderm pileipellis. *Lactarius lanceolatus* 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*.

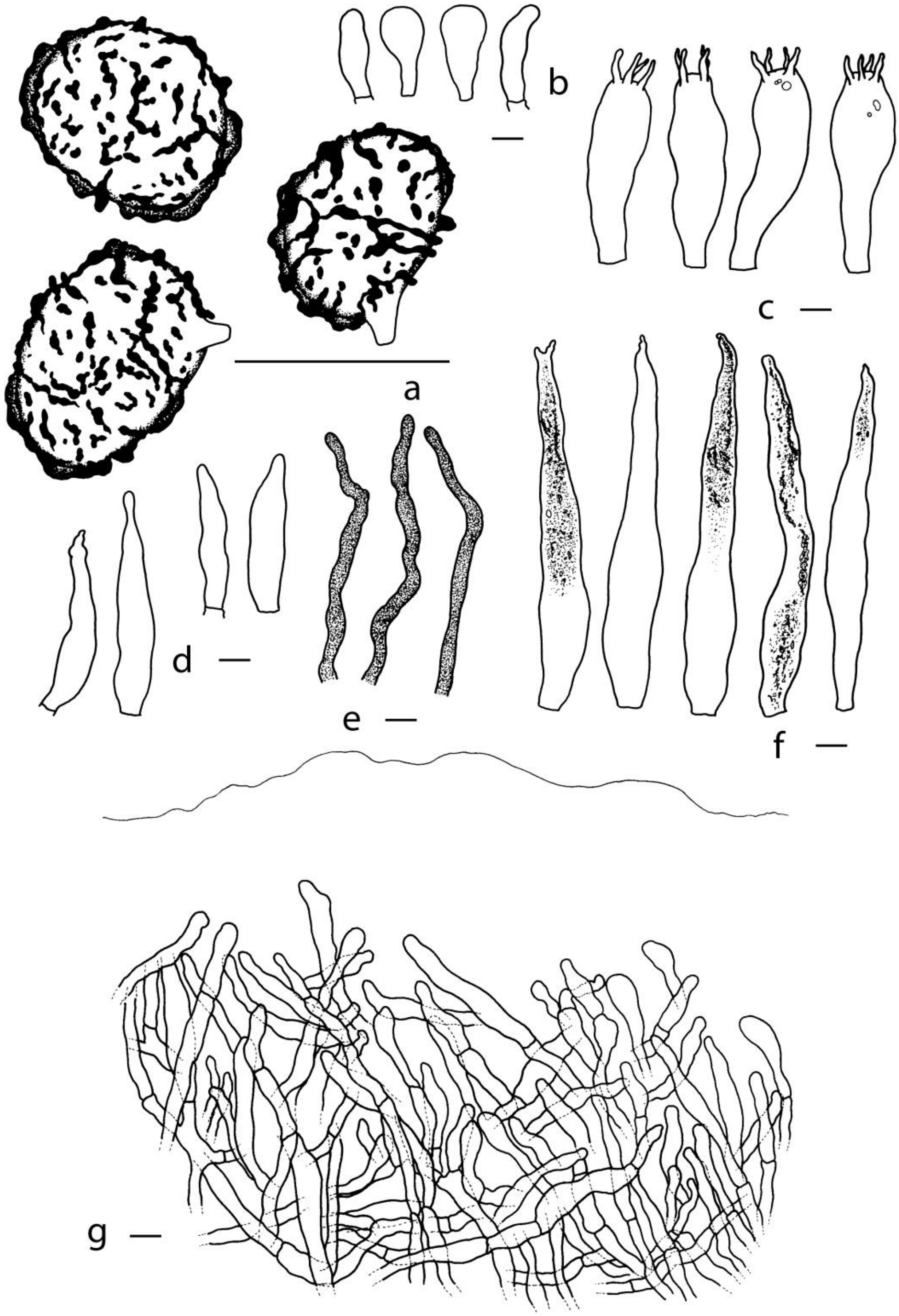

Figure 6.6: *Lactarius aurantionitidus* (a–g: xp3-20120905-06, holotype): a. basidiospores, b. marginal cells, c. basidia, d. cheilocystidia, e. pseudocystidia, f. pleuromacrocystidia, g. pileipellis (scale bar = 10µm).

Lactarius dombangensis Verbeken & Van de Putte sp. nov. (Figure 6.7)

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 *Tsuga*, *Abies*, *Juniperus*, *Picea* and *Larix,* 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 x 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 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 South-west of Dombang Valley, mixed coniferous forest with *Rhododendron* sp., *Tsuga*, *Abies densa*, *Juniperus*, *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 *L. obscuratus* in Europe: *L. obscuratus*, *L. cyathuliformis* and *L. omphaliformis*. 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.

L. crenulatulus and *L. perparvus* are also similar but they grow in tropical evergreen forest dominated by members of Fagaceae. *Lactarius crenulatulus* is somewhat different 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 flavigalactus Verbeken & K. Das sp. nov. (Figure 6.8 and 6.11c)

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 for pileipellis structure.

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, 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 x 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

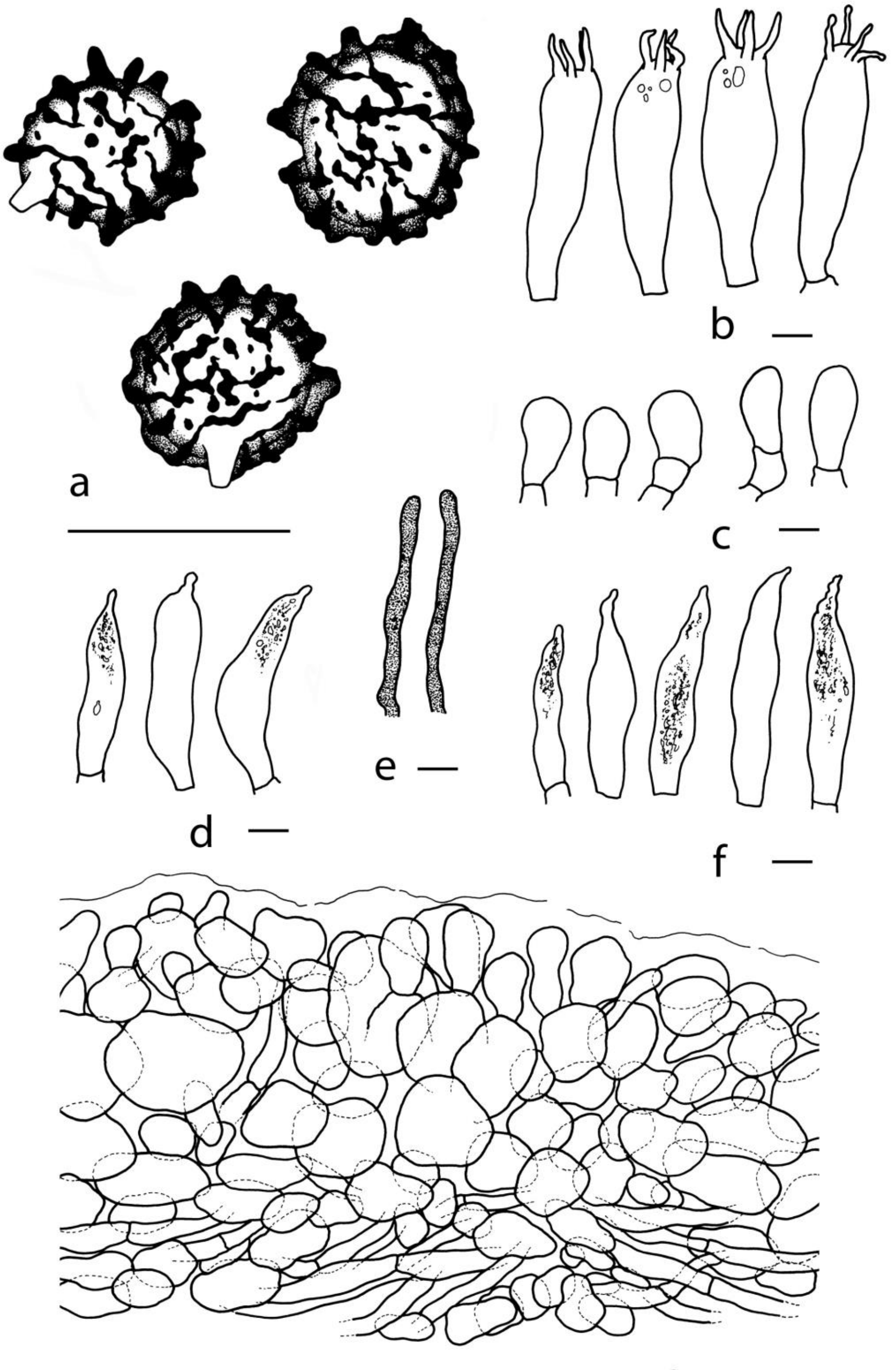

Figure 6.7: *Lactarius dombangensis* (a–g: AVKDKVP09-020, holotype): a. basidiospores, b. basidia, c. marginal cells, d. cheilocystidia, e. pseudocystidia, f. pleuromacrocystidia, g. pileipellis (scale bar = 10µm).

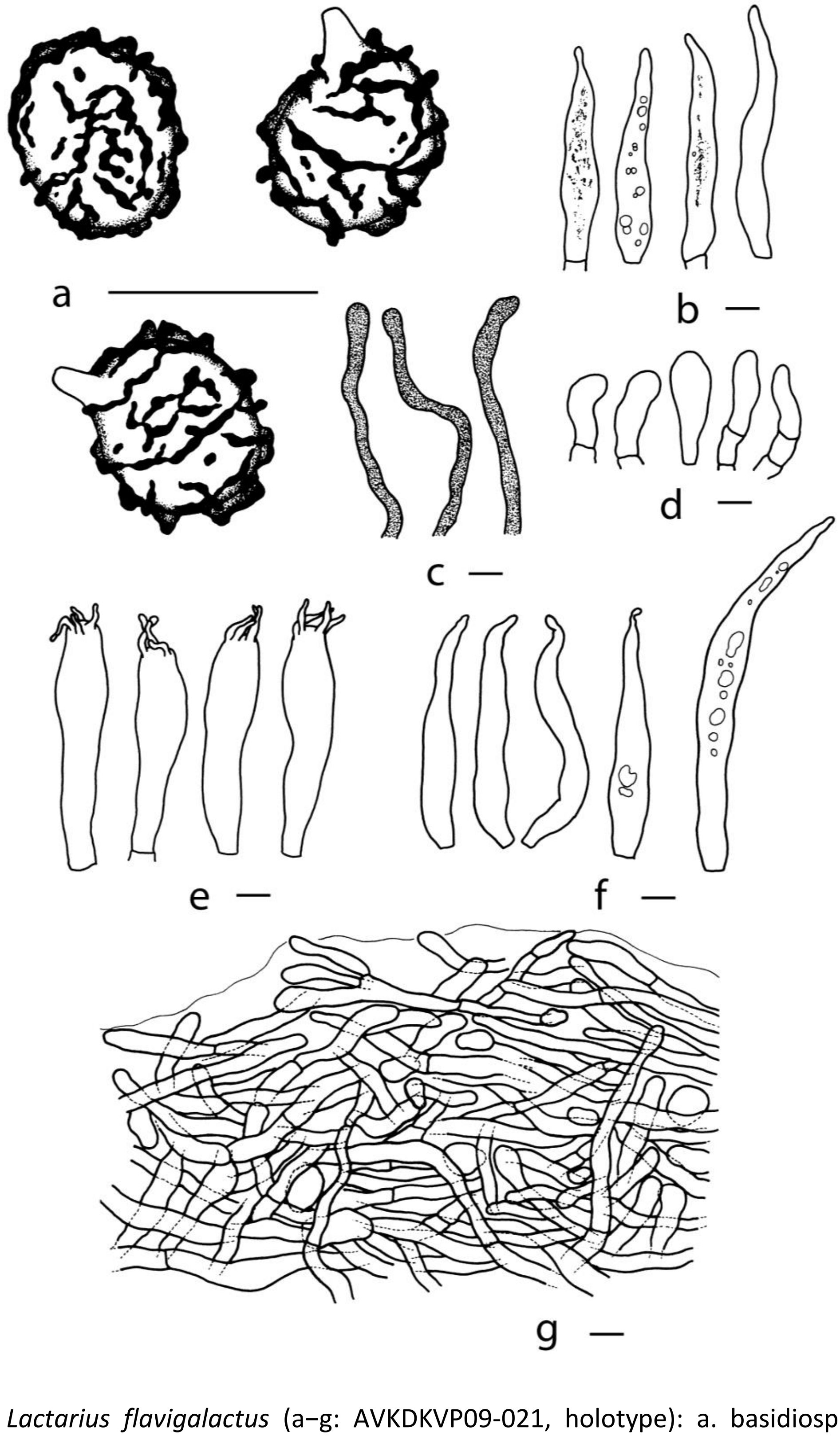

Figure 6.8: *Lactarius flavigalactus* (a–g: AVKDKVP09-021, holotype): a. basidiospores, b. cheilocystidia, c. pseudocystidia, d. marginal cells, e. basidia, f. pleuromacrocystidia, g. pileipellis (scale bar = 10µm).

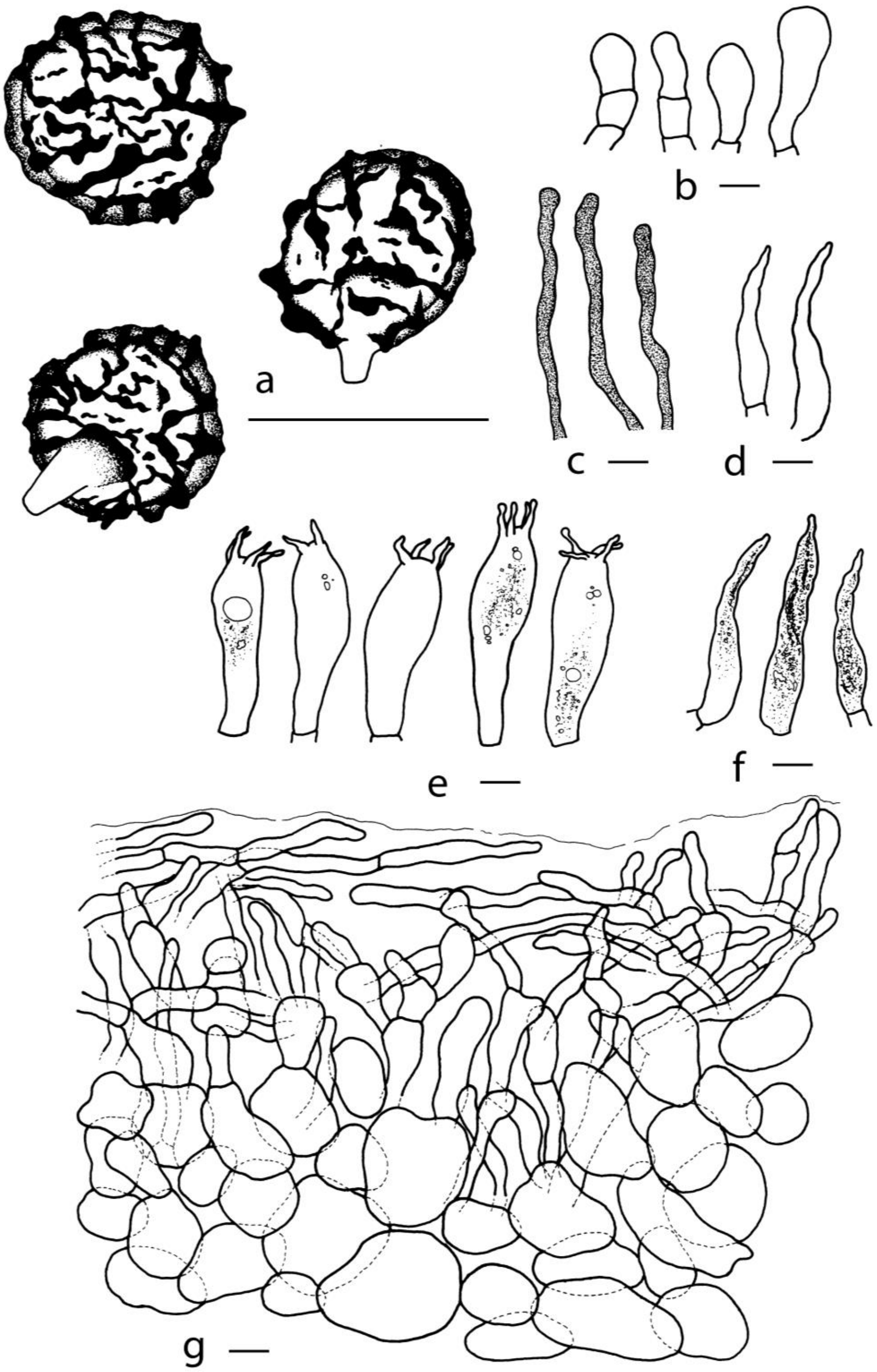

Figure 6.9: *Lactarius lachungensis* (a–f: AVKDKVP09-009, holotype, g: AVKDKVP09-010): a. basidiospores, b. marginal cells, c. pseudocystidia, d. cheilocystidia, e. basidia, f. pleuromacrocystidia, g. pileipellis (scale bar = 10µm).

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°28.45'99°51.63', 3212m 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', 3215m alt., 08/07/2013, ZD084 (KUN!, GENT!).

Comment: In the field *L. flavigalactus* reminds of the European *L. badiosanguineus* by 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* is also similar but this species has a paler pileus margin, unchanging milk and completely reticulate basidiospores. *Lactarius hepaticus* has similar colour 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. (Figure 6.9 and 6.11d)

Mycobank number: MB812834 **Facesoffungi number:** FoF 00857

Diagnosis: Medium-sized, warm orange brown basidiocarps, 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, with lamellulae of different lengths, without regular pattern, pink, orange white (6A2); edge entire, concolourous. *Stipe* 35−40 x 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.

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 to 1.4 µm high which are irregular, obtuse to subacute and sometimes crenulate, forming an incomplete reticulum to subreticulum, connected by thin ridges; isolated wart 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: In the field this species reminds of the European *L. tabidus*. *Lactarius 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*.

Lactarius sikkimensis Verbeken & K. Das sp. nov. (Figure 6.10 and 6.11e)

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, with lamellulae of different lengths, without regular pattern, pink, orange white (6A2) to pale orange (6A3); edge entire, concolourous. *Stipe* 40−75 x 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, growing in 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!); Sikkim, 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., 14/08/2009, AVKDKVP09−025 (GENT!);−ibid.14/08/2009, AVKDKVP09-026 (GENT!); Sikkim Prov., 2 km South-west of Dombang Valley, mixed coniferous forest with *Tsuga*, *Abies densa*, *Juniperus*, *Picea* and *Larix s*, N27°44.13' E88°45.96, 2840 m alt., 14/08/2009, AVKDKVP09−027 (GENT!, CAL!); Sikkim Prov., Dombang Valley, 5−6 km from Lachung, mixed coniferous forest (*Picea*, *Tsuga*, *Juniperus*) with few *Rhododendrons*, N27°44.51' E88°46.32, 2940 m alt., 15/08/2009, AVKDKVP09−058 (GENT!, CAL!).

Comment: In the field *Lactarius sikkimensis* is very similar to *L. lachungensis,* but it differs by the brick-red tinges which are absolutely absent in *L. lachungensis*. The other significant difference is the basidiospores that are larger and more ellipsoid in collections of *L. sikkimensis*.

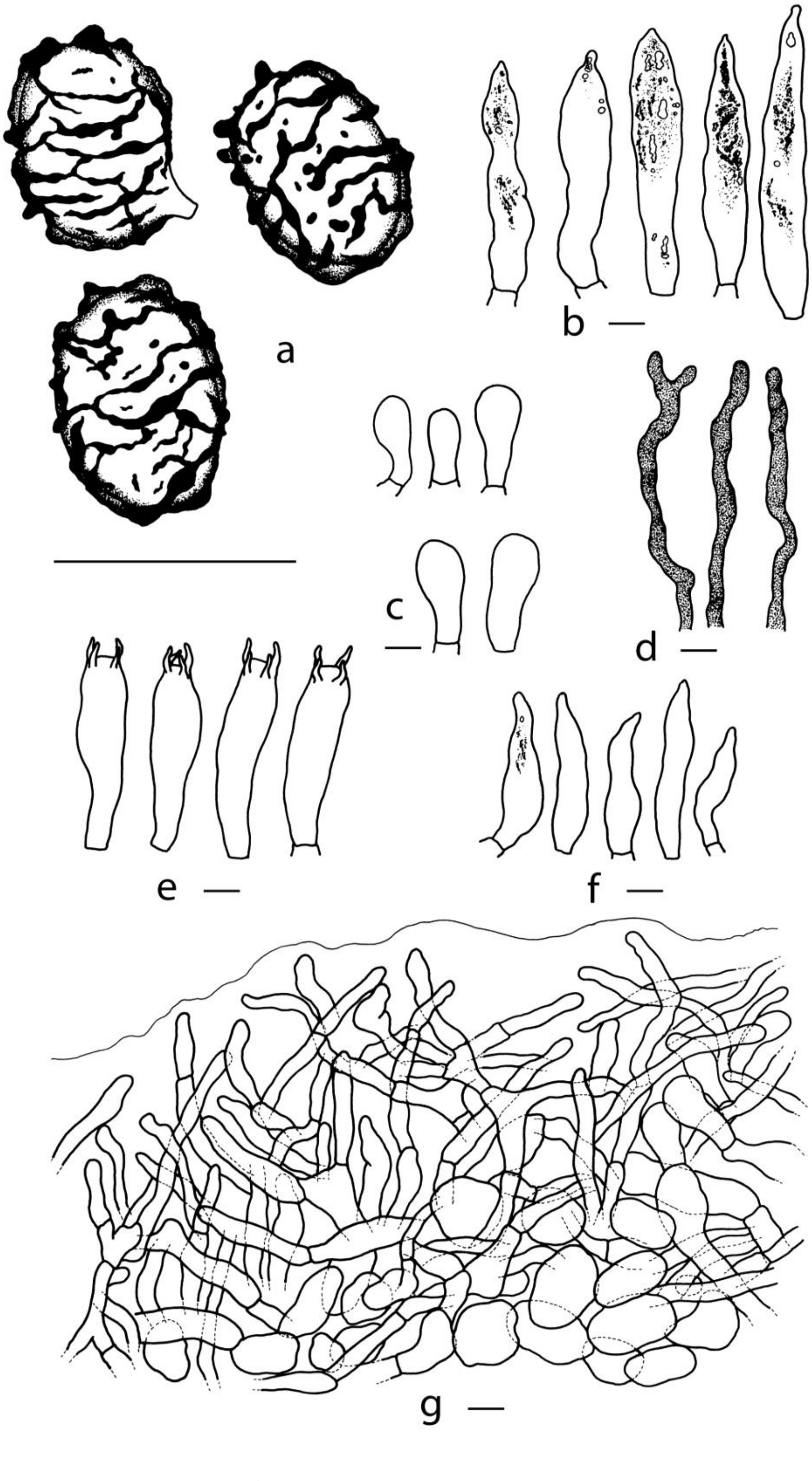

Figure 6.10: *Lactarius sikkimensis* (a–f: AVKDKVP09-024, holotype, g: AVKDKVP09-058): a. basidiospores, b. pleuromacrocystidia, c. marginal cells, d. pseudocystidia, e. basidia, f. cheilocystidia, g. pileipellis (scale bar = 10µm).

Figure 6.11: 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).

Discussion

Taxonomy

Pileus zonation is a striking field character that is considered typical for many members of *L.* subg. *Lactarius* (Heilmann-Clausen et al, 1998). Molecular results from this study show that strongly zonated caps can also occur in *L.* subg. *Russularia.* Two species traditionally treated as members of *L.* subg. *Lactarius*, *L. chrysorrheus* and *L. vinaceorufescens* are firmly placed in *L.* subg. *Russularia* and are thus classified as members of *L.* subg. *Russularia* in our study. *Lactarius chrysorrheus* is described from Europe but also reported 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, also 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 *L. indochrysorrheus* and an undescribed, related species from North America (collections AV04-212 and AV05-359, GENT).

The classification of *L. helvus* and *L. rufus* are contradictory in different monographs. Both species are treated as members of *L.* subg. *Russularia* by Hesler and Smith (1979) and Basso (1999). By many authors they have been classified in a separate subgenus *L.* 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 *L. 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 in the 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 *L.* 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,

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

the presence or absence of true cystidia and the presence or absence of well-developed cellular layer in pileipellis were proposed as discriminative characters.

We evaluated five characteristics that are considered important for species delimitation and classification in *L.* subg. *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 (Figure 6.2). 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 *L.* subg. *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 Heilmann-Clausen et al. (1998) and Basso (1999) are considered. Our result differs from the classification of Basso (1999) because here *L.* subg. *Rhysocybella* Bon species sensu Basso (1999) are included within *L.* subgenus *Russularia*. Pileipellis structure and true cystidia were mentioned as an important character in many studies (Bon 1983, Heilmann-Clausen et al. 1998, Basso 1999). In clade a (see figure 6.2), all species have true cystidia. 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 *L. dombangensis* and the *Alnus*-associated taxa: *L. cyathuliformis*, *L. obscuratus*, *L. brunneohepaticus*, *L. herrerae* and *L. 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: *L. quietus* and *L. aquosus*. Both species have an inconspicuous zonation and true cystidia.

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 *L.* 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 *L. chrysorrheus* (in clade g, figure 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 (Figure 6.2).

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. *L. chichuensis* (zebroid), *L. crenulatulus* (complete reticulum), *L. politus* (ridges up to 2.5 µm forming a complete reticulum), *L. helvus* (complete reticulum) (Figure 6.2). The same is true for an ornamentation completely composed of isolated warts (occurring in *L. falcatus*, *L. atrii* and *L. aurantionitidus*).

Concerning the general habitus, 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* and *L. borzianus* 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 *L. 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 (*L. sublaccarioides*, *L. pasohensis*, *L. atrobrunneus* and *L. laccaroides*) 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 characterization 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 brusing 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.

We hypothesize that the ectomycorrhizal host association might have played an important role in species evolution within this subgenus. 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). This study shows that most *Alnus*-associated species of *L.* subg. *Russularia* form a supported clade (Figure 6.2). This is in accordance with Rochet et al. (2011) and confirms that *L. brunneohepaticus*, *L. obscuratus*, *L. cyathuliformis* and *L. herrerae* appear to have evolved from the same common ancestor while *L. omphaliformis* has an independent origin. Most of these species are associated with hosts of *Alnus* section *Alnus*, except for *Lactarius brunneohepaticus* which is found in association with *A. alnobetula* (*A*. section *Alnobetula*). *Lactarius obscuratus* and *L. cyathuliformis* are associated with *A. incana*, *A. glutinosa* and *A. cordata* (*A.* section *Alnus*). Montoya et al. (2015) described the ectomycorrhizal association of the Mexican *L. herrerae* with *A. 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. *L. aurantiacus*, *L. fulvissimus* etc. 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 about ecology and symbiotic interaction of ECM species.

Intercontinental specificity

The discussion of distribution patterns is necessarily limited by the sampling of mainly European and Asian representatives. Especially representatives from the New World are 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, northern versus southern hemisphere etc.). 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 between Europe/Asia and North America/Europe. The European and Chinese specimens identified as *L. badiosanguineus* group together in a clade with high support (98% bs and 1.00 pp, in Figure 6.2) 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* (Franch.) E. Pritz. 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 *L. tabidus* (Figure 6.2). 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 *L. 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* (Figure 6.1). 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*, *L. helvus* and *L. camphoratus* are known to occur in North America (Koljalg et al. 2013). *Lactarius subdulcis* has been recorded in the United States and Iran (Koljalg et 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 *L.* subg. *Russularia* species.

Divergence time estimation

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 (Figure 6.3 and Table 6.2). 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). Hibbett & Methany (2009) suggest that the age of the Boletales and Russulales might be young enough to have been plesiomorphically associated with either rosids or Pinaceae.

The estimated crown age of Russulales is 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 mid Miocene (dataset2, see Figure 6.4). 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 *L. aquosus*/*L. quietus* group remains uncertain due to its low supports (19% bs/0.48 PP). This clade appeared 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 with 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. We annotated possible host ranges in the dated phylogeny of *L.* subg. *Russularia* in figure 6.4.

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 *L. 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. *L. hepaticus*, *L. badiosanguineus* and *L. rufus* as well as the newly proposed species in this study. All newly proposed species and some tropical lowland species, e.g. *L. sublaccarioides*, *L. inconspicuus* and *L. tangerinus*, 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 and diversified towards subfamilies and genera 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 Ecocene 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 the Beringia Land Bridge (BLB) during the Mid 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 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). The ancestral *Quercus* species might early diverged between North America and Asia via the BLB. White oaks (section *Quercus* s.s.) might have a New World origin (Manos et al. 1999). The migration events might be occurred in the late Eocene (~40 mya) and the Mid Miocene (~15 mya) (Tiffney 1985). The other dominant vegetation in Southeast Asia is Dipterocarpaceae. Molecular dating analyses suggested that the origin of ancestral species of Dipterocarpaceae might be Africa in the early Cretaceous and might be migrated to the Deccan plate and Southeast Asia. Fossil records showed 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*.

Many studies proposed hypotheses on the major factors of the migration of ancestral species of ECM trees. Two major events during Neogene and Quaternary periods that could have led to the migration or isolation of populations of ancestral *L.* subg. *Russularia* species are given here.

1. The formation of high mountains may have profound effects on historical climate, for example, the uplift of the Himalayan-Tibetan complex. Climatic changes could have triggered the migration or range expansion of ECM host trees. The height of the Himalayan mountains and the Tibetan plateau are thought to affect the climates of Asia. Zhisheng et al. (2001) proposed that the formation of Himalayan mountains began in the end of the Paleocene epoch, about 50 mya.

A global climatic change was occurred in the end of Eocene (~34 mya) (Millar 1999). In both Northern and Southern hemisphere climate shifted to a zonal condition more similar to the present-day climate. The climate in the middle latitudes shifted from a zonal climate pattern in the Paleogene into a monsoon dominated pattern in the early Miocene. The Southwestern and Southeastern summer monsoons brought humidity to the continent (Guo et al. 2008). The Southeastern monsoon from the South China Sea influences the humidity for macrofungi in Southeast Asia and Southern China. The distribution areas of ectomycorrhizal trees in subtropical region in Asia shrunk because of the formation of the inland deserts. In the early of Miocene, the onset of loess deposition in the central part of China was influenced by the northerly dust carrying winter winds from Northern sources (Liu 1985, An et al. 1990, Ding et al. 1995, Liu and Ding 1998, Guo et al. 2002 and Guo et al. 2008). This phenomenon has led to the extension of drylands in formerly subtropical regions in China. The significant increases in altitude of Tibetan plateau might have occurred about 10–8 mya. These geographic barriers may have caused the isolation of population of ancestral species. The uplift in these areas has resulted in inland aridification in China (Millar 1999, Guo et al. 2008) and might have a large effect in estabishment of the south Asian monsoon (Dong & Wen 2013) and intensification of East Asian summer and winter monsoon (Zhisheng et al. 2001).

2. Quaternary glaciations have led to an increase of isolation and migration of plant species at a global scale (Willis & McElwain 2002). Quaternary glaciation is a series of glacial events separated by interglacial events during Pleistocene epoch. During these events, continental glaciers covered large part of Europe, Siberia and North America. Comes and Kadereit (1998) explained that the isolation of species during glacial period, expansion during interglacial and postglacial period might played a major role in plant distribution and diversification. Linares (2011) explained that the glacial cycles might have caused the migration of ancient *Abies* species in the Mediterranean basin. The possible refugia pattern of European *Quercus* spp. might have spread southwards to Italy, Iberia and Balkan Peninsula (Dumolin-Lapègue et al. 1997 and Ferris et al. 1998). In Southeast Asia, lowered-sea level during the last glacial maximum resulted in a continuous corridor, called Sundaland (Wurster et al. 2010). This corridor might be a land bridge between the Malaysian Peninsula and the present-day major islands, e.g. Sumatra, Java and Borneo (Bird et al. 2005).

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CHAPTER 7

GENERAL DISCUSSION AND CONCLUSION

7.1 Species concept and cryptic species

Species are the fundamental units of organisms. Therefore the criteria of species delimitation need to be practical and accurate. A number of different species concepts have been used in mycology. Especially in the field of macrofungi, species recognition and species delimitation has a strong tradition of following the morphological species concept (MSC) where differences and similarities in morphological features form the basis of species boudary. This concept brought about some problems because morphological characters are often rather simple in some groups of fungi. Morphology can mislead in evolutionary relationship or species delimitation of morphologically similar species in cases of convergent evolution. Hence similarity in morphology often harbours cryptic species. Cryptic species means that two or more species are morphologically so similar that it is not possible two tell them apart by using morphological tools only. Consequently, they are erroneously treated under one species name (Bickford et al. 2006). On the other hand, pseudo-cryptic species are morphological similar species that have been identified as separate species when appropriate methods were applied (Luttikhuizen & Dekker 2010).

Besides the MSC, the biological species concept (BSC) is frequently used. The BSC defines species as populations that interbreed and reproduce in nature, not according to similarity of appearance. Many morphological species have been discovered using the BSC (Anderson & Ullrich 1978). However, the ability of reproduction is not always testable in fungi, e.g. in fungi that reproduce only asexually, in fungi that cannot be coaxed into mating in cultivation and in obligate mycorrhizal fungi that are not growing in cultures. The latter is the case for *Lactarius*. Although a few species of *Lactarius* have been cultivated on culture media, e.g. *L. deliciosus* (Barros et al. 2006, Akata et al. 2012), little studies on *In Vitro* culture of *Lactarius* are available. Therefore the BSC has not been primarily applied for *Lactarius* spp. The Ecological Species Concept (ESC) emphasizes adaptation to a particular niche. Since the introduction of molecular tools, the Phylogenetic Species Concept (PSC) has been used in many groups of fungi. The PSC uses genetic information to define species. A phylogenetic species independently evolved in a strongly supported clade. This approach is currently being widely used in species delimitation for fungal species. Recently the genealogical concordance of multiple genes has been applied in species delimitation for certain groups of fungi, e.g. *Lactifluus*, because species boundary based on a single locus can sometimes be subjective or uncertain in closely related clades or cryptic species when they receive low phylogenetic supports. Therefore Genealogical Concordance Phylogenetic Species Recognition (GCPSR) has become widely accepted and used as a practical and functional species concept in mycological communities (O'Donnell et al. 1998, Taylor et al. 2000, Dettman et al. 2003a, 2003b, de Queiroz 2007, Cai et al. 2011). To avoid subjective conclusion from a single species concept, a polyphasic approach using conclusions of the MSC, ESC and PSC, named the Consolidate Species Concept (CSC), has been proposed and used (Quaedvlieg et al. 2014).

As in many macrofungi, species delimitation in *Lactarius* subg. *Russularia*, was mainly and only based on the MSC. Most recent monographs (Hesler and Smith 1979, Heilmann-Clausen et al. 1998 and Basso 1999) were using a taxonomy and classification purely based on macro- and micromorphology, to a lesser extent adding some macrochemical and ecological characters. However, when using a morphological based criterion alone, species boundaries of some species and classification systems are uncertain because it evidently depends on the precision of the collector's observation, as well as a number of considered characteristics. *Lactarius* subg. *Russularia* has remarkably similar morphological features, especially when compared to other groups of milkcaps, species boundaries of some species remain problematic. This is especially true in European taxa where there has been a longer mycological tradition resulting in many different opinions. For some characters there has been long discussion whether the variation was inter- or intraspecific (e.g. in the group around *L. serifluus*: colour of the cap, shape of the paracystidia). Some collections have been defined as new species without in depth comparison to the type of extant species, which later resulted as synonymous names. Some names of extant species have been used for superficially similar species. In tropical regions we have fewer names available and hence fewer synonyms, but we are confronted with the problem that names of temperate taxa are used for tropical taxa based on (superficial) morphological similarities without in depth comparison or molecular support. Thus, a combination of multiple species concepts can strengthen species boundaries of *L.* subg. *Russularia*.

In this PhD work, I aimed at using the CSC, combining macromorphological, micromorphological, ecological and molecular data to define species delimitation and evaluate whether cryptic species do occur in this subgenus or not. Cryptic species are defined when species are genetically distinct, but morphologically indistinguishable. Pseudocryptic species are similar at first sight but can be discriminated when the suitable (and often traditionally not studied) characteristics are used. New species in this study were described when it was clear that they were morphologically well-defined and formed a distinct phylogenetic group with high support (bootstrap value that exceeds 95%). Highly similar or identical sequences are to be expected for specimens of the same species.

Morphological characteristics of collected specimens were described from fresh specimens or obtained from the notes of collectors or the original species description. In this phase I am already confronted by the first problem using morphological characters, which is the reliability of observations. The accurate and consequent observation and description of particular features is necessary and no strict protocol for these observations exists, e.g. how to define the time interval needed for latex to stain yellow, how to isolate latex from the flesh, how to observe latex on white tissue, which concentration of KOH to use to test the latex colour change with this reagens etc. Furthermore, in order to record morphological variation, it is necessary to collect different stages of developing fruiting bodies. If only one fruiting body was used to document a collection, I prefer not to describe it as a new taxon until we obtained more collections. A protocol to standardize the macromorphological descriptions of *Lactarius* subg. *Russularia* representatives is given in the appendix 4.

As I used molecular as well as morphological data to compare taxa, I observed that in some cases the evidences given by both types of characters were not congruent. Ecological data were gathered but not used to the same extent as the morphological ones, because identifying the host tree was not always straightforward and these data are very often lacking in descriptions of other collectors. The actual ectomycorrhizal association between a fruiting body and its host tree has not been tested or confirmed molecularly in this work. Of course, the dominant vegetation in a sampling site can be helpful for identifying species and might give clues about the ectomycorrhizal host tree.

So far, the results in this study show that there are no cryptic species in Asian *L.* subg. *Russularia*. Some of the molecularly recognized species are strongly supported by unique and striking morphological characters, for example, the distant gills and dark brown cap in *L. atrobrunneus*, or the sticky surface and bright orange cap in *L. aurantionitidus*. Pseudocryptic species occur, they look very much a-like at first, but after an intensive search we found morphological characters to separate them. For example *L. fuscomaculatus and L. rubrobrunneus* were first separated based on molecular data, but are morphologically very similar and hard to distinguish both in the field and under the microscope. The slight differences between both species are the presence of distinct dark brown discolouration in the cap of mature *L. fuscomaculatus* and the greater frequency of macrocystidia in this species.

Despite the fact that DNA sequence data have become widely used in species delimitation of fungi, there is a little information on DNA sequence data of *L.* subg. *Russularia* before this study and most of the former data are restricted to sequences of the ITS regions. One of the aims in this thesis is to generate reliable DNA sequence data on both ITS and *rpb2*, and contribute these sequences in public sequence database. In this study I generated DNA sequences of ITS and *rpb2* genes from European and Asian taxa as well as a lesser number of North American samples. A total of 238 ITS and 121 *rpb2* sequences has been deposited in GenBank.

From the molecular data I can conclude that both ITS and *rpb2* are useful for species delimitation. The two parts of the genome give a concordant signal. Unfortunately, many European specimens failed in *rpb2* amplification, especially those samples that are more than 10 years. The success rate of PCR amplification is dependent on the size of the amplified fragments and the storage duration of samples (Rachmayanti et al. 2009). In addition, the overheating in the drying process of the fruiting bodies could also damage the DNA molecule. Therefore I realize that material needs to be fresh. Fresh tissues of fruiting body should be conserved in CTAB buffer in order to have a more successful amplification for larger molecular markers, like *rpb2* gene.

7.2 Biodiversity

Before this PhD study, the knowledge on the biodiversity of *L.* subg. *Russularia* was mainly restricted to the temperate region, Europe and North America, but very poor in tropical regions. The number of *L.* subg. *Russularia* species described from Southeast Asia before this study was six species: *L. chichuensis* and *L. hirtipes* from China; *L. falcatus* from Thailand; *L. caulocystidia*, *L. javanicus* and *L. sulphurescens* from Indonesia (Table 7.1). One of the objectives in this study was to fill these gaps by conducting extensive sampling in five different places in Southeast Asia and South Asia for which provisional exploring suggested that they were biodiversity hotspots for this or related groups of ectomycorrhizal macrofungi: 1) Northern Thailand, mainly in Chiang Mai and Chiang Rai provinces, 2) Pasoh National Forest Reserve, Malaysia, 3) the regions around Dalat in Vietnam, 4) Sikkim province in India and 5) Yunnan province in China. Further exploration of the African continent was conducted by other members of the mycology research group, but as suspected before the start of this study, the subgenus seems to be completely absent in sub-Saharan Africa (except for the introduced *L. hepaticus* growing with *Pinus* in South Africa).

This study revealed twenty three new species, with nineteen of them described from Southeast Asia. This result highlights the high diversity of this subgenus and makes the total number of *L.* subg. *Russularia* species in Southeast Asia now twenty-five species (Table 7.1). Fifteen new species were discovered in Thailand, of which two are also found to be common in Vietnam (*L. kesiyae* and *L. rubrocorrugatus*) and recently *L. kesiyae* was also collected in Laos (Verbeken, unpubl.). The remaining new species are described from Malaysia (two species), India (four species) and China (two species which one is also found in India).

The high number of new species known from Thailand does not necessarily suggest that the biodiversity of *L.* subg. *Russularia* in Thailand is much richer than in the other mentioned areas, but is also reflects the high exploration rate in Northern Thailand. I was able to capture the peak fruiting period in the mushroom season because I was mostly in Northern Thailand during the complete rainy season. During shorter expeditions to other countries it is always difficult to capture the very right moment. For example, the expeditions in Vietnam were not successful and consequently yielded only two species of *L.* subg. *Russularia*, both from coniferous forest. Unfortunately I was unable to obtain collections from evergreen forest because the habitat was exceptionally dry. Further sampling in evergreen forest in Vietnam would be recommended. The same is true for Laos, where the subgenus also seems to be well represented. There are some reasons to believe that there are numerous new species still waiting to be discovered in this subcontinent. Most species discovered so far seem to be restricted to some habitats and regions, i.e. evergreen forest with Fagaceae trees in Northern Thailand and temperate coniferous forest in India and China, suggesting that exploration of other habitats will reveal a separate diversity. Some species are very small and often grow among the leaf litter, making them difficult to be noticed in the field, like *L. glabrigracilis*, *L. perparvus* and *L. crenulatulus*. Some species are very similar to other species, thus without careful observation, they can be overlooked in the field, like *L. sublaccarioides* and *L. laccarioides*. If further mycological expeditions were carried out in habitats with different ectomycorrhizal trees (i.e. Dipterocarpaceae, Betulaceae, Fabaceae) or in other underexplored regions, many more new species might be discovered.

Table 7.1. The number of described species of *L.* subg. *Russularia* in different regions of Asia and Oceania before and after this study.

Geographic regions	Before this study	After this study
East Asia	2	2
Southeast Asia	6	25
South Asia	4	8
Oceania	9	9

I am convinced that this research will be a basis and motivation for the further study in biodiversity of *L.* subg. *Russularia* in Southeast Asia and neighbouring regions. I propose an identification keys to all species known in Southeast Asia and Australasia as follows.

Identification key to species of *L.* subg. *Russularia* in Southeast Asia, South Asia and Australasia

1. Basidiocarp angiocarpic; latex white, turning bright yellow; basidiospores echinulate……………………***L. fulcatus***

1. Basidiocarp agaricoid……………………………………………………………………………………(2)

2. Pileus large sized, up to 190 mm diam., surface venose or wrinkled; distant gills; pileipellis a trichoepithelium (palisade)……………………………………………………………………………………***L. javanicus***

2. Pileus small to medium sized, never larger than 100 mm; not with this combination of characters……………………………………………………………………………………(3)

3. Pileus distinctly small sized, with the average size not exceeding 20 mm in mature specimens; stipe small and narrow……………………………………………………………………………………(4)

3. Pileus medium sized, with average size of the mature specimens distinctly large than 20 mm…………………(11)

4. Pileus margin typically with triangle tufts in young and mature specimens……………………………***L. gracilis***

4. Pileus margin without tufts……………………………………………………………………………………(5)

5. Pileipellis with a slime layer, ixohymenoepithelium to ixoepithelium; pileus warm reddish orange, crenulate margin; basidiospore ornamentation obtuse to rounded connecting by lower ridges, incomplete reticulum……………………………………………………………………………………***L. dombangensis***

5. Not with this combination of characters, pileipellis never with a slime layer……………………………(6)

6. Basidiospore ornamentation strikingly inamyloid, very low ridges forming almost complete reticulum; pileipellis a trichoepithelium……………………………………………………………………………***L. inamyloideus***

6. Basidiospore ornamentation amyloid……………………………………………………………………………(7)

7. Basidiospore ornamentation forming a complete and dense reticulum; true cystidia absent; stipe mostly with whitish pruinose at base……………………………………………………………………………***L. crenulatulus***

7. Basidiospore ornamentation forming an incomplete reticulum or isolated warts……………………………(8)

8. Basidiospore ornamentation composed of isolated rounded obtuse warts up to 1 µm high; a pointed papilla on pileus; true cystidia absent; pileipellis a trichoepithelium……………………………………***L. liliputianus***

8. Basidiospore ornamentation forming an incomplete reticulum, with some short isolated ridges present…..(9)

9. Pileus about 8 mm diam., never with striate margin; latex transparent; true cystidia absent; pileipellis an epithelium……………………………………………………………………………………***L. perconicus***

9. Pileus larger or smaller, but with a striate margin; white latex; true cystidia present……………………(10)

10. Pileus very small, 5−7 mm diam., without a pointed papilla; stipe 9−12 mm length; pileipellis a cutis……………………………………………………………………………………***L. perparvus***

10. Pileus larger, ranging from5 to 40 mm diam., with a distinct pointed papilla; stipe 10−50 mm length; pileipellis an epithelium……………………………………………………………………***L. glabrigracilis***

11. Latex transparent, completely watery, unchanging on exposure……………………………………………(12)

11. Latex white……………………………………………………………………………………(20)

12. True cystidia absent; pileipellis an epithelium……………………………………………………………(13)

12. True cystidia present……………………………………………………………………………………(14)

13. Pileus medium sized, about 45 mm diam., orange brown to reddish brown, basidiospore ornamentation zebroid……………………………………………………………………**_L. corrugatus_**

13. Pileus rather small sized, 6−31 mm diam., dark reddish brown to dark brown, wrinkled;; basidiospore ornamentation subreticulate……………………………………………………………**_L. atrobrunneus_**

14. Pileipellis a hyphoepithelium, with a layer of swollen to isodiametric cells………………………………(15)

14. Pileipellis a cutis or trichoderm, completely composed out of hyphae, without isodiametric cells…………(17)

15. Basidiospore ornamentation high with ridges up to 2.5 µm, forming a complete reticulum; pileus polished, but locally wrinkled, dark brown at center, paler towards margin…………………………………**_L. politus_**

15. Basidiospore ornamentation composed of ridges lower than 2.0 µm, forming an incomplete to almost completereticulum……………………………………………………………………(16)

16. Pleuromacrocystidia with a rostrate apex, remarkably protruding up to 40 µm; pileus dark reddish brown to dark brown…………………………………………………………………**_L. austrorostratus_**

16. Pleuromacrocystidia with a shorter apex, mucronatet;, reddish brown…………………………**_L. rubrocorrugatus_**

17. Pileus often with inconspicuous zonation, reddish brown to brown, paler towards margin…………**_L. aquosus_**

17. Pileus without zonation……………………………………………………………………(18)

18. Stipitipellis with caulocystidia……………………………………………………………**_L. caulocystidiatus_**

18. Stipitipellis without caulocystidia……………………………………………………………(19)

19. Basidiospores 6.1−7.9 × 5.4−7.0 µm; pileipellis a trichoderm……………………………………**_L. tangerinus_**

19. Basidiospores 8.4−9.8 × 7.6−9.0 µm; pileipellis a trichopalisade to trichoderm………………………**_L. pennulatus_**

20. Latex white, then turning chrome yellow within a few minute; pileus glossy and shiny, reddish brown; pileipellis an ixocutis……………………………………………………………………**_L. flavigalactus_**

20. Latex white, unchanging or with subtle colour change, slowly turning pale cream to pale yellow within an hour……………………………………………………………………………(21)

21. Species reminding a *Laccaria* species, with gill spacement distant, mostly less than 8 L+l/cm, pileus margin translucently striate and sulcate……………………………………………………………(22)

21. Species not reminding a *Laccaria* species, gill spacement much more crowded to subdistant and pileus without sulcate and translucent striations……………………………………………………………(24)

22. Pleuromacrocystidia present……………………………………………………………………(23)

22. Pleuromacrocystidia absent……………………………………………………………………(25)

23. Pleuromacrocystidia strikingly protruding from the hymenium; pileus orange brown to reddish brown, hygrophanous; basidiospores ornamentation complete reticulum…………………………………**_L. sublaccarioides_**

23. Pleuromacrocystidia not protruding from the hymenium; pileus warm brown to liver brown; basidiospores ornamentation incomplete reticulum……………………………………………………………**_L. stubbei_**

24. Pileus surface non-velutinous; stipe long (26−71 mm) and often very hispid ……………………**_L. laccarioides_**

24. Pileus surface minutely velutinous; stipe shorter (9−22 mm) and hairs only at the base…………**_L. pasohensis_**

25. Pileipellis without a thin slime layer; pileus dry……………………………………………………(26)

25. Pileipellis covered with a thin slime layer; pileus slightly viscid to viscid……………………………(31)

26. Pileus with distinct cinnamon brown zonation……………………………………………………………………...…***L. subzonarius***

26. Pileus not zonate, basidiocarp reddish brown……………………………………………………………………………………(27)

27. Pileipellis a loosely arranged cutis; basidiospore ornamentation completely reticulate; true cystidia absent; pileus felty or fibrillose; latex milky, unchanging on exposure…………………………………………………...***L. condimentus***

27. Pileipellis with a hyphoepithelium; basidiospore zebroid or incomplete reticulate; true cystidia present; pileus non-fibrillose; latex white, unchanging on exposure…………………………………………………………………………(28)

28. Basidiospore ornamentation zebroid……………………..………………………………………………………..***L. chichuensis***

28. Basidiospore ornamentation incompletely reticulate to almost completely reticulate…………….……………(29)

29. True cystidia abundant, protruding 10−30 µm from the hymenium; pileipellis an epithelium to hymenoepithelium………..***L. hirtipes***

29. True cystidia rare or absent, often not protruding from the hymenium; pileipellis a hyphoepithelium……(30)

30. Pileipellis a hyphoepithelium, with a layer of terminal hyphae about 10−20 µm thick; pileus typically with distinct dark brown discolourations and spots...***L. fuscomaculatus***

30. Pileipellis a transition between hyphoepithelium and epithelium, without or with a layer of terminal hyphae up to 10 µm thick; pileus more unicolour, without dark brown spots…………………….……..***L. rubobrunneus***

31. Pileus with paler colours, yellowish orange to light orange, rather unicolourous, glossy and viscid; basidiospore ornamentation composed of isolated warts and short ridges; pileipellis an ixotrichoderm ……***L. aurantionitidus***

31. Pileus with darker colours, liver brown to brownish orange to reddish orange………………………………………(32)

32. Basidiospore ornamentation composed of isolated obtuse warts or knots, seldom forming a partial reticulum………(33)

32. Basidiospore ornamentation typically incompletely reticulate to completely reticulate……….……………(34)

33. Basidiospore ornamentation composed of thick and rounded knots; pileus reddish brown; pileipellis an ixocutis………***L. guttisporus***

33. Basidiospore ornamentation composed of isolated and obtuse warts; pileus greasy, light brown with reddish brown spots; pileipellis an ixocutis……………………………………………………………………………….…***L. atrii***

34. Latex watery white, slowly turning to pale yellow on exposure and drying; pileipellis an ixotrichoderm; growing with *Castanopsis*………………………………………………………………………………………......***L. inconspicuus***

34. Latex watery white, unchanging on exposure…………………………………………………………………………………(35)

35. Pileus with distinctive obtusely conical papilla, reddish brown to orange brown; latex staining yellow on white handkerchief; basidiospore almost complete reticulum; pileipellis an ixotrichoderm to ixocutis……***L. austrotabidus***

35. Pileus without conical papilla……(36)

36. Growing with *Eucalyptus*; pileus viscid, brownish red; pileipellis an ixocutis………………………………***L. eucalypti***

36. Growing with Pinaceae……(37)

37. Growing with *Pinus kesiyae*; latex turning yellow on white handkerchief…......................................***L. kesiyae***

37. Growing with *Abies*, *Picea* or *Larix*………………………………………………………………………………………………(38)

38. Pileus orange brown without reddish brown tinge; pileipellis a transition between ixohyphoepithelium to ixotrichoepithelium………***L. lachungensis***

38. Pileus dark liver brown with an abruptly pale pinkish margin; pileipellis an ixotrichopalisade…………………….. ……***L. sikkimensis***

7.3 Classification and evolutionary relationships

Systematic mycologists have classified species of *L.* subg. *Russularia* based on some morphological characteristics. Difference of used morphological characters for classification systems by different mycologists have led to contradictions in their infrasubgeneric classification. Classifications proposed by different mycologists (Hesler and Smith 1979, Heilmann-Clausen et al. 1998 and Basso 1999) are shown in the appendix 2. Hesler and Smith (1979) proposed five sections, mainly based on the pileus structure and presence or absence of incrustations in the pileipellis. Heilmann-Clausen et al. (1998) classified species based on pileipellis structure and presence or absence of true cystidia and recognize 3 sections. The classification of Heilmann-Clausen is slightly different from Hesler and Smith's monograph. The sections *Subsquamulosi* and *Trivialis* were excluded from *L.* subg. *Russularia* and transferred to *L.* subg. *Lactarius*. Basso (1999) proposed a different classification based on pileipellis structure. The author has transferred some representatives, which are characterized by a cellular pileipellis and reticulate basidiospore ornamentation, to *L.* subg. *Rhysocybella* Bon.

One of the aims of this PhD study was to see whether one of the existing classification systems was supported by molecular data or whether a new system was needed. The results show that ITS and *rpb2* phylograms are highly similar. Morphological characters in general have a low phylogenetic signal for taxonomic ranks that are higher than the species level. The combination of microscopic characteristics, pileipellis and the presence or absence of macrocystidia, tend to be phylogenetic informative in many clades.

Evaluating Hesler and Smith's classification turned out to be impossible because of the lack of sufficient molecular data of North American species. What we can conclude is that the classification of Basso (1999) is not supported because all species which were treated in *L.* subg. *Rhysocybella* fall in *L.* subg. *Russularia*, in both single and concatenated analyses. The phylogeny based on concatenated dataset is also different from the classification system of Heilmann-Clausen et al. 1998. Species treated in *L.* sect. *Russularia* and *L.* sect. *Tabidi* are nested in clade a (Figure 6.2 in chapter 6), except *L. quietus* which appeared to be sister to species in clade a and diverged in a separate lineage. Species treated in *L.* sect. *Olentes* spread in different clades: *L. camphoratus* in clade d, *L. subumbonatus* in clade c and *L. atlanticus* in clade f. In my opinion, cellular pileipellis might be relevant in species lineage within this subgenus, but it is still hard to clearly group the species with cellular pileipellis in a separate section or subgenus as suggested in recent monographs (Heilmann-Clausen et al. 1998 and Basso 1999). The result of concatenated analysis (figure 6.2 in chapter 6) shows that a combination of two morphological characteristics; the presence/absence of true cystidia and pileipellis structure, appeared to be useful for infrasubgeneric classification. However, the phylogram obtained from concatenated data comprises low supported nodes which indicate uncertain position of particular clades. A multiple locus analysis might confirm their taxonomic positions and allow to show a more explicit classification scheme of the subgenus.

The molecular results in this study revealed that the morphological concept for this subgenus should be extended after the inclusion of some species, e.g. *L. chrysorrheus*, *L. helvus*, *L. rufus* and *L. vinaceorufescens* (see more details in chapter 6). On the other hand, an ITS phylogram including representatives of *L.* subg. *Russularia* revealed that *L. surphurescens* should not be a member of *L.* subg. *Russularia* (data not shown in this thesis). The taxonomic position of this species remains unclear. A further phylogenetic study including representatives of other subgenera of *Lactarius* will address this question.

Similarly we see that there is no geographical support for the clades and that species in the same geographical area do not cluster together. In other words, a species can be sister species with a species in a distant continent. For example, the *chrysorrheus* clade, a group where we do see very striking supporting molecular features (milk soon turning sulphur yellow and zonations in the cap), contains species from different geographic regions: North America (*L. vinaceorufescens* and *L. sp.*), Europe (*L. chrysorrheus*) and Asia (*L. indochrysorrheus*).

The evolutionary relationships and diversification pattern of *Russularia* species are poorly understood. The ancestral species of *L.* subg. *Russularia* might have originated in the Mid Miocene period (18 mya). The multitude of intercontinental sister relationships of North American, European and Asian taxa might be possibly due to allopatric speciation.

This evolutionary process might occur when populations of the same species become isolated by migration or vicariance. Geographic change and physical barrier, like uplift of a mountain chain and separation of continents, could hinder gene flow of a single species which lead to divergence of genetic information. In addition, the distribution of *L.* subg. *Russularia* might be related with the distribution of their symbiont partners. The migration of flora between Asia and Europe possibly occurred via a continuous landmass between these continents. The distribution of boreal floras should be via the Beringia Land Bridge (Brubaker et al. 2005) or North Atlantic land bridges (Akhmetiev et al. 1978, Elddholm et al. 1994, Tiffney & Manchester 2001, Denk et al. 2005, Denk et al. 2010).

In other cases, new species can be evolved through parapatric and sympatric speciation. Parapatric speciation is the evolution of geographically adjacent organisms into a new species. Whereas sympatric speciation could explain the cause of diversification of different species evolved from a common ancestor inhabiting the same geographical region. Intensive samplings on a global scale are necessary in order to understand the pattern of geographical distribution of *L.* subg. *Russularia*.

7.4 Phylogenetic signal of the morphological characters

Integration of morphological and molecular data offers the opportunity to test the taxonomical value or phylogenetic signal of some traditionally used morphological characters. In the concatenated analysis, I have focused on some characteristics that are frequently mentioned in previous literature and are used to delimitate species as well as to distinguish infrasubgeneric taxa. The result indicates that there is no such thing as a single characteristic that can be used to distinguish groups within this subgenus. On the other hand, a combination of two characters: pileipellis structure and the presence or absence of macrocystidia, has a significant value as a phylogenetic tool and helps to characterize clades.

Because this subgenus shows a diverse range of pileipellis structures, many authors already used the character to delimitate species or characterize infrageneric taxa. The same is true for the presence or absence of macrocystidia. This study reveals that the absence of macrocystidia occurred multiple times in species diversification, for example, in members of *L.* section *Olentes*, and in tropical Asian taxa such as *L. laccarioides*, *L. atrobrunneus* and *L. crenulatulus*. Pileipellis structures without a distinct layer of isodiametric cells are most common in the subgenus. It can be entirely composed of filamentous hyphae or a mixture of hyphae and inflated cells, e.g. (ixo-)cutis, (ixo-)trichoderm, (ixo-)trichopalisade. Remarkably all species with a hyphal pileipellis types develop macrocystidia. The minority of species have a pileipellis with a distinct layer of isodiametric cells, e.g. (ixo-)hyphoepithelium, (ixo-)trichoepithelium, epithelium and ixohymenoepithelium. They have arisen in an independent lineage which is subdivided by the presence or absence of macrocystidia.

Glutinous layers on top of a cutis and a trichoderm have evolved several times for several species in clade a, e.g. *L. aurantionitidus*, *L. kesiyae*, *L. atrii*, *L. flavigalactus L. lachungensis* (Figure 6.2). This character seems to have occurred more frequently in temperate regions or highland rainforest. However, the glutinous layer found in species within this subgenus never develops as strongly as in many members of *L.* subg. *Lactarius*, where it is a very common character. Since *L.* subg. *Lactarius* is a subgenus that is very dominant in temperate and boreal zones rather than in tropical areas, I assume there is a relation between climate and thickness of the slime layer to some extent.

Thanks to the typically amyloid ornamentation, the basidiospores are usually useful for identification at species level. As previously mentioned in Verbeken and Horak (2000), only *L. inamyloideus* lacks amyloid ornamentation. Most species in the subgenus have an incompletely reticulate basidiospore with ornamentation up to 1.5 µm high. Generally, the ornamentation is composed of warts or ridges interconnected by fine connective lines forming incomplete or subreticulum. The complete and dense reticulum and isolated rounded warts ornamentation have arisen multiple times among species in this subgenus and species with these characteristics do not fall in a monophyletic clade. Only two species, *L. chichuensis* and *L. corrugatus*, have a zebroid spore ornamentation. So although basidiospore ornamentation remains a very reliable character for species delimitation, the phylogenetic signal at higher level is very low.

The colour reaction of the latex on exposure is traditionally an important character for the classification of several groups of milkcaps (*Lactarius* and *Lactifluus*). However, within this subgenus, this feature is often subtle among several species. Most species lack colour reaction. In other cases, the colour reaction is very weak and hard to observe, from white latex slowly turning yellowish white or pale yellow. Hyaline latex is not characteristic for phylogenetic lineages while strong yellowing milk seems to be informative. *Lactarius chrysorrheus*, *L. vinaceorufescens* and *L. indochrysorrheus* form a distinct monophyletic clade (Figure 6.1). The yellowing milk together with the presence of the strong pileus zonation allows to easily discriminate the group of *L. chrysorrheus* from the other species. The strong zonation has arisen in other lineages independently, e.g. *L.* cfr. *subzonarius*.

Traditionally, the basidiocarp type was considered a very important phylogenetic character. Angiocarpic species were formerly placed in genera other than *Lactarius* and *Lactifluus* (*Arcangeliella*, *Zelleromyces* and *Gastrolactarius* J.M. Vidal). It has been shown before (Desjardin 2003, Verbeken et al. 2014a, 2014b) that the angiocarpic fruitbody evolved several times in *Lactarius* and occurs in the three subgenera. A recent study revealed that there are more tropical angiocarpic species than expected (Verbeken et al. 2014a). Angiocarpy evolved at least three times within *L.* subg. *Russularia*. *Lactarius falcatus*, *L. borzianus* and *L. hispanicus* have separate positions in the tree. The fact that *L. falcatus* was discovered in tropical rainforest indeed subscribes the fact that they might be more commonly occurring in tropical conditions than was assumed before. Up to now, pleurotoid species are unknown in the complete genus *Lactarius*. All known pleurotoid milkcaps belong to *Lactifluus*.

The other morphological characters are not useful on higher levels. The lamellar density is rather consistent among collections in a species. Most species in this subgenus have crowded to subdistant gills. Very distant gills are rare and I hypothesized that this character might have evolved in one clade, but as shown in chapter 2, this is not the case. Many species in this subgenus have remarkably small basidiocarps. However, they do not fall in one clade: the species in the *Obscuratus* group belong to clade a, species in the *Gracilis* group belong to clade e, *L. crenulatulus* and *L. atrobrunneus* belong to clade c. The presence of strongly grooved pileus develops in several species in the subgenus, e.g. *L. laccarioides*, *L. sublaccarioides*, *L. perparvus*, *L. crenulatulus*, *L. glabrigracilis* and species in the *Obscuratus* clade. This character has multiple origins and has no phylogenetic signal. The presence of hairs at pileus margin is not a common morphological feature in the subgenus as it is only found in *L. gracilis*.

An important ecological feature, ectomycorrhizal host association, most probably played important roles in evolution and diversification. The ectomycorrhizal association is phylogenetic informative for sections of *Leccinum*, e.g. sect. *Scabra* and *Fumosa* (dec Bakker et al. 2004). In case of *L.* subg. *Russularia*, several species have been recorded with a wide range of host plants. Ecology of many species are missing or uncertain in original descriptions. In vegetation types with abundant different ectomycorrhizal hosts such as tropical rain forest or mixed forest of Fagaceae and Pinaceae, etc., it is often difficult to define the exact host tree.

7.5 Intercontinental conspecificity

Since *Lactarius* is a genus of ectomycorrhizal fungi, species distribution is influenced by geological or ecological barriers, climatic conditions and vegetation type, mainly by the presence of possible host species. One of the taxonomic debates is whether previous records of intercontinental conspecificity of *Russularia* species are true or not. In most cases, European names were used in Asian and North American literature. So far real conspecificity was only supported by molecular data in the case of *L. controversus*, a *Populus*- and *Salix*-associated representative of *L.* subg. *Lactarius* (D'Hooge 2007).

Since there seem to be look-alikes between Europe and tropical Southeast Asian, e.g. *L. tabidus* and *L. inconspicuus*, *L. rostratus* and *L. rubrocorrugatus* or even between three continents, e.g. *L. chrysorrheus*, *L. vinaceorufescens* and *L. indochrysorrheus*, we aimed at assessing eventual intercontinental conspecificity for representatives in *L*. subg. *Russularia*. Unfortunately very little DNA sequence data of North American species are available. Results suggested that there is no conspecificity between temperate European species and tropical Asian species, but there is a case of conspecificity between temperate Europe and the high mountain areas in Asia. A Chinese *L. badiosanguineus* is found in highland mixed forest with *Betula*, *Pinus* and *Picea* in Diqing Tibetan Autonomous Prefecture, China (3302 m alt.). The wide distribution of *L. badiosanguineus* might be possibly due to the distribution of coniferous trees through the continuous landmass of Eurasia. In general, species are not conspecific between continents and former records suggesting this are based on look-alikes occurring in the different areas. Many Southeast Asian species are reminiscent of European and North American taxa, for example, the recently described Asian species *L. indochrysorrheus* (Das et al. 2015) recalls the European *L. chrysorrheus* and the North American *L. vinaceorufescens* by the similar fruiting body with conspicuous zonation and latex colour change, which is quickly turning sulphur yellow. *Lactarius sublaccarioides*, a species we described from Thailand, resembles the North American *L. herrerae* which also reminds very strongly a *Laccaria* species. *Lactarius rubrocorrugatus* reminds the European *L. rostratus* in some morphological aspects: both species have a small habit and are reddish brown, and have transparent latex and smell of Pentatomidae bug. As a general conclusion I can say that this PhD research confirms what was the hypothesis in *Lactarius*: each continent has its particular mycoflora. *Lactarius badiosanguineus* is the odd one out.

On the other hand, within the continent, several Asian species show a wide distribution. This was already suspected for *Lactarius gracilis* with records from Japan (Hongo 1945), Southern China (Wang 2007) and Thailand (Wisitrassameewong et al. 2014). The molecular and morphological evidences confirmed that it is indeed the same species (Wisitrassameewong et al. 2014). The species is originally described from Japan but in this study I also observed that this species is a common one in Thailand. *Lactarius chichuensis* spreads over Southern China and Northern Thailand in similar habitat, Fagaceae in Southern China and mixed forest between Fagaceae and *Pinus* trees in Northern Thailand. *Lactarius kesiyae*, a species associates with *P. kesiyae*, presents in Northern Thailand, Laos and Vietnam. *Lactarius rubrocorrugatus* occurs in Northern Thailand and Vietnam. The species seems to have a broad ectomycorrhizal association: with Fagaceae and Dipterocarpaceae in Thailand and with *Pinus* trees in Vietnam (Wisitrassameewong et al. 2015). *Lactarius flavigalactus* associates with coniferous trees in Sikkim, Himalaya region in India and Yunnan province in Southern China.

7.6 Future perspectives

This thesis reveals a huge diversity of *L.* subg. *Russularia* in Southeast Asia, and also provides a standardized identification key and description form. In addition, one of the important contributions in this study is submission of DNA sequence data. ITS and *rpb2* sequence data are deposited in GenBank. I realize that the DNA database of *L.* subg. *Russularia* is very important for metagenomics, which is a genetic approach to address the entire community of organism (Handelsman 2004, Thomas et al. 2012). Hence I believe that these generated DNA sequences will be useful in future studies.

Although the overview of *L.* subg. *Russularia* is more complete now, however, some perspectives of this subgenus are necessary to address.

7.6.1 Biodiversity and taxonomy

Many areas remain understudied, particularly Asia and South America. This study is a primary step to fill these hiatus, starting with some regions in Southeast Asia. More expeditions in undersampled areas need to be carried out in order to obtain morphological, molecular and ecological data. Several known species lack DNA sequence data, therefore, more sequencing is needed. For older material and type specimens, new tools have been developed recently; they could help in resolving some of the pending nomenclatural and taxonomic questions, also in a rather well studied area as Europe. Many species still need typification. Of the thirty European taxa, eleven taxa are not typified.

The taxonomy of *L.* subg. *Russularia* has been controversial, resulting in several classification systems. Although this study provided a recent morphological and molecular overview of the subgenus, low supported clades still remain in the two locus analysis. More genetic markers are necessary to establish more reliable hypothesis of the evolutionary relationships between most taxa and lineages at higher levels.

7.6.2 Reevaluation and resampling of North American specimens

Although diversity of North American *Russularia* species are well-documented by a significant monograph of Hesler & Smith (1979), reevaluation of specimens is necessary because their monograph lacks illustration of microscopic characters and does not mention host association which makes it difficult to compare the descriptions with collected samples. The varieties of species are necessary to investigate whether they are intraspecific variation in the same species or they should be treated as different species. Resampling of known species is also needed in order to examine morphological features and obtain DNA sequence data because several European names were applied in the monograph, i.e. *L. subdulcis*, *L. tabidus*, *L. obscuratus*. A molecular analysis could assess intercontinental conspecificity of these species and reveal biogeographical pattern of European and North American species. This study will also clarify the exact number of described species in the continent, and extending the phylogeny with more North American samples will give a more complete overview and infrasubgeneric classification of the subgenus.

7.6.3 Biogeography and dating phylogeny

Due to the fact that most representatives in this study were restricted in particular regions in Europe and Asia, it is impossible to test biogeography of species in *L.* subg. *Russularia*. In order to test biogeography of *Russularia* species, global sampling of extant species and a multiple locus data are necessary. An analysis of ancestral species reconstruction would infer the distribution and evolutionary pattern of *Russularia* species.

7.6.4 Ectomycorrhizal interaction and ecology

Host specificity of several species is unknown in mixed vegetation habitat. Since many species are morphologically similar, I believe that knowing the exact ectomycorrhizal association can be helpful for species delimitation. Ectomycorrhizal rootlets should be studied and sequenced to verify the fungal species and ectomycorrhizal host tree.

7.7 General conclusion

Lactarius subgenus *Russularia* is considered as one of the important ectomycorrhizal groups in terms of wide distribution and number of species. The main focus points of this thesis are diversity, taxonomy, evolutionary relationships of *L.* subg. *Russularia*. Southeast Asia has been chosen as the study area in this thesis because the region is undersampling and it is suitable habitat which habours many ectomycorrhizal hosts. Prior to this study, *L.* subg. *Russularia* has been unknown to locals in this subcontinent. Therefore this study emphasizes their ecological importance because *L.* subg. *Russularia* are common indigenous mycoflora in Southeast Asia. This thesis describes 23 new species from Southeast Asia and India, with illustrations and identification keys. All new species described in this thesis are supported both morphologically, ecologically and by phylogenetic characteristics.

Regarding taxonomy of *L.* subg. *Russularia*, uncertainty on species boundaries is evident and relationships of taxa are unclear. Species delimitation of *L.* subg. *Russularia* has been traditionally used morphological characters. In this study I delimitate species based on morphological, ecological and phylogenetic supports. The latter approach uses a phylogram based on ITS data. Most important features for the identification of members of this subgenus are orange or brownish or reddish fruitbody, fragile in texture and display a negative or weak latex discolouration, generally from white to pale yellow, while the extremity of the colour reaction is strong yellow. However, the morphological concept of the subgenus should be broaden after the inclusion of species that were formerly classified in *L.* subg. *Lactarius*: *L. chrysorrheus*, *L. vinaceorufescens*. The molecular result also clarified the exact taxonomic positions of *L. helvus* and *L. rufus*. Results suggested that *ITS* gene marker coupled with morphological characteristics work fine in delimitation at species level within the subgenus.

Phylogenetic analyses indicated that a combination of some morphological characteristics is possibly phylogenetic informative for certain groups: pileipellis structure, presence or absence of true cystidia and colour reaction of latex. The phylogeny contains sister intercontinental clades in which could be the result of migration across land bridges. Allopatric speciation possibly plays an important role in species diversification. Conspecificity between temperate and tropical taxa does not exist, therefore, European or American names adopted for tropical taxa in this thesis should be rejected. The wide distribution of the European *L. badiosanguineus* (between Europe and highland forest in China) is supported by morphological, ecological and molecular data. The other case is the wide distribution of *L. gracilis*, the species originally described from Japan but the species was one of the common flora in Northern Thailand in this study. However, intercontinental conspecificity of taxa across distant geographical areas or continents in temperate or tropical zones is uncommon. This aspect should be further investigated with more samplings, both morphologically, ecologically and moleculary.

The age of *L.* subg. *Russularia* is estimated to be in the Miocene epoch (~18 mya). The early ancestor of *L.* subg. *Russularia* might have evolved into at least 2 major lineages: clade a (12 mya) and clade b (16 mya). The multitude of mycorrhizal association (Betulaceae, Pinaceae and Fagaceae) is found in clade a, whereas some species have a generalised mycorrhizal association, e.g. *L. aurantiacus*, *L. tabidus*, *L. fulvissimus*, etc. In clade b, most species are presented in forest dominated with Fagaceae. This might possibly suggest a tropical origin of this clade.

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SUMMARY

Although *Lactarius* subg. *Russularia* is one of the groups of ectomycorrhizal fungi that is dominant in many ecosystems worldwide, the knowledge on its biodiversity (74 species before the start of this PhD), is mainly restricted to temperate regions in North America and Europe. The subgenus appears to be understudied in other regions, particularly in tropical Asia, which is supposed to be one of the biodiversity hotspots due to the high concentration of optimal host trees and climate conditions. *Lactarius* subg. *Russularia* is one of the three currently accepted subgenera in the genus *Lactarius*. Species within this subgenus are rather uniformous in terms of fruiting body colour and latex features, which lead to confusion in species delimitation and classification when only the morphological species concept was used. This thesis aims to standardize species delimitation by integrating morphological and molecular, and to a lesser extent ecological data and explore the adaptation of other species concepts. The main focus is on biodiversity in Southeast Asia as the lack of knowledge in this region resulted in a very incomplete sampling for phylogenetic analyses. The thesis provides insights in species delimitation and evolutionary relationships of *L.* subg. *Russularia* using the consolidated species concept. This results in the description of twenty-three new species (fifteen from Thailand, two from Malaysia, five from India and one from China) which raises the total number of species in the subgenus from six to twenty-four in Southeast Asia and four to nine species in South Asia.

Chapters 2-5 explore new diversity of *L.* subg. *Russularia* in Northern Thailand and Vietnam using the concordance between morphology, ecology and ITS phylogeny. All species are discovered in montane rainforest dominated by Fagaceae, Dipterocarpaceae and Pinaceae. Two morphologically distinct groups are proposed: a group of species with remarkably distant gills (in chapter 2, where *L. laccarioides*, *L. pasohensis*, *L. stubbei* and *L. sublaccarioides* are proposed as species new to science) and a group of species with very small basidiocarps (in chapter 3 where *L. crenulatulus*, *L. glabrigracilis* and *L. perparvus* are proposed as new species). Chapter 4 describes eight novel species present in montane rainforest of Thailand and Vietnam: *L. aquosus*, *L. austrorostratus*, *L. fuscomaculatus*, *L.*

inconspicuus, *L. kesiyae*, *L. rubrobrunneus*, *L. rubrocorrugatus* and *L. tangerinus*. Chapter 5 described *L. atrobrunneus* and *L. politus* as new species from Thailand.

Chapter 6 starts by exploring new diversity of *L.* subg. *Russularia* in highland coniferous forest of India and Southwest China. Six new species are proposed, supported by both morphological characters and molecular data: *L. atrii*, *L. aurantionitidus*, *L. dombangensis*, *L. flavigalactus*, *L. lachungensis* and *L. sikkimensis*.

This chapter also focuses on the overall phylogeny of the subgenus and the testing of the traditional classification schemes. Phylogenetic results based on an ITS-*rpb2* dataset suggested unnatural classification schemes in the most recent literature, revealing the need of reclassification. Although previously classified in other subgenera, *Lactarius chrysorrheus*, *L. rufus*, *L. helvus* and *L. vinaceorufescens* should be included in *L.* subg. *Russularia* to make the subgenus a monophyletic group.

Five morphological characters (latex colour, latex colour change, basidiospore ornamentation, presence or absence of true cystidia and pileipellis structure) are plotted on an ITS-*rpb2* phylogram to show the correspondence between morphology and phylogeny. A combination of morphological characters (e.g. pileipellis structure and presence or absence of true cystidia) can be linked with evolutionary lineages of species whereas other characters, e.g. spacing of the gills, size of the basidiocarp, and latex colour change do not give evident phylogenetic information. Strikingly the geographical distribution of the species also has a low phylogenetic signal, since species from different continents are mixed in most clades. Our analysis reveals that there is no conspecificity between tropical Asian and temperate European representatives, in contrast to what was suggested before in literature. The wide distribution of temperate species spanning Eurasia or Europe/North America is a rare occasion. A thorough examination of morphological, ecological and molecular information of species is necessary for confirming this perspective. This chapter also proposes a preliminary result of divergence time estimation of *L.* subg. *Russularia*. The ancestral species might have emerged in the mid Miocene (18.67 mya, 15.85−28.28 mya; 95%HPD) and evolved in at least two major clades. The dating tree is annotated with species distribution and possible host range.

Chapter 7 is a general discussion of the thesis. A summary of total number of species before and after this thesis and identification keys of all species described in Southeast Asia and

Australasia are given. The overview of the subgenus in this thesis partly differs from previous classification systems. However, this result has not been translated into a new infrasubgeneric classification system yet, because of uncertainty in several phylogenetic clades and insufficient data of extant species. A multiple locus analyses with a wider sampling would reveal a more plausible classification system.

SAMENVATTING

Lactarius subg. *Russularia* is één van de groepen ectomycorrhizavormende fungi die dominant zijn in veel ecosystemen over de hele wereld, maar toch is de kennis van de biodiversiteit van deze groep (74 soorten voor de start van deze studie) hoofdzakelijk beperkt tot de gematigde gebieden van Europa en Noord-Amerika. De kennis van dit subgenus vertoonde duidelijke hiaten in andere regio's, en vooral in tropisch Azië, dat nochtans op basis van de aanwezigheid van geschikte gastheerbomen en optimale klimaatomstandigheden, verondersteld wordt een hotspot aan diversiteit te zijn.

Lactarius subg. *Russularia* is één van de drie momenteel aanvaarde subgenera in het melkzwammengenus *Lactarius*. Soorten in dit subgenus vertonen weinig variatie in kenmerken als kleuren van het basidiocarp en kleur en verkleuring van de melk, wat geleid heeft tot taxonomische en systematische verwarring wanneer enkel het morfologisch soortconcept wordt gebruikt. Deze thesis heeft tot doel soortafbakening in deze groep te standardiseren door morfologische en moleculaire (en in beperktere mate ecologische) kenmerken te integreren en andere soortconcepten aan te wenden. De focus ligt op biodiversiteit in Zuidoost- Azië omdat het kennishiaat in deze regio resulteerde in een zeer incomplete dataset voor fylogenetisch analyses.

Betere inzichten in soortafbakening en evolutionaire verwantschappen binnen *L.* subg. *Russularia* worden in deze thesis verworven door gebruik te maken van het "consolidated species concept". Dit resulteert in de beschrijving van 23 nieuwe soorten (15 van Thailand, 2 van Malaysia, 5 van Indië en één van China) wat het totaal aantal soorten in het subgenus in Zuidoost-Azië doet toenemen van 6 tot 24 en in Zuid-Azië van 4 tot 9.

Hoofdstukken 2-5 beschrijven nieuwe diversiteit van *L.* subg. *Russularia* in Noord-Thailand en Vietnam, gebaseerd op integreren van morfologie, ecologie en ITS fylogenie. Alle soorten werden ontdekt in bergregenwoud gedomineerd door Fagaceae, Dipterocarpaceae en Pinaceae. Twee morfologisch goed herkenbare groepen worden apart behandeld: een groep van soorten met opvallend ver uit elkaar staande plaatjes (in hoofdstuk 2, waar *L. laccarioides*, *L. pasohensis*, *L. stubbei* en *L. sublaccarioides* worden voorgesteld als nieuwe soorten) en een groep van soorten met opvallend kleine basidiocarpen (in hoofdstuk 3 waar *L. crenulatulus*, *L. glabrigracilis* en *L. perparvus* worden voorgesteld als nieuwe soorten). Hoofdstuk 4 beschrijft acht nieuwe soorten van bergregenwoud in Thailand en Vietnam: *L. aquosus*, *L. austrorostratus*, *L. fuscomaculatus*, *L. inconspicuus*, *L. kesiyae*, *L. rubrobrunneus*, *L. rubrocorrugatus* en *L. tangerinus*. Hoofdstuk 5 beschrijft *L. atrobrunneus* en *L. politus* als nieuwe soorten.

Hoofdstuk 6 begint met exploratie van nieuwe diversiteit in bergnaaldbossen in India en Zuidwest-China. Zes nieuwe soorten worden voorgesteld, ondersteund door zowel morfologische als moleculaire data *L. atrii*, *L. aurantionitidus*, *L. dombangensis*, *L. flavigalactus*, *L. lachungensis* en *L. sikkimensis*.

In dit hoofdstuk wordt ook een algemene fylogenie van het subgenus voorgesteld en worden de traditionele classificaties hieraan getoetst. De fylogenie gebaseerd op een ITS-*rpb2* dataset suggereert onnatuurlijke classificaties van het subgenus in alle recente literatuur; een nieuwe indeling in secties en subsecties gebaseerd op de moleculaire data, dringt zich op. Soorten die eerder in andere subgenera werden geplaatst zoals *Lactarius chrysorrheus*, *L. rufus*, *L. helvus* en *L. vinaceorufescens* moeten opgenomen worden in *L.* subg. *Russularia* om de groep monofyletisch te maken.

Vijf morfologische kenmerken (latex kleur, latex verkleuring, basidiospore ornamentatie, aan- of afwezigheid van echte cystidia en structuur van de pileipellis) worden geplot op het ITS-*rpb2* fylogram om hun fylogenetisch signaal te toetsen. Een combinatie van morfologische kenmerken (vb. pileipellis structuur en aan- of afwezigheid van echte cystidia) kan gelinkt worden aan evolutionaire clades terwijl andere kenmerken zoals afstand tussen de plaatjes, formaat van basidiocarp en latex verkleuring geen fylogenetische informatie geven.

Opvallend is dat ook de geografische verspreiding van de soorten een erg zwak fylogenetisch signaal geeft omdat soorten van verschillende continenten gemengd voorkomen in de meeste clades. Onze analyse toont aan dat er geen conspecificiteit is tussen tropisch Azië en gematigd Europa, in tegenstelling tot wat eerder werd gesuggereerd in diverse literatuur. Een wijde verspreiding van gematigde soorten in Eurazië of Europa/Noord-Amerika is zeldzaam. Deze conclusies zijn gebaseerd op zorgvuldig onderzoek van morfologische, ecologische en moleculaire data.

Dit hoofdstuk stelt ook de preliminaire resultaten van een “divergence time estimation” van *L.* subg. *Russularia* voor. De voorouderlijke soort dook vermoedelijk op in het midden Miocene (18.67 miljoen jaar terug, 15.85−28.28 miljoen jaar terug; 95%HPD) en evolueerde in minstens twee grote clades. De geschatte leeftijd van de belangrijke aftakkingen is consistent met de hypotheses van paleoklimatologische veranderingen. De gedateerde boom wordt becommentarieerd met verspreiding van de soorten en mogelijke gastheerswitches.

Hoofdstuk 7 is een algemene discussie gebaseerd op de bevindingen van dit onderzoek. Een overzicht van het totaal aantal soorten in het subgenus voor en na deze studie wordt gegeven, alsook een identificatiesleutel tot alle soorten beschreven van Zuidoost-Azië en Australazië. Dit overzicht verschilt op enkele punten van eerdere traditionele classificatieschema’s, alhoewel nog niet alle resultaten werden vertaald in een nieuw infrasubgenerisch classificatiesysteem omdat er nog te veel onzekere factoren zijn in bepaalde clades en omdat de dataset nog niet volledig is. Een multiple locus analyse van een nog grotere groep soorten zou in de toekomst wel kunnen leiden tot een nieuwe classificatie.

APPENDICES

APPENDIX 1

PREPARATION OF CHEMICALS AND PROTOCOLS FOR DNA EXTRACTION, PCR AMPLIFICATION

1.1 CTAB extraction protocol for fresh materials

1) Take a mushroom and cut a piece of ca. 200-300 mg from the hood with a sterilized scalpel. Put this in a 2.2 ml tube with 2x CTAB buffer.

2) Put the samples for 2.5 min in the fridge (-20 °C) and directly afterwards 2.5 min in a 65 °C water bath and repeat this 2 times more (3 freeze-thaw cycles).

3) Take a piece of the stored material and place it, together with 1000 µl of the CTAB solution (from the tube) in a mortar, grind it and pour it into a new 2.2 ml tube.

Note: don’t forget to refill the original CTAB tubes with 1000 µl 2x CTAB.

4) Incubate the samples in a 65 °C water bath for one hour.

5) Add 450 µl of Chloroform-iso-amylalcohol to each sample, vortex it till it appears milky and centrifuge the samples at 14000 rpm for 10 min.

6) Three phases must be visible now:

- Bottom layer: chloroform with dissolved proteins, lipides and chlorophyll
- Middle layer: cell rests and dissolved green cell walls
- Top layer: nucleid acids and dissolved polysaccharides

7) Take 800 µl of highest phase and put in a new 2.2 ml tube. Be careful and stay away from middle phase. Remove your pipette if you see stirring in the middle phase.

8) Add another 450 µl chloroform-iso-amylalcohol (24:1) and mix by energetic shaking 5-10 min in fume hood.

9) Centrifuge samples again for 10 min at 14000 rpm. Then take 500 µl of upper phase and put in new 1.5 ml tube.

10) Add 500 µl cold isopropanol. Mix by shaking for 5-10 min at room temperature, so that tiny white flakes appear (not always visible).

1.2 DNA extraction protocol for dried materials

Note: do every step in fume hood.

1) Take 0.5 – 1 g of the herbarium specimen (e.g. part of lamella) and put it together with two 2 glass beads in a 2 ml eppendorf tube.

2) Freeze tubes in liquid nitrogen and put tubes in bead beater: 3 runs of 1 min 30 sec at speed 30.

3) Add 1000 µl extraction-buffer and 50 µl 10 % SDS to each sample and vortex.

4) Leave it for 1 h at 65°C and occasionally vortex to dissolve most of the material.

5) Add 2 µl of proteinase K, mix and leave over night at 50-55°C.

6) Centrifuge for 10 min at max speed (13200-14000 rpm). Transfer supernatant to a new 2 ml eppendorf tube.

7) Add an equal volume (~ 1000 µl) of Iso-propanol, mix by inverting the tube.

8) Centrifuge for 10 min at max speed (13200-14000 rpm) and pour off the supernatant.

9) Wash the DNA pellet by adding 200 µl 70 % EtOH, leave it for 20 min.

10) Centrifuge for 10 min at max speed (13200-14000 rpm).

11) Use a pipette to clear away the supernatant and air dry the DNA pellet.

12) Dissolve the DNA pellet in 100 µl milliQ H_2O, pipette up and down until DNA is dissolved.

1.3 Preparation of solutions for molecular works

0.1 M Tris.Cl, 100 ml (pH=8)

1. Add 12.114 g in 50 ml of MilliQ H_2O.
2. Adjust pH using HCl until pH reach 8.
3. Adjust the volume to 100 ml with MilliQ H_2O.

0.5 M NaCl, 100 ml

1. Add 2.92 g of NaCl and add 100 ml of extraction buffer
2. Mix the solution until it is clear.

0.05 EDTA, 100 ml

1. Add 1.86 g of EDTA and add 100 ml of extraction buffer
2. Mix the solution until it is clear.

2% CTAB buffer (100 ml)

1. Add 2 g of CTAB in 80 ml of MilliQ H_2O.

Note: dissolve CTAB on a hot plate until the solution is clear.

2. Add 20 ml of Tris.Cl.

2x CTAB buffer (100 ml)

1. Add 2 g of CTAB and add 10 ml of 1M Tris (pH 8-9).
2. Add 8.18 g of NaCl and add 0.58 g of EDTA.
3. Fill to a total volume of 95 ml with MilliQ H_2O and adjust pH to 8 using HCl or NaOH.
4. Fill to a total volume of 100 ml with MilliQ H_2O.

2 M NaCl

1. Add 11.688 g of NaCl and add 100 ml of CTAB buffer.
2. Mix the solution until it is clear.

10% SDS

1. Add 10 g of SDS and add 100 ml of MilliQ H_2O.
2. Mix the solution until it is clear.

0.2 M Tris.Cl (pH = 7.5)

1. Add 12.114 g of Tris in 50 ml of MilliQ H_2O.
2. Mix the solution until it is clear.
3. Check pH and adjust pH to 7.5 using HCl.
4. Adjust the volume to 100 ml using MilliQ H_2O.

EtOH solutions

1. 70% EtOH: add 70 ml of absolute EtOH and add additional 30 ml of MilliQ H_2O.
2. 96% EtOH: add 96 ml of absolute EtOH and add additional 4 ml of MilliQ H_2O.

CTAB extraction buffer for fresh materials

1. Make a solution of 50 ml 1M Tris (pH 8.0), 20 ml 0.5M EDTA (pH 8.0), 142 ml 5M NaCl,

10g PVP-40 and 10g CTAB.

2. Add MilliQ H_2O to a volume of 400 ml and heat in a waterbath until PVP-40 and CTAB are dissolved.

3. Add MilliQ H_2O to a final volume of 500 ml.

4. Dispense into aliquots (not more than 2/3 of bottle volume) and sterilize by autoclaving. The PVP-40 gives the solution a yellow colour.

5. Add 2-mercapto-ethanol (1-2%) just before use.

1.4 DNA quantity measurement

1. Put 1 µl of sterilized MilliQ H_2O and click on Blank.
2. Add 2 µl of sample and then clean pedestal with tissue and MilliQ H_2O each time.

Note: Good DNA concentration: concentration of 1000 ng per µl and best DNA concentration for PCR: 100−200 ng per µl.

1.5 PCR

1.5.1 Master mix ingredients

ITS for 30 µl

Master mix (per sample)		Master mix (for __ samples **+** 1 blanco **+** 10%)		
Amplification buffer	3 µl	Amplification buffer	__	µl
MgCl2 (25mM)	0.3 µl	MgCl2	__	µl
dNTPs (10mM)	0.6 µl	dNTPs	__	µl
ITS1 (10µM)	0.6 µl	Primer 1	__	µl
ITS4 (10µM)	0.6 µl	Primer 2	__	µl
H2O MilliQ	21.72 µl	H2O MilliQ	__	µl
Taq (5u/µl)	0.18 µl	Taq	__	µl
Total volume:	27 µl	**Total volume:**	__	µl
DNA volume	3 µl			

rpb2 for 30 µl

Master mix (per sample)		Master mix (for __ samples + 1 blanco + 10%)		
Amplification buffer	3 µl	Amplification buffer	__	µl
MgCl2 (25mM)	0.3 µl	MgCl2	__	µl
dNTPs (10mM)	0.6 µl	dNTPs	__	µl
fRPB2-5F (10µM)	2.4 µl	Primer 1	__	µl
fRPB2-7cR (10µM)	2.4 µl	Primer 2	__	µl
H2O MilliQ	18.12 µl	H2O MilliQ	__	µl
Taq (5u/µl)	0.18 µl	Taq	__	µl
Total volume:	27 µl	Total volume:	__	µl
DNA volume	3 µl			

1.5.2 PCR program

Lid at 105°C

1. preheating 94°C -- 10 sec
2. pause – place samples – press enter to proceed
3. Initial denaturation 94°C -- 1-5 min.
4. Denaturation 94°C -- 30 sec.
5. Annealing 55°C -- 30 sec.
6. Extension 70°C -- 30-60 (45) sec.
7. Step 4–6: 25–35 cycles (= 34 repeats)
8. Final extension 70°C -- 5-10 (7) min.
9. End (4°) 20°C

1.6 Agarose gel preparation and electrophoresis

1. Prepare 1% agarose gel in 1xTAE.
2. Heat the solution in microwave until it is clear.
3. Let Erlenmeyer flask cool down.
4. Poor agarose mixture on gel mold, put spacers and wait for about 15-20 min.
5. Remove spacers.
6. Load 3-5 µl of molecular weight marker (one slot/row).

7. Load 5 µl of PCR products into gel slots.
8. Run gel electrophoresis at 120 mV for 30 min.

1.7 Preparation of chemicals for morphological studies

10% KOH, 100 ml

1. Add 1 g of KOH and adjust the volume to 100 ml with MilliQ H_2O.
2. Mix the solution until it is clear.

Congo red stain, 100 ml

1. Add 1 g of Congo Red in 99 ml of MilliQ H_2O and filter the excess dye.

Melzer's reagent, 100 ml

1. Add 1.5 g of Iodine, 5 g of potassium iodide and 100 g of Chloral hydrate to 100 ml of MilliQ H_2O.
2. Warm the solution on hot plate.

APPENDIX 2

INFRASUBGENERIC CLASSIFICATION OF *LACTARIUS* SUBGENUS *RUSSULARIA* IN MOST RECENT MONOGRAPHS

Author	Subgenus	Section	Species
Hesler and Smith 1979	*Russularia*	*Trivialis*	*L. midlandensis*
			L. vinaceopallidus
			L. trivialis
			L. pallidus
			L. affinis var. *viridilactis*
			L. affinis var. *affinis*
			L. pseudoaffinis
			L. subtestaceus
			L. coleoteris
			L. hysginus ss. *Romagnesi*
			L. hysginus ss. *Neohoff*
			L. hysginus var. *americanus*
			L. hysginus var. *subroseus*
			L. hysginus var. *hysginus*
		Russularia	*L. rufus* var. *rufus*
			L. rufus var. *parvus*
			L. atrobadius
			L. subflammeus
			L. splendens
			L. hepaticus
			L. luculentus var. *laetus*
			L. luculentus var. *luculentus*
			L. bryophilus
			L. minusculus
			L. subumbrinus
			L. lanceolatus
			L. moschatus
			L. parvulus
			L. subolivaceus
			L. alachuanus var. *amarissimus*
			L. alachuanus var. *alachuanus*
			L. mutabilis
			L. cognoscibilis
			L. quietus var. *quietus*
			L. quietus var. *incanus*
		Pseudo-aurantiaci	*L. frustratus*
			L. subviscidus
			L. duplicatus

Appendix 2. Continued.

Author	Subgenus	Section	Species
Hesler and Smith 1979	*Russularia*	*Pseudo-aurantiaci*	*L. substriatus*
			L. umbrinopapillatus
			L. sublacustris
			L. oculatus
			L. imperticus
		Subsquamulosi	*L. subzonarius*
			L. aquifluus
			L. helvus
			L. alpinus var. *alpinus*
			L. alpinus var. *mitis*
		Thejogalus	*L. camphoratus*
			L. fragilis var. *fragilis*
			L. fragilis var. *rubidus*
			L. subserifluus
			L. thiersii
			L. rufulus
			L. occidentalis
			L. areolatus
			L. rimosellus
			L. highlandensis
			L. canadensis
			L. thejogalus
			L. nimkeae
			L. nancyae
			L. badiopallescens
			L. carbonicola
Heilmann-Clausen et al. 1998	*Russularia*	*Russularia*	*L. aurantiacus*
			L. lanceolatus
			L. duplicatus
			L. badiosanguineus
			L. sphagneti
			L. hepaticus
			L. decipiens
			L. quietus
			L. subdulcis
			L. fulvissimus
			L. lacunarum
		Tabidi	*L. tabidus*
			L. rubrocinctus
			L. obscuratus
			L. cyathuliformis
			L. omphaliformis
Heilmann-Clausen et al. 1998	*Russularia*	*Olentes*	*L. camphoratus*
			L. rostratus
			L. serifluus
			L. subumbonatus
Basso 1999	*Russularia*	*Colorati*	*L. alpinus*
			L. glyciosmus
			L. helvus
			L. lepidotus
			L. lilacinus
			L. mammosus

Appendix 2. Continued.

Author	Subgenus	Section	Species
Basso 1999	*Russularia*	*Colorati*	*L. rufus*
		Russulares	*L. quietus*
			L. subdulcis
			L. badiosanguineus
			L. sphagneti
			L. decipiens
			L. hepaticus
			L. lacunarum
			L. lapponicus
		Mitissimi	*L. mitissimus*
			L. aurantiacus
			L. fulvissimus
			L. lanceolatus
	Rhysocybella	*Ichorati*	*L. rubrocinctus*
			L. britannicus var. *britannicus*
			L. britannicus var. *pseudofulvissimus*
		Olentes	*L. cremor*
			L. camphoratus
			L. atlanticus
			L. subumbonatus
			L. serifluus
		Rhysocybella	*L. tabidus*
			L. brunneohepaticus
			L. obscuratus var. *subalpinus*
			L. obscuratus var. *obscuratus*
			L. obscuratus var. *radiatus*
			L. omphaliformis
			L. cyathuliformis

APPENDIX 3

A NEW ANGIOCARPOUS *LACTARIUS* SPECIES FROM THAILAND

Abstract

Lactarius bisporus sp. nov. is described from primary tropical forest in Thailand. Morphological characters and DNA sequence data are given. Comparisons with the closely related angiocarpous taxon *Lactarius pomiolens* are provided.

Keywords: basidiomycetes, ectomycorrhiza, taxonomy, truffle-like Russulales

Modified from: Verbeken, A., Hampe, F., Wisitrassameewong, K., Hyde, K.D., Eberhardt, U. & Nuytinck, J. (2014). A new angiocarpous *Lactarius* species from Thailand. *Phytotaxa* 181(3): 163−170.

Introduction

As in numerous other agaricomycete groups, it is now accepted that also in the *Russulales* angiocarpous species have evolved many times from gymnocarpous species, and that the shape of the basidiocarps has long been overestimated as a phylogenetic character (Miller *et al.* 2001, Desjardin 2003, Eberhardt & Verbeken 2004, Nuytinck *et al.* 2004, Verbeken *et al.* 2014). After the recent division of the milkcaps in three genera: *Multifurca* Buyck & V. Hofst. (Buyck *et al.* 2008, 2010), *Lactarius* Pers., and *Lactifluus* (Pers.) Roussel (Buyck *et al.* 2008, 2010, Norvell 2011, Barrie 2011), all truffle-like milkcap species known so far seem to belong to the genus *Lactarius.* Before the inclusion of angiocarpous Russulales in agaricoid genera was accepted, a number of genera were erected to include sequestrate species. Milk-exuding species were often, but not exclusively, described in *Arcangeliella* Cavara or *Zelleromyces* Singer & A.H. Sm. (Miller et al. 2001, Eberhardt & Verbeken 2004, Nuytinck *et al.* 2004).

The angiocarpous habit evolved in the three main subgenera: *L.* subg. *Plinthogalus* (Burl.) Hesler & A.H. Sm., *L.* subg. *Russularia* (Fr. ex Burl.) Kauffman and *L.* subg. *Piperites* (Fr. ex J. Kickx f.) Kauffman (Verbeken *et al.* 2014). Angiocarpous Russulales are mainly known from North America and Australia, but also occur in the tropics where their diversity is probably underestimated (Eberhardt & Verbeken 2004, Verbeken *et al.* 2014). A recent expedition in Northern Thailand revealed another new truffle-like milkcap, which is proposed here as *Lactarius bisporus* sp. nov**.**

Material and Methods

Morphological study

Macroscopic characters are all based on fresh material. Microscopic features were studied from dried material mainly in Congo Red in L4 (Clémençon 1973). Spore ornamentation is described and illustrated as observed in Melzer's reagent. For details on terminology we refer to Verbeken (1996) and Verbeken & Walleyn (2010). Line-drawings were made by A. Verbeken, with the aid of a drawing tube at original magnifications: 6000 × for spores, 1000 × for individual elements and sections. Basidia length excludes sterigmata length. Spores were measured in side view in Melzer's reagent, excluding the ornamentation, and measurements are given as {(MIN) [AV-2×SD]–*AV*–[AV+2×SD] (MAX)}$_{\text{length}}$ × {(MIN) [AV-

2×SD]−AV−[AV+2×SD](MAX)}$_{\text{width}}$ in which AV = mean value for the measured collection and SD = standard deviation. Q corresponds to spore "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 specimens, respectively. Colour pictures of this species will be available at http://www2.muse.it/russulales-news/.

DNA extraction, PCR amplification and sequencing

DNA was extracted from fresh material stored in 2×CTAB buffer, using the methods described by Nuytinck & Verbeken (2003) with slight modifications (Van de Putte *et al.* 2010). The internal transcribed spacer region of the nuclear ribosomal DNA (ITS) was amplified and sequenced using primers ITS1-F and ITS4 (White *et al.* 1990, Gardes & Bruns 1993). Sequencing (using the same primers) was conducted by MACROGEN (Amsterdam, The Netherlands). Sequences were assembled and edited with the software Sequencher$^{\text{TM}}$ v5.0 (GeneCodes Corporation, Ann Arbor, Michigan, USA).

Alignment and phylogenetic analyses

Table 1 shows an overview of all specimens and sequences used in the phylogenetic analyses, including GenBank accession numbers. Alignments were constructed with PRANK (Löytynoja & Goldman 2008). The alignment was manually refined in BioEdit v7.0.9.0 (Hall 1999) and made available in TreeBASE (http://purl.org/phylo/treebase/ phylows/study/TB2:S15099). Maximum Likelihood (ML) analyses (Figure 1) were performed in RAxML v7.0.3 (Stamatakis 2006), combining a ML search with the Rapid Bootstrapping algorithm for 1000 replicates and using the GTRGAMMA model.

Result

The results of the ML analyses (Figure 5.1) show that angiocarpous species are included in all major clades of the genus *Lactarius*, corresponding to *L.* subgen*. Russularia*, *L*. subgen. *Piperites* and *L*. subgen. *Plinthogalus*. The newly described species *L. bisporus* is not included in either of these subgenera, but belongs to *Lactarius,* as the placement in a joined and well-supported clade with *L. pomiolens* Verbeken & Stubbe shows. The majority of angiocarpous species are included in mixed clades together with agaricoid taxa. The clade of *L. bisporus* with two angiocarpous taxa is an exception.

TABLE 1. Specimens and GenBank accession numbers of DNA sequences used in the molecular analyses.

Species	Voucher collection	Origin	ITS accession no.
Arcangeliella camphorate		USA	EU644701
crassa		USA	AY558740
A. sp.		Australia, Tasmania	JF960610
Lactarius acris	EU014 (UPS)	Germany	DQ421988
L. akahatsu	AV2004-141 (GENT)	Thailand	KF133269
L. albocarneus	AV98-080 (GENT)	France	KF241545
L. alboscrobiculatus	LTH175 (CMU, SFSU, GENT)	Thailand	EF141538
L. angiocarpus	DA00-448 (GENT)	Zambia	AY606942
L. atroviridis	AV05-306 (GENT)	USA	KF133270
L. auriolla	RW1601 (GENT)	Sweden	KF133257
L. azonites	AV00-124 (GENT)	Belgium	KF241540
L. baliophaeus	AV05-155 (GENT)	Malawi	GU258277
L. bisporus **spec. nov.**	FH 12-160 (type, GENT)	Thailand	KF856293
L. borzianus		Switzerland	AF373599
L. camphoratus	UE04.09.2004 (UPS)	Sweden	DQ422009
L. chichuensis	Wang1236 (HKAS)	China	KF241541
L. chromospermus	AV99-174 (GENT)	Zimbabwe	KF133260
L. chrysorrheus	UE04.10.2002-8 (UPS)	Italy	KF133261
L. citriolens	UE20.09.2004-03 (UPS)	Sweden	DQ422003
L. controversus	AV00-117 (GENT)	Italy	KF241544
L. crassiusculus	LTH369 (GENT)	Thailand	EF560684
L. cyanescens	DS06-058 (GENT)	Malaysia	GU258278
L. cyathuliformis	UE04.09.2004-2 (UPS)	Sweden	KF133266
L. deliciosus	JN2001-046 (GENT)	Slovakia	KF133272
L. echinellus	AV07-169 (GENT)	Sri Lanka	KF133287
L. echinus	AV07-168 (type, GENT)	Sri Lanka	KF133273
L. falcatus	KVP08-038 (type, GENT)	Thailand	KF133274
L. flexuosus	UE06.09.2002-1 (UPS)	Sweden	DQ421992
L. formosus	LTH382 (type, CMU, SFSU, GENT)	Thailand	EF141549
L. fuliginosus	MTB97-24 (GENT)	Sweden	JQ446111
L. helvus	UE08.09.2004-1 (UPS)	Sweden	KF133263
L. hispidulus	AB152 (GENT)	Guinea	KF133258
L. kabansus	AV99-205 (GENT)	Zimbabwe	KF133259
L. lignyotus	UE06.09.2003-5 (UPS)	Sweden	DQ421993
L. luridus	OB11-011 (GENT)	Belgium	KF241547
L. mairei	AV00-118 (GENT)	Italy	AY336950
L. mammosus	UE09.09.2004-5 (UPS)	Sweden	KF133265
L. montoyae	KD1065 (type, BSHC)	India	EF560673
L. necator	AV04-231 (GENT)	France	KF133276
L. peckii	JN2004-020 (GENT)	USA	KF133277
L. pomiolens	AV07-159 (type, GENT)	Sri Lanka	KF133282
L. pubescens	AV96-931 (GENT)	Norway	AY336958
L. quieticolor	UE10.09.2004-1 (UPS)	Sweden	DQ422002

TABLE 1. Continued.

Species	Voucher collection	Origin	ITS accession no.
L. quietus	UE16.09.2004 (UPS)	Sweden	KF133264
L. romagnesii	UE29.09.2002-6 (UPS)	France	DQ421989
L. rubriviridis	DED7312 (SFSU)	USA	EF685088
L. rufus	JN2002-008 (GENT)	Norway	KF241543
L. saturnisporus	DS07-490 (GENT)	Sri Lanka	KF133285
L. shoreae	AV07-164 (type, GENT)	Sri Lanka	KF133278
L. sphagneti	PL2805 (pers. herb. P. Leonard)	UK	KF133268
L. spinosulus	AT2003068 (UPS)	Sweden	KF133262
L. stephensii	RW2930 (GENT)	Belgium	AY331012
L. subdulcis	JV2006-024 (GENT)	Belgium	KF133279
L. subplinthogalus	AV04-219 (GENT)	USA	KF241539
L. subsericatus	UE11.10.2004-8 (UPS)	Sweden	DQ422011
L. tenellus	DKA3598 (BR)	Benin	KF133280
L. thyinos	A.Voitk23-08-2004 (GENT)	Canada	KF133271
L. torminosus	RW3183 (GENT)	Czech Republic	KF133281
L. trivialis	UE27.08.2002-17a (UPS)	Sweden	DQ421991
L. uvidus	KVP10-027 (GENT)	Russia	KF241546
L. vietus	UE11.19.2004-1 (UPS)	Sweden	KF133267
L. vinaceorufescens	JN2007-018 (GENT)	Canada	KF241542
Multifurca furcata	RH7804 (NY)	Costa Rica	DQ421994
M. ochricompacta	BB02.107 (PC)	USA	DQ421984
M. zonaria	DED7442 (PC)	Thailand	DQ421990
Zelleromyces gardneri		USA	DQ453696
Z. giennensis		Spain	AF230900
Z. hispanicus		Spain	AF231913
Z. sp.		Australia, Tasmania	JF960852
Z. sp.		Australia, Tasmania	JF960853
Z. sp.		Australia, Tasmania	JF960854

Taxonomy

Lactarius bisporus Verbeken & F. Hampe sp. nov. (Figure 2 and 3)

Mycobank number: MB 807217

Diagnosis: Basidiomata 10–35 mm diam., 10–15 mm alta, subglobosa, subregularia. Peridium leviter velutinosum, ochraceum ad luteobrunneum. Stipes absens. Gleba labyrinthuloidea, albida, cum locis parvis, cum paucis venis gelatinosis. Columella absens. Latex excarsus, albidus, immutabilis. Sporae globosae ad subglobosae, 9.8–*11.3*–13.0 × 9.8–*11.2*–12.5 µm, amyloidae, spinis et verrucis usque ad 2(3) µm altis ornatae, macula suprahilaris non amyloidea. Basidia 45−55 ×12−18 µm, bispora, subcylindrata ad clavata. Cystidia 25−55 × 12−20 µm, cylindrata, clavata vel utriformia. Peridiopellis ex hyphis periclinis.

Typus: Thailand. Chiang Mai Province, Mae On District, about 3 km from Tharnthong Lodges, 725 m elev. N18°51’55”, E99°17’23”, 17 July 2012, leg. Felix Hampe, FH 12–160 (holotype, GENT!), MFLU 12–0650 (isotype, MFLU!).

Etymology:—referring to the strictly 2-spored basidia.

Basidiocarps angiocarpous, semihypogeous, 10–35 mm diam., 10–15 mm high, subglobose, rather regular. *Peridium* with minutely velutinous surface, chamois-leather-like, ochraceous yellow to yellow-brown. *Stipe* absent. *Columella* absent. *Gleba* whitish, strongly labyrinthuloid, with small loci, with some, but very few, gelatinous veins between the loci. *Latex* scarce, whitish hyaline, unchanging on the gleba and unchanging on white paper, unchanging with KOH. *Taste* bitter to astringent, disagreeable but not burning acrid. *Smell* not remarkable.

Basidiospores globose to subglobose, 9.8–*11.3*–13.0 × 9.8–*11.2*–12.5 µm, Q = 1.00–*1.01*–1.04, n = 20; ornamentation amyloid, consisting of isolated, up to 2(3) µm high spines and isolated warts; spines conical, subacute, with rounded to tapering, rarely forked apex; apiculus up to 2.5(3) µm long; plage not distinct, inamyloid. *Basidia* 2-spored, subcylindrical to clavate, sometimes irregular and somewhat tortuous, 45–55 × 12–18 µm, with 5–12 µm long sterigmata. *Macrocystidia* very variable in shape, clavate, subcapitate, ventricose or lageniform, 25–55 × 12–20 µm, sometimes slightly but distinctly thick-walled. *Pseudocystidia* present. *Peridiopellis* consisting of 2–3 µm broad hyphae which are rather periclinally arranged in the subpellis, but form a densely intricate and interwoven layer on top and are embedded in a gelatinous matrix.

Habitat:—primary tropical forest with *Dipterocarpus tuberculatus* Roxb. Known only from the type collection.

FIGURE 1. The obtained ML topology based on ITS sequences. Bootstrap values are indicated if they exceed 50%. Names in orange are angiocarpous milk cap species for which ITS sequences are available on GenBank. (KF856293). *Lactarius bisporus* sp. nov. holotype is indicated in red. The scale bar represents the expected number of nucleotide changes per site.

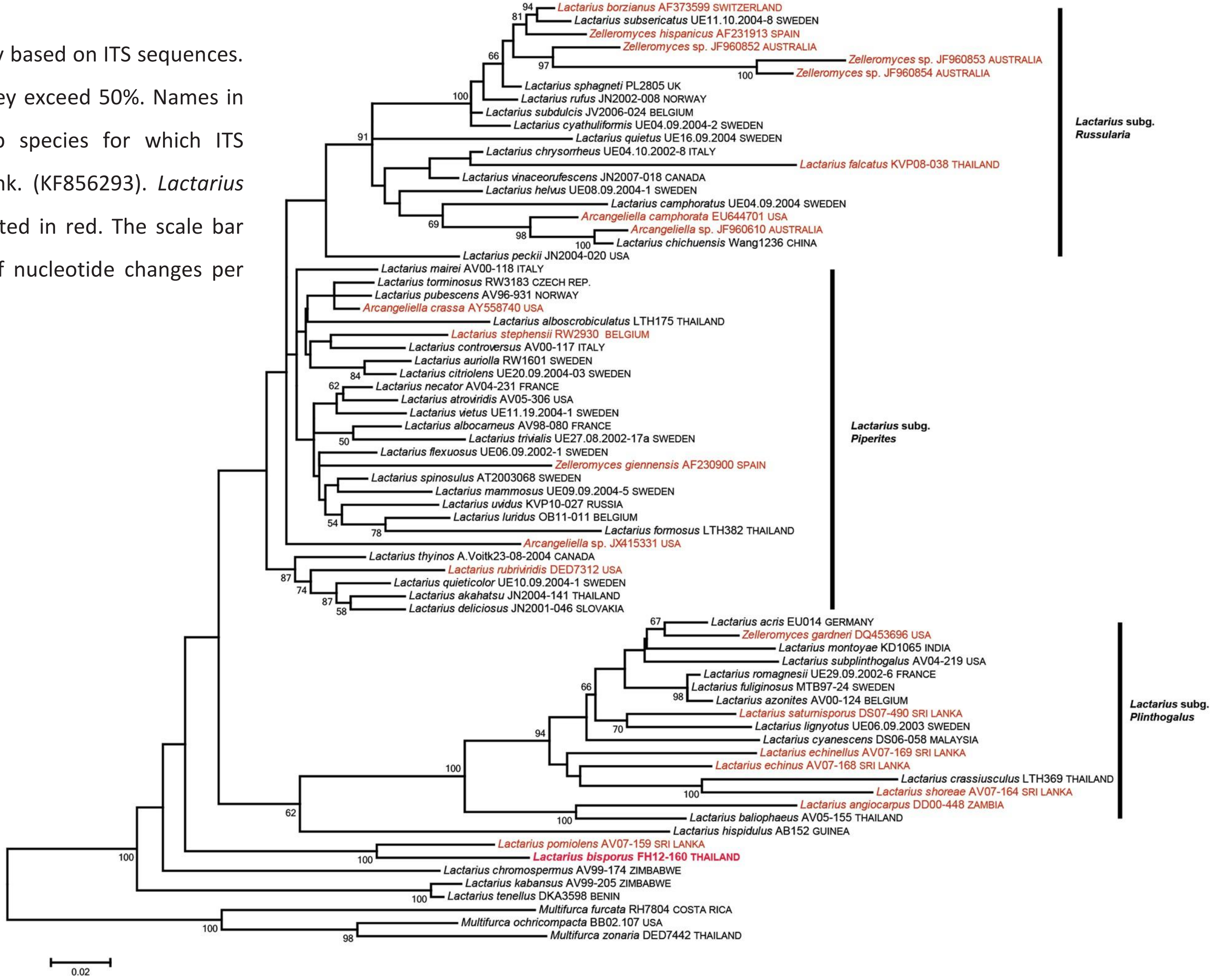

FIGURE 2. Basidiocarps of *Lactarius bisporus* (FH 12–160, holotype, photo by Felix Hampe).

Discussion

The ITS analysis shows that this new species of truffle-like Russulales belongs to the genus *Lactarius*, as all other known angiocarpous milkcaps known so far. While it is obvious from the tree that the angiocarpous habit evolved several times in the three major clades of the genus, representing *L.* subg. *Piperites*, *L.* subg. *Russularia* and *L.* subg. *Plinthogalus*, we see that this new Thai species is most closely related with *L. pomiolens*, a species from Sri Lanka (Verbeken *et al.* 2014). *Lactarius pomiolens,* also an angiocarpous taxon*,* forms one of the earlier diverging lineages in the genus and has not been assigned to any of the existing subgenera. Both angiocarpous species *L. bisporus* and *L. pomiolens* form a separate isolated small clade as does the group around *L. kabansus* Pegler & Piearce, a tropical African species showing some superficial resemblances with *L.* subg. *Plinthogalus* and the morphologically very isolated dark-spored *L. chromospermus* Pegler.

Morphologically, this new species clearly differs from *L. pomiolens* by the 2-spored basidia and the isolated spines and warts as spore ornamentation. The spores in *L. pomiolens* are extremely large for spores formed by 4- spored basidia (10.5–*12.0*–13.3 ×

10.2–*11.5*–12.8 µm) and have very high wings. As already stated before (Verbeken *et al.* 2014) the spore ornamentation within truffle-like milkcaps shows large differences: isolated spines or warts on one hand, high ridges on the other hand, but this character does not seem to contain much phylogenetic signal. Furthermore, *L. pomiolens* has latex that turns immediately sulphur yellow while the latex in *L. bisporus* is white and unchanging.

As the name indicates, *L. pomiolens* is characterized by a strong and sweet apple-like smell. Many species with normally 4-spored basidia show a low percentage of 2-spored ones, but exclusively 2-spored basidia are rare in the genus. It is remarkable that though exclusively 2-spored basidia are very exceptional in agaricoid milkcaps (such as e.g. *L. acerrimus* Britzelm.), they seem to be more common in angiocarpous representatives. Other Asian 2-spored angiocarpous milkcap species are *L. echinus* Stubbe & Verbeken, *L. falcatus* Verbeken & Van de Putte, *Arcangeliella lactifera* (B.C. Zhang & Y.N. Yu) J.M. Vidal and *A. nanjingensis* (B. Liu & K. Tao) J.M. Vidal. Both *L. echinus* and *A. lactifera* differ from the newly proposed species by the longer (up to 4 µm) and more regular spines. *Lactarius falcatus* has lower spines, at most 2 µm long, which are typically curved at the apex and also differs macroscopically by latex that is turning immediately bright pale yellow. *Martellia nanjingensis* has smaller spores (8–11.5 × 7.5–10 µm) with spines up to 1.5 µm long and differs macroscopically by the brown to dark brown basidiocarps (Tao *et al.* 1993).

Acknowledgement

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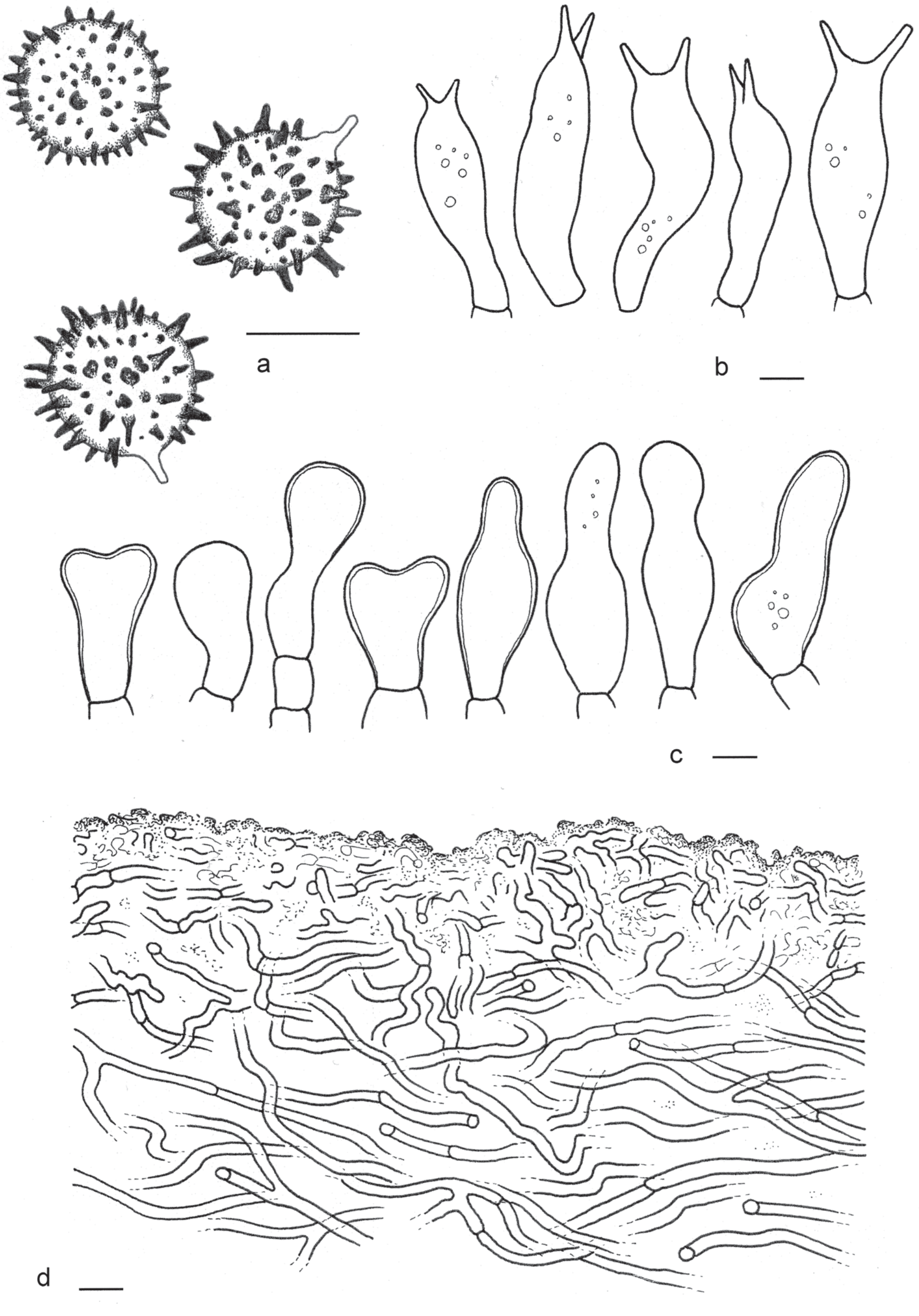

FIGURE 3. a. Basidiospores (FH 12–160, holotype), b. basidia (FH 12–160, holotype), c. cystidia (FH 12–160, holotype), d. peridiopellis (FH 12–160, holotype), (scale bar = 10 µm).

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APPENDIX 4

DESCRIPTION FORM FOR *LACTARIUS* SUBGENUS *RUSSULARIA*

Species: **Collector:** **Collection code:**

Sample: ☐ dried specimen ☐ fresh tissue in CTAB buffer ☐ root sample ☐ picture

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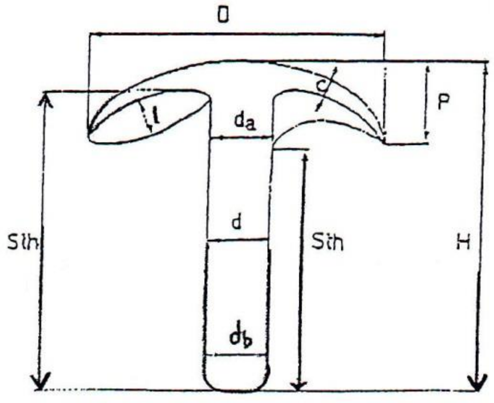
LOCALITY AND ECOLOGY

Collecting site:

Host tree:

Altitude: **Date:**



DIMENSION: all in mm Dev.: Development

J: Juvenile Y: Young F: Full-grown O: Old

			Pileus		Stipe						Remarks
No.	**Dev.**	**H**	**D**	**P**	**Sth**	**da**	**d**	**db**	**Context**	**Lam.**	
1											
2											
3											
4											
5											

PILEUS

Shape: ☐ plano-convex ☐ infundibuliform

Surface: ☐ dry ☐ sticky ☐ smooth ☐ wrinkled

☐ zonation ☐ hygrophanous ☐ with a papilla

Colour: ☐ orange ( ) ☐ orange brown ( ) ☐ brown ( )

☐ red ( ) ☐ reddish brown ( ) ☐ dark brown ( )

Remark: please specify colour code in brackets

Note:

Discolouration: ☐ absent ☐ orange ☐ brown ☐ dark brown

Striation: ☐ absent ☐ short ☐ strong and reach to the center

Margin: ☐ even ☐ crenulate ☐ hairy

CONTEXT

Context	**Pileus**	**Stipe**
Colour		
Changes when cut		
Changes with age		

LAMELLAE

Colour: ☐ cream ( ) ☐ orange ( ) ☐ brown ( )

Discolouration: ☐ absent ☐ orange ( ) ☐ brown ( ) ☐ dark brown ( )

Attachment: ☐ adnate ☐ decurrent

Density: ☐ crowded ☐ subdistant ☐ distant ☐ forked

Series of lamellulae: **L+l/cm at mid-radius:**

Note:

STIPE

Shape: ☐ cylindrical ☐ tapering downward ☐ tapering upward

Surface: ☐ dry ☐ sticky ☐ smooth ☐ wrinkled

Colour: ☐ orange ( ) ☐ orange brown ( ) ☐ brown ( )

☐ red ( ) ☐ reddish brown ( ) ☐ dark brown ( )

Base: ☐ naked ☐ strigose hairs ☐ whitish pruinose

Note:

LATEX

Colour: ☐ transparent ☐ watery whitish ☐ white

Colour change:

☐ Unchanging on exposure

☐ Slowly turn to pale yellow or pale cream within an hour

☐ Immediately turn to bright yellow or strong yellow

Note:

With 10% KOH: ☐ unchanging ☐ pale yellow ( ) ☐ yellow ( )

On white tissue paper: ☐ unchanging ☐ pale yellow ( ) ☐ yellow ( )

Taste: ☐ mild ☐ bitter ☐ acrid ☐ burning acrid

CHEMICAL REACTION ON FLESH

10%KOH: **$FeSO_4$:**

TASTE AND ODOUR

Taste: ☐ mild ☐ bitter ☐ acrid ☐ burning acrid

Odour: ☐ indistinct ☐ Pentatomidae bug ☐ sweetish

☐ fishy ☐aromatic ☐ fruity ☐ coconut

☐ etc. ____

Drawing of basidiocarp:

APPENDIX 5

COMPARISON BETWEEN ITS and *rpb2* PHYLOGRAMS

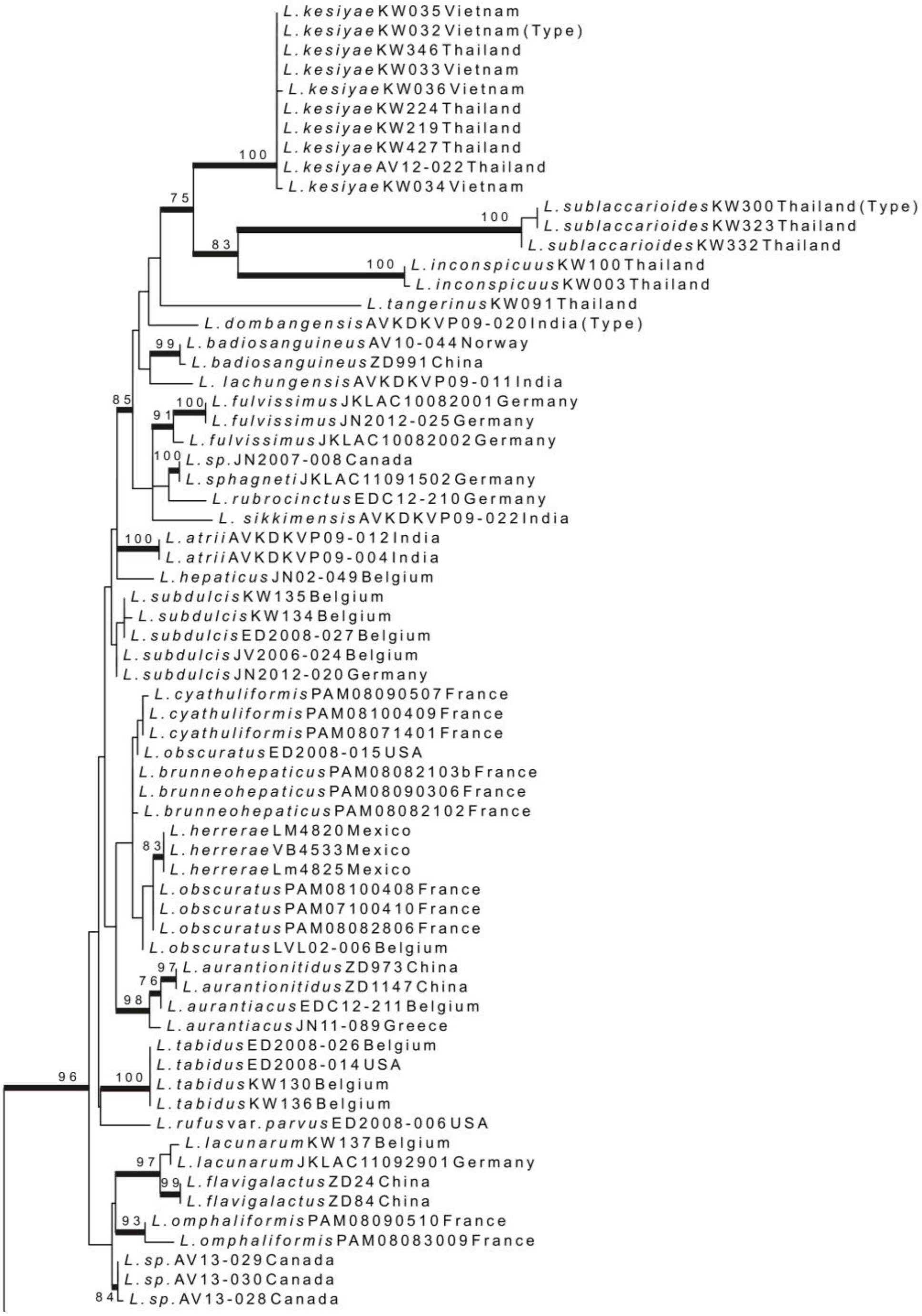


Figure 1. Continued.

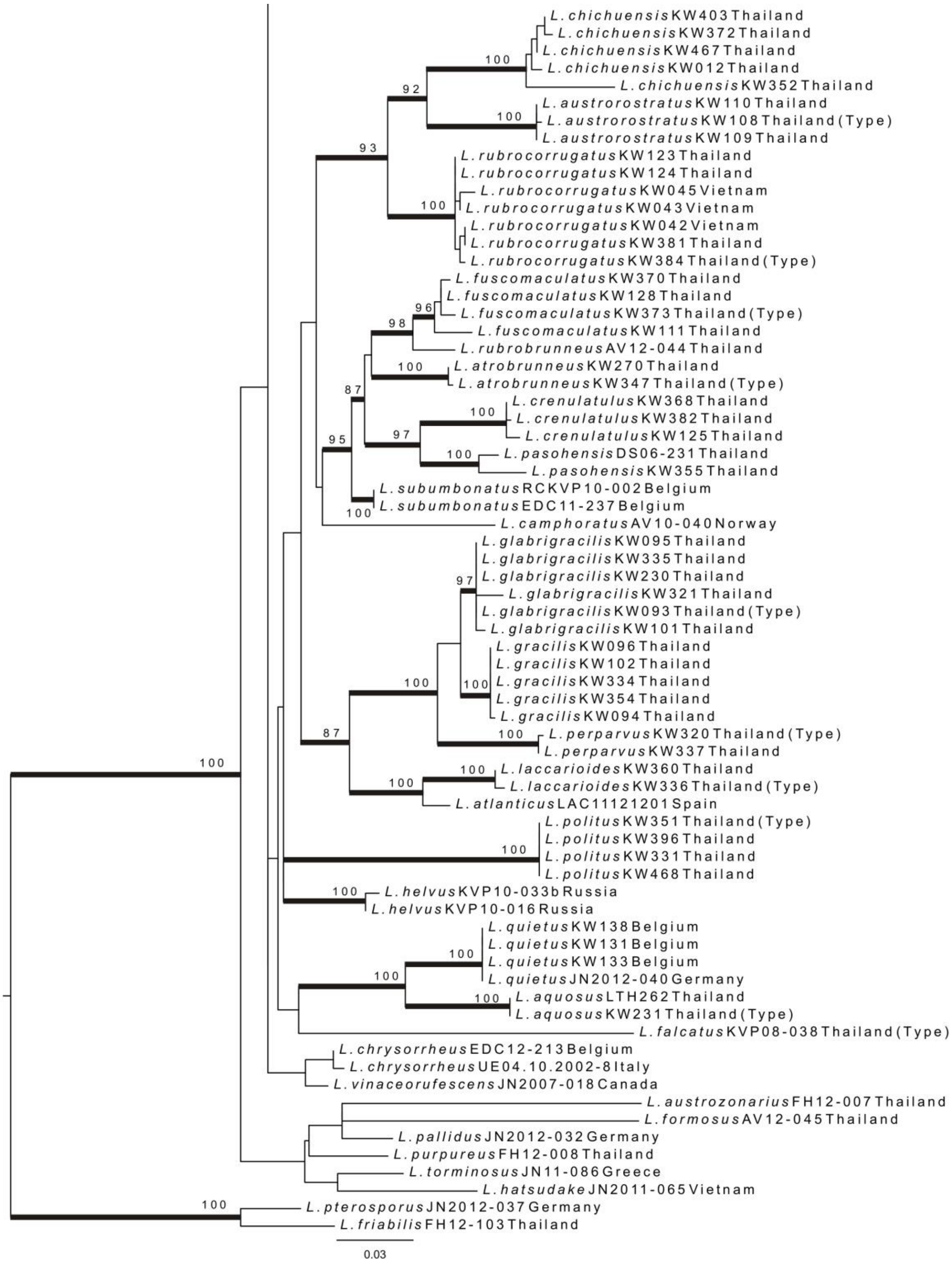


Figure 1. ML phylogram based on ITS sequence data.

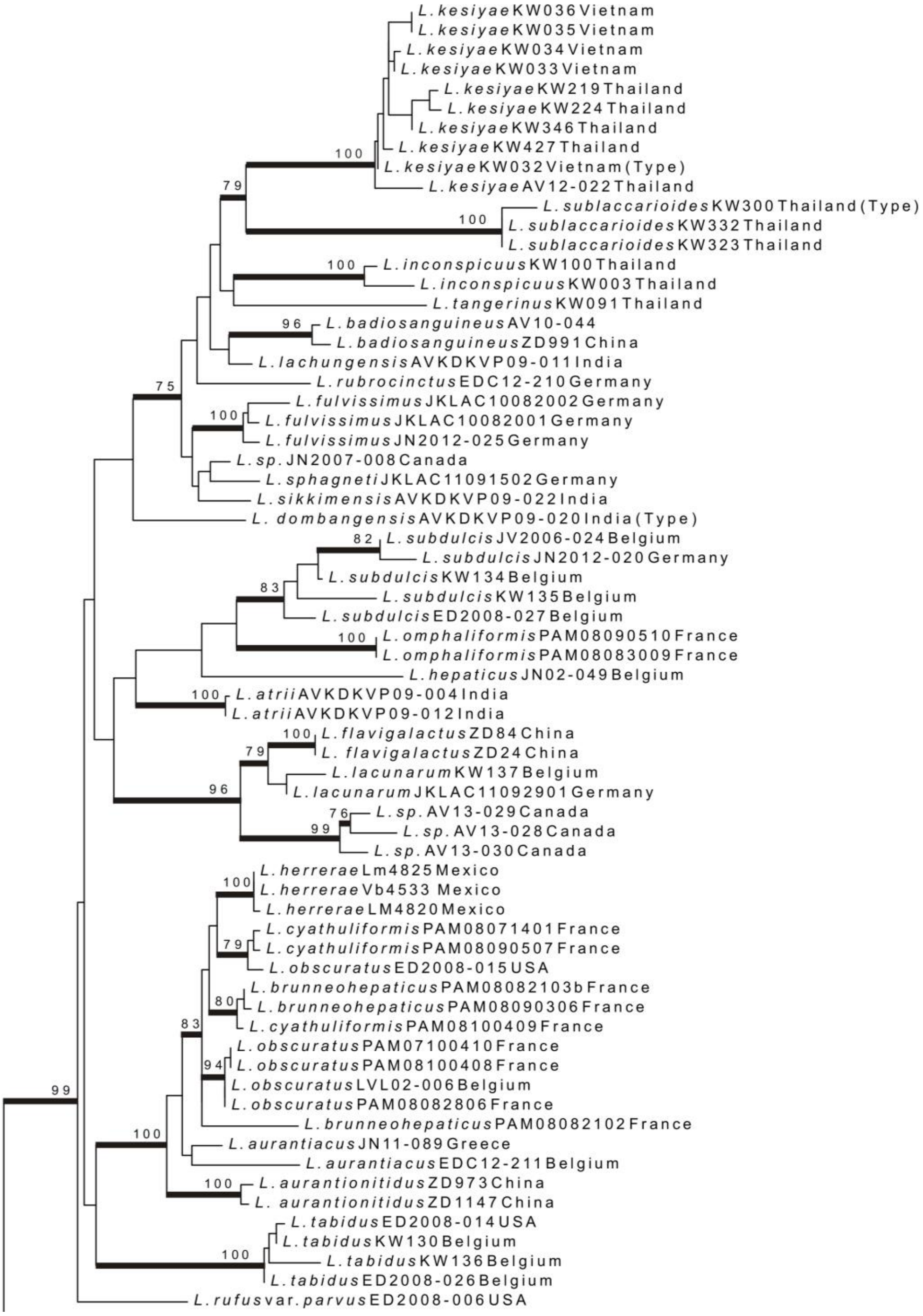

L. kesiyae KW036 Vietnam
L. kesiyae KW035 Vietnam
L. kesiyae KW034 Vietnam
L. kesiyae KW033 Vietnam
L. kesiyae KW219 Thailand
L. kesiyae KW224 Thailand
L. kesiyae KW346 Thailand
L. kesiyae KW427 Thailand
100
L. kesiyae KW032 Vietnam (Type)
79
L. kesiyae AV12-022 Thailand
L. sublaccarioides KW300 Thailand (Type)
100
L. sublaccarioides KW332 Thailand
L. sublaccarioides KW323 Thailand
100
L. inconspicuus KW100 Thailand
L. inconspicuus KW003 Thailand
L. tangerinus KW091 Thailand
96
L. badiosanguineus AV10-044
L. badiosanguineus ZD991 China
L. lachungensis AVKDKVP09-011 India
L. rubrocinctus EDC12-210 Germany
75
L. fulvissimus JKLAC10082002 Germany
100
L. fulvissimus JKLAC10082001 Germany
L. fulvissimus JN2012-025 Germany
L. sp. JN2007-008 Canada
L. sphagneti JKLAC11091502 Germany
L. sikkimensis AVKDKVP09-022 India
L. dombangensis AVKDKVP09-020 India (Type)
82
L. subdulcis JV2006-024 Belgium
L. subdulcis JN2012-020 Germany
L. subdulcis KW134 Belgium
83
L. subdulcis KW135 Belgium
L. subdulcis ED2008-027 Belgium
100
L. omphaliformis PAM08090510 France
L. omphaliformis PAM08083009 France
L. hepaticus JN02-049 Belgium
100
L. atrii AVKDKVP09-004 India
L. atrii AVKDKVP09-012 India
100
L. flavigalactus ZD84 China
L. flavigalactus ZD24 China
79
L. lacunarum KW137 Belgium
L. lacunarum JKLAC11092901 Germany
96
76
L. sp. AV13-029 Canada
99
L. sp. AV13-028 Canada
L. sp. AV13-030 Canada
L. herrerae Lm4825 Mexico
100
L. herrerae Vb4533 Mexico
L. herrerae LM4820 Mexico
L. cyathuliformis PAM08071401 France
79
L. cyathuliformis PAM08090507 France
L. obscuratus ED2008-015 USA
L. brunneohepaticus PAM08082103b France
80
L. brunneohepaticus PAM08090306 France
L. cyathuliformis PAM08100409 France
83
L. obscuratus PAM07100410 France
94
L. obscuratus PAM08100408 France
L. obscuratus LVL02-006 Belgium
L. obscuratus PAM08082806 France
L. brunneohepaticus PAM08082102 France
99
100
L. aurantiacus JN11-089 Greece
L. aurantiacus EDC12-211 Belgium
100
L. aurantionitidus ZD973 China
L. aurantionitidus ZD1147 China
L. tabidus ED2008-014 USA
L. tabidus KW130 Belgium
100
L. tabidus KW136 Belgium
L. tabidus ED2008-026 Belgium
L. rufus var. parvus ED2008-006 USA

Figure 2. Continued.

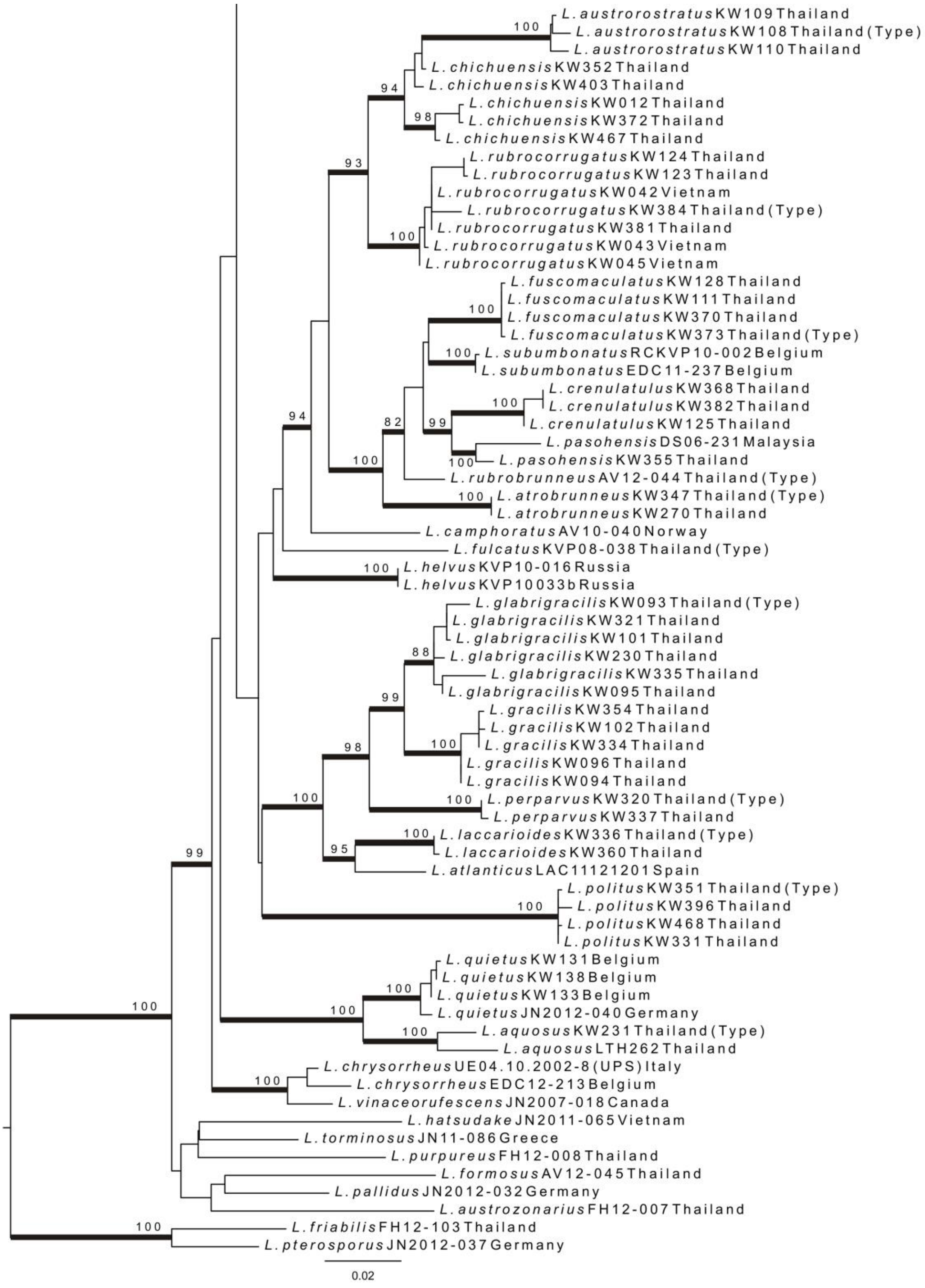


Figure 2. ML phylogram based on *rpb2* sequence data

APPENDIX 6

LIST OF ACCEPTED SPECIES OF *LACTARIUS* SUBGENUS *RUSSULARIA*

The overview of known and accepted *Lactarius* subg. *Russularia* around the world based on the current knowledge. The country indicated is the country from which the species is originally described.

Geographic region	**Country**	**Species**	**Host tree**	**Reference**
Asia	Japan	*L. gracilis*	Fagaceae	Hongo 1957a
	Japan	*L. subzonarius*	*Abies*	Hongo 1957b
	China	*L. chichuensis*	Fagaceae	Chiu 1945
	China	*L. hirtipes*	Fagaceae	Ying 1991
	Indonesia	*L. caulocystidiatus*	Fagaceae	Verbeken et al. 2001
	Indonesia	*L. javanicus*	Fagaceae	Verbeken et al. 2001
	Indonesia	*L. sulphurescens*	Fagaceae	Verbeken et al. 2001
	India	*L. indochrysorrheus*	*Picea, Abies*	Das et al. 2015
	India	*L. mukteswaricus*	*Quercus*	Das et al. 2004
	India	*L. sanjappae*	*Quercus*	Das et al. 2004
	India	*L. verbekenae*	*Quercus*	Das et al. 2004
	Malaysia	*L. pasohensis*	*Shorea*	Wisitrassameewong et al. 2014a
	Malaysia	*L. stubbei*	Deciduous forest	Wisitrassameewong et al. 2014a
	Thailand	*L. atrobrunneus*	Fagaceae	Liu et al. 2015
	Thailand	*L. austrorostratus*	Fagaceae	Wisitrassameewong et al. 2015
	Thailand	*L. aquosus*	Fagaceae	Wisitrassameewong et al. 2015
	Thailand	*L. crenulatulus*	Fagaceae	Wisitrassameewong et al. 2014b
	Thailand	*L. falcatus*	Fagaceae	Wisitrassameewong et al. 2014b
	Thailand	*L. fuscomaculatus*	Fagaceae	Wisitrassameewong et al. 2015
	Thailand	*L. glabrigracilis*	Fagaceae	Wisitrassameewong et al. 2014b
	Thailand	*L. inconspicuus*	Fagaceae	Wisitrassameewong et al. 2015
	Thailand, Vietnam	*L. kesiyae*	*Pinus*	Wisitrassameewong et al. 2015
	Thailand	*L. laccarioides*	Fagaceae	Wisitrassameewong et al. 2014a
	Thailand	*L. perparvus*	Fagaceae	Wisitrassameewong et al. 2014b
	Thailand	*L. politus*	Fagaceae	Liu et al. 2015
	Thailand	*L. rubrobrunneus*	Fagaceae	Wisitrassameewong et al. 2015
	Thailand, Vietnam	*L. rubrocorrugatus*	Fagaceae	Wisitrassameewong et al. 2015
	Thailand	*L. sublaccarioides*	Fagaceae	Wisitrassameewong et al. 2014a

Appendix 6 Continued.

Geographic region	Country	Species	Host tree	Reference
Asia	Thailand	*L. tangerinus*	Fagaceae	Wisitrassameewong et al. 2015
Oceania	Papua New Guinea	*L. austrotabidus*	Fagaceae	Verbeken and Horak 2000
	Papua New Guinea	*L. condimentus*	Fagaceae	Verbeken and Horak 2000
	Papua New Guinea	*L. corrugatus*	Fagaceae	Verbeken and Horak 2000
	Papua New Guinea	*L. guttisporus*	Fagaceae	Verbeken and Horak 2000
	Papua New Guinea	*L. inamyloideus*	Fagaceae	Verbeken and Horak 2000
	Papua New Guinea	*L. liliputianus*	Fagaceae	Verbeken and Horak 2000
	Papua New Guinea	*L. pennulatus*	Fagaceae	Verbeken and Horak 2000
	Papua New Guinea	*L. perconicus*	Fagaceae	Verbeken and Horak 2000
	Australia	*L. eucalypti*	*Eucalyptus*	Miller and Hilton 1987
Europe	France	*L. atlanticus*	*Pinus*	Basso 1999
	Western Europe	*L. aurantiacus*	Diverse	Heilmann-Clausen et al. 1998
	France	*L. badiosanguineus*	*Picea, Abies*	Heilmann-Clausen et al. 1998
	Italy	*L. borzianus*	*Picea, Abies*	Cavara 1900, Vidal 2004
	Austria	*L. brunneohepaticus*	*Alnus*	Basso 1999
	France	*L. camphoratus*	Mixed forest	Heilmann-Clausen et al. 1998
	Sweden	*L. chrysorrheus*	*Quercus*	Heilmann-Clausen et al. 1998
	France	*L. cyathuliformis*	*Alnus*	Heilmann-Clausen et al. 1998
	France	*L. decipiens*	Deciduous forest	Heilmann-Clausen et al. 1998
	France	*L. fulvissimus*	*Fagus, Quercus, Tilia, Populus, Corylus*	Heilmann-Clausen et al. 1998
	France	*L. hepaticus*	*Pinus, Picea*	Heilmann-Clausen et al. 1998
	Sweden	*L. helvus*	*Pinus, Picea*	Heilmann-Clausen et al. 1998
	Spain	*L. hispanicus*	*Pinus*	Calonge and Pegler 1998
	Great Britain	*L. lacunarum*	*Alnus, Populus, Betula*	Heilmann-Clausen et al. 1998
	Germany	*L. obscuratus*	*Alnus*	Heilmann-Clausen et al. 1998
	France	*L. omphaliformis*	*Alnus*	Heilmann-Clausen et al. 1998
	Sweden	*L. quietus*	*Quercus*	Heilmann-Clausen et al. 1998
	Denmark	*L. rostratus*	*Fagus*	Heilmann-Clausen et al. 1998
	Sweden	*L. rubrocinctus*	*Fagus*	Heilmann-Clausen et al. 1998
	Slovania	*L. rufus*	*Pinus, Picea*	Heilmann-Clausen et al. 1998
	France	*L. serifluus*	Deciduous forest	Heilmann-Clausen et al. 1998
	Sweden	*L. sphagneti*	*Picea*	Heilmann-Clausen et al. 1998
	Western Europe	*L. subdulcis*	*Fagus*	Heilmann-Clausen et al. 1998
	Sweden	*L. subumbonatus*	*Quercus*	Heilmann-Clausen et al. 1998
	Sweden	*L. tabidus*	*Betula, Quercus, Picea*	Heilmann-Clausen et al. 1998
North and Central America	USA	*L. alachuanus var. alachuanus*	Indeterminate	Hesler and Smith 1979
	USA	*L. alachuanus var. amarissimus*	Indeterminate	Hesler and Smith 1979
	USA	*L. areolatus*	*Quercus*	Hesler and Smith 1979
	USA	*L. atrobadius*	*Pinus, Picea, Alnus*	Hesler and Smith 1979
	USA	*L. badiopallescens*	*Fagus*	Hesler and Smith 1979
	USA	*L. bryophilus*	Indeterminate	Hesler and Smith 1979

Appendix 6: Continued.

Geographic region	Country	Species	Host tree	Reference
North and Central America	USA	*L. canadensis*	Indeterminate	Hesler and Smith 1979
	USA	*L. carbinocola*	Indeterminate	Hesler and Smith 1979
	USA	*L. cognoscibilis*	Indeterminate	Hesler and Smith 1979
	Canada	*L. duplicatus*	*Betula*	Hesler and Smith 1979
	USA	*L. fragilis*	Indeterminate	Hesler and Smith 1979
	USA	*L. frustratus*	*Quercus*	Hesler and Smith 1979
	Mexico	*L. herrerae*	*Alnus*	Montoya et al. 2014
	USA	*L. highlandensis*	*Pinus*	Hesler and Smith 1979
	USA	*L. imperceptus*	*Quercus*	Hesler and Smith 1979
	USA	*L. lanceolatus*	*Salix*	Hesler and Smith 1979
	USA	*L. luculentus var. laetus*	*Conifer, Alnus*	Hesler and Smith 1979
	USA	*L. luculentus var. luculentu*s	*Abies*	Hesler and Smith 1979
	USA	*L. minusculus*	Indeterminate	Hesler and Smith 1979
	USA	*L. moschatus*	Mixed forest, *Pinus*	Hesler and Smith 1979
	USA	*L. mutabilis*	Conifer	Hesler and Smith 1979
	USA	*L. nancyae*	*Pinus, Acer*	Hesler and Smith 1979
	USA	*L. nimkeae*	*Quercus*	Hesler and Smith 1979
	USA	*L. occidentalis*	*Thuja, Alnus*	Hesler and Smith 1979
	USA	*L. oculatus*	Indeterminate	Hesler and Smith 1979
	USA	*L. parvulus*	Indeterminate	Hesler and Smith 1979
	USA	*L. rimosellus*	*Pinus*	Hesler and Smith 1979
	USA	*L. rubidus*	Indeterminate	Hesler and Smith 1979, Kuo et al. 2013
	USA	*L. rufulus*	*Quercus*	Hesler and Smith 1979
	USA	*L. splendens*	Conifer	Hesler and Smith 1979
	Mexico	*L. strigosipes*	*Carpinus*	Montoya and Bandala 2008
	USA	*L. subflammeus*	*Pinus*	Hesler and Smith 1979
	USA	*L. sublacustris*	Indeterminate	Hesler and Smith 1979
	USA	*L. subolivaceus*	*Pinus*	Hesler and Smith 1979
	USA	*L. subserifluus*	*Quercus, Carya*	Hesler and Smith 1979
	USA	*L. substriatus*	Conifer	Hesler and Smith 1979
	USA	*L. subumbrinus*	*Pinus*	Hesler and Smith 1979
	USA	*L. subviscidus*	Conifer	Hesler and Smith 1979
	USA	*L. thiersii*	*Pinus*	Hesler and Smith 1979
	USA	*L. umbrinopapillatus*	*Pinus*	Hesler and Smith 1979
	USA	*L. vinaceorufescens*	*Pinus*	Hesler and Smith 1979
South America	Argentina	*L. alni*	*Alnus*	Singer 1962

APPENDIX 7

DNA SEQUENCE DATA USED FOR PHYLOGENETIC ANALYSES IN CHAPTER 6

Appendix 7.1: Information on samples used in ITS global and concatenated analysis based on ITS-*rpb2*.

Species names	Voucher collections	Country	*ITS*	*rpb2*
Lactarius decipiens	AV2000-137	Italy	KF432973	
Lactarius decipiens	KW497	Slovakia	KT165315	
Lactarius decipiens	KW496	Slovakia	KT165316	
Lactarius decipiens	KW472	Belgium	KT165313	
Lactarius inconspicuus	KW100	Thailand	KF433001	KR025677
Lactarius inconspicuus	KW003	Thailand	KR025583	
Lactarius sp.	OTA62000	New Zealand	JQ279512	
Lactarius eucalypti	TU103696	Australia	UDB002670	
Lactarius sublaccarioides	KW300	Thailand	KF432996	KR025678
Lactarius sublaccarioides	KW323	Thailand	KF432997	
Lactarius sublaccarioides	KW332	Thailand	KF432998	
Lactarius imperticus	JMP0044	USA	EU819485	
Lactarius imperticus	AM2BB6F3	USA	JQ272401	
Lactarius kesiyae	AV12-022	Thailand	KR025618	
Lactarius kesiyae	KW427	Thailand	KR025614	
Lactarius kesiyae	KW033	Vietnam	KF432994	
Lactarius kesiyae	KW032	Vietnam	KR025619	KR025644
Lactarius kesiyae	KW036	Vietnam	KR025620	
Lactarius sp.	TU115274	Puerto Rico	UDB017819	
Lactarius tangerinus	KW091	Thailand	KR025626	KR025675
Lactarius tangerinus	LTH203	Thailand	KR025627	
Lactarius tangerinus	EDC14-475	Thailand	KR025625	
Lactarius badiosanguineus	AV04-235	France	KF432983	
Lactarius badiosanguineus	ZD991	China	KT165257	KR025654
Lactarius badiosanguineus	AV10-044	Norway	KR025578	KR025655
Lactarius badiosanguineus	AV97-568	Sweden	KT165255	
Lactarius badiosanguineus	JN2001-63	Slovakia	KT165256	
Lactarius badiosanguineus	AV97-578	Sweden	KT165254	
***Lactarius dombangensis* sp. nov.**	AVKDKVP-09-020	India	KT165258	KR025664
***Lactarius lachungensis* sp. nov.**	AVKDKVP-09-019	India	KT165284	
***Lactarius lachungensis* sp. nov.**	AVKDKVP-09-009	India	KT165283	
***Lactarius lachungensis* sp. nov.**	AVKDKVP-09-017	India	KT165281	

Appendix 7.1. Continued.

Species names	Voucher collections	Country	*ITS*	*rpb2*
Lactarius lachungensis **sp. nov.**	AVKDKVP-09-011	India	KT165282	KR025659
Lactarius lachungensis **sp. nov.**	AVKDKVP-09-010	India	KT165285	
Lactarius rubrocinctus	JKLAC19982201	Germany	KR025575	
Lactarius rubrocinctus	Walleyn4025	Belgium	KT165246	
Lactarius rubrocinctus	EDC12-210	Germany	KF432977	KR025663
Lactarius badiopallescens	LM4629F	Mexico	FJ348706	
Lactarius badiopallescens	LM4630F	Mexico	FJ348705	
Lactarius sp.	JN2007-008	Canada	KT165247	KR025657
Lactarius sphagneti	JKLAC11091502	Germany	KF432975	KR025658
Lactarius sikkimensis **sp. nov.**	AVKDKVP-09-024	India	KT165252	
Lactarius sikkimensis **sp. nov.**	AVKDKVP-09-023	India	KT165250	
Lactarius sikkimensis **sp. nov.**	AVKDKVP-09-022	India	KT165248	KR025656
Lactarius sikkimensis **sp. nov.**	AVKDKVP-09-026	India	KT165253	
Lactarius sikkimensis **sp. nov.**	AVKDKVP-09-027	India	KT165249	
Lactarius fulvissimus	JKLAC10082001	Germany	KF432970	KR025660
Lactarius fulvissimus	JN2012-025	Belgium	KR025576	
Lactarius fulvissimus	f322	Italy	UDB000381	
Lactarius fulvissimus	KW485	Slovakia	KT165243	
Lactarius borzianus		Switzerland	AF373599	
Lactarius atrii **sp. nov.**	AVKDKVP-09-012	India	KT165242	
Lactarius atrii **sp. nov.**	AVKDKVP-09-004	India	KT165239	KR025635
Lactarius atrii **sp. nov.**	AVKDKVP-09-065	India	KT165240	
Lactarius atrii **sp. nov.**	AVKDKVP-09-066	India	KT165241	
Lactarius hepaticus	JN02-049	Belgium	KF432980	KR025674
Lactarius hepaticus	JV2006-025	Belgium	KF025574	
Lactarius hepaticus	JV2006-021	Belgium	KF025573	
Lactarius subdulcis	ED2008-27	Belgium	KR025572	KR025670
Lactarius subdulcis	KW498	Slovakia	KT165260	
Lactarius subdulcis	KW482	Slovakia	KT165259	
Lactarius subdulcis	JN2012-020	Germany	KR025571	
Lactarius lacunarum	JKLAC11092901	Germany	KF432982	KR025638
Lactarius lacunarum	EDC11-231	Belgium	KR025570	
Lactarius lacunarum	KW479	Slovakia	KT165262	
Lactarius flavigalactus **sp. nov.**	AVKDKVP-09-021	India	KT165264	
Lactarius flavigalactus **sp. nov.**	ZD024	China	KT165265	KR025639
Lactarius flavigalactus **sp. nov.**	ZD084	China	KT165266	
Lactarius flavigalactus **sp. nov.**	Xp4-20120929-05	China	KT165267	
Lactarius sp.	AV13-029	Canada	KT165271	KR025642
Lactarius sp.	AV13-021	Canada	KT165268	
Lactarius sp.	AV13-030	Canada	KT165269	KR025641
Lactarius sp.	AV13-028	Canada	KT165270	KR025643
Lactarius omphaliformis	PAM08083009	France	HQ714719	
Lactarius omphaliformis	PAM08090505	France	HQ714728	
Lactarius omphaliformis	PAM08090510	France	HQ714730	HQ714861
Lactarius sp.	TU110012	Ecuador	UDB014060	
Lactarius sp.	TU110028	Ecuador	UDB014072	
Lactarius rufus	KVP10-030	Russia	KT165277	
Lactarius rufus	RW1575bis	Sweden	KT165273	
Lactarius rufus	KW500	Belgium	KT165272	
Lactarius rufus	JN2012-022	Germany	KT165276	
Lactarius rufus var. *parvus*	ED2008-06	USA	KT165278	KR025669
Lactarius tabidus	KW130	Belgium	KR025582	KR025666

Appendix 7.1. Continued.

Species names	**Voucher collections**	**Country**	***ITS***	***rpb2***
Lactarius tabidus	Noordeloose9386	Sweden	KT165310	
Lactarius tabidus	ED2008-26	Belgium	KT165309	
Lactarius tabidus	MN2	Netherland	KT165308	
Lactarius tabidus	ED2008-14	USA	KT165311	KR025665
Lactarius tabidus	AV13-001	Canada	KT165307	
Lactarius tabidus	VT2443	USA	AF349716	
Lactarius aurantiacus	UE179	Germany	AF157412	
Lactarius aurantiacus	hue179	Germany	UDB000312	
Lactarius lanceolatus	IA-F20	Norway	UDB002454	
Lactarius aurantiacus	MA-Fungi53340	Spain	AJ555565	
Lactarius aurantiacus	EDC12-211	Belgium	KT165235	
Lactarius aurantiacus	JN2001-60	Slovakia	KF432974	
Lactarius aurantiacus	JN2011-89	Greece	KR025580	KR025633
Lactarius aurantionitidus **sp. nov.**	ZD973	China	KT165236	KR025631
Lactarius aurantionitidus **sp. nov.**	ZD1147	China	KT165238	
Lactarius aurantionitidus **sp. nov.**	Xp3-20120905-06	China	KT165237	
Lactarius sp.	ED2008-12	USA	KT165234	
Lctarius luculentus var. *laetus*		Canada	FJ845419	
Lactarius substriatus	Src438	USA	DQ974746	
Lactarius cfr. duplicatus	UE483	Sweden	UDB000819	
Lactarius herrerae	LM4820	Mexico	KF891371	
Lactarius herrerae	VB4533	Mexico	KF891372	
Lactarius herrerae	LM4825	Mexico	KF891370	KF891377
Lactarius cyathuliformis	TU118282	Estonia	UDB015448	
Lactarius cyathuliformis	TU106143	Estonia	UDB011523	
Lactarius cyathuliformis	TU101572	Estonia	UDB017855	
Lactarius cyathuliformis	PAM08071401	France	HQ714708	HQ714843
Lactarius sp.	TU110008	Ecuador	UDB014057	
Lactarius obscuratus	LVL02-006	Belgium	KF432978	KR025629
Lactarius obscuratus	EDC14-548	Slovakia	KT165233	
Lactarius obscuratus	TU113028	Slovenia	UDB016722	
Lactarius obscuratus	ED2008-15	USA	KR025579	
Lactarius brunneohepaticus	PAM08082103b	France	HQ714773	
Lactarius brunneohepaticus	PAM06090207	Switzerland	HQ714702	
Lactarius brunneohepaticus	PAM08082102	France	HQ714771	HQ714895
Lactarius crenulatulus	KW125	Thailand	KR025605	
Lactarius crenulatulus	KW383	Thailand	KR025604	
Lactarius crenulatulus	KW368	Thailand	KJ458979	
Lactarius crenulatulus	KW382	Thailand	KJ458980	KR025723
Lactarius pasohensis	DS06-231	Malaysia	KF432987	KR025725
Lactarius pasohensis	DS06-245	Malaysia	KF432986	
Lactarius fuscomaculatus	KW370	Thailand	KR025600	
Lactarius fuscomaculatus	KW373	Thailand	KR025603	KR025718
Lactarius fuscomaculatus	KW128	Thailand	KR025601	
Lactarius fuscomaculatus	KW111	Thailand	KF433201	
Lactarius rubrobrunneus	LTH334	Thailand	KR025598	
Lactarius rubrobrunneus	LTH149	Thailand	KR025599	
Lactarius rubrobrunneus	AV12-044	Thailand	KF432985	KR025719
Lactarius rubidus	KGP102	USA	DQ822820	
Lactarius rubidus	M.Kuo01131106	USA	KC691205	
Lactarius atrobrunneus	KW270	Thailand	KP744443	
Lactarius atrobrunneus	KW347	Thailand	KP744442	KR025728

Appendix 7.1. Continued.

Species names	**Voucher collections**	**Country**	***ITS***	***rpb2***
Lactarius serifluus	20.09.2004-15	Sweden	UDB000868	
Lactarius subumbonatus	26.07.2002-35	Sweden	UDB000867	
Lactarius subumbonatus	RCKVP10-002	Belgium	KF432981	KR025721
Lactarius serifluus	JV2006-28	Belgium	KR025597	
Lactarius serifluus	TU101620	Finland	UDB018079	
Lactarius serifluus	KW484	Slovakia	KT165294	
Lactarius subumbonatus	JKLAC110902	Germany	KR025596	
Lactarius subumbonatus	EDC11-237	Belgium	KR025595	
Lactarius serifluus	KW480	Slovakia	KT165293	
Lactarius subumbonatus	KW477	Belgium	KT165292	
Lactarius subumbonatus	UE50797	Slovakia	KT165291	
Lactarius camphoratus	JV2006-51	Belgium	KT165318	
Lactarius camphoratus	JV2006-20	Belgium	KR025610	
Lactarius camphoratus	AV10-40	Norway	KF432971	KR025697
Lactarius rubrocorrugatus	KW384	Thailand	KR025590	KR025698
Lactarius rubrocorrugatus	KW042	Vietnam	KF433010	
Lactarius rubrocorrugatus	KW043	Vietnam	KR025589	
Lactarius rubrocorrugatus	KW045	Vietnam	KR025591	
Lactarius rostratus	691	Italy	JF908276	
Lactarius rostratus	EDC14-570	Slovakia	KT165323	
Lactarius chichuensis	KW372	Thailand	KT881541	KR025705
Lactarius chichuensis	KW012	Thailand	KF433008	
Lactarius chichuensis	XHW1236	China	KF475766	
Lactarius austrorostratus	KW108	Thailand	KF433012	KR025710
Lactarius austrorostratus	KW110	Thailand	KF433014	
Lactarius austrorostratus	KW109	Thailand	KF433013	
Lactarius glabrigracilis	KW101	Thailand	KT165321	
Lactarius glabrigracilis	KW093	Thailand	KR025606	KR025684
Lactarius glabrigracilis	KW230	Thailand	KJ458984	
Lactarius gracilis	KW096	Thailand	KR025609	KR025693
Lactarius gracilis	KW102	Thailand	KF433016	
Lactarius gracilis	KW354	Thailand	KR025608	
Lactarius gracilis	KW334	Thailand	KF433017	
Lactarius gracilis	KW094	Thailand	KT165322	
Lactarius perparvus	KW320	Thailand	KJ458981	KR025695
Lactarius perparvus	KW337	Thailand	KJ458982	
Lactarius sp.	TU110589	Papua New Guinea	UDB013198	
Lactarius hirtipes	ZHW1243	China	KF433007	
Lactarius laccarioides	KW360	Thailand	KF432992	
Lactarius laccarioides	KW336	Thailand	KF432991	KR025683
Lactarius atlanticus	JKLAC13122801	Portugal	KR025611	
Lactarius atlanticus	LAC11121201	Spain	KF432976	KR025681
Lactarius strigosipes	Lamus136	Mexico	JN859141	
Lactarius strigosipes		Mexico	JN003629	
Lactarius subserifluus	JMP0046	USA	EU819486	
Lactarius chrysorrheus	AV13-049	Italy	KT165289	
Lactarius chrysorrheus	368	Spain	EU423918	
Lactarius chrysorrheus	JV2006-008	Belgium	KT165287	
Lactarius chrysorrheus	EDC12-213	Belgium	KT165290	KR025729
Lactarius chrysorrheus	KW488	Slovakia	KT165286	
Lactarius chrysorrheus	KW495	Slovakia	KT165288	

Appendix 7.1. Continued.

Species names	**Voucher collections**	**Country**	***ITS***	***rpb2***
Lactarius sp.	AV05-359	USA	KJ742399	
Lactarius sp.	AV04-212	USA	KJ742398	
Lactarius indochrysorrheus	KD11-002	India	KJ742391	
Lactarius vinaceorufescens	JN2007-018	USA	KF241542	KR025730
Lactarius cfr. subzonarius	LTH145	Thailand	KF433006	
Lactarius cfr. subzonarius	LTH324	Thailand	KF433005	
Lactarius politus	KW351	Thailand	KF433019	KR025743
Lactarius politus	KW331	Thailand	KF433020	
Lactarius politus	KW396	Thailand	KF433018	
Lactarius helvus	KVP10-033b	Russia	KT165298	
Lactarius helvus	KVP10-016	Russia	KT165301	KR025713
Lactarius quietus	KW131	Belgium	KF432972	
Lactarius quietus	KW133	Belgium	KR025624	
Lactarius quietus	KW138	Belgium	KT165231	
Lactarius quietus	KW481	Slovakia	KT165230	
Lactarius quietus	JN2012-040	Germany	KR025623	KR025736
Lactarius quietus	KW493	Slovakia	KT165232	
Lactarius cfr. quietus	ASIS22652	South Korea	KM052571	
Lactarius cfr. quietus	EMF27	China	JF273529	
Lactarius aquosus	LTH262	Thailand	KR025622	
Lactarius aquosus	KW231	Thailand	KF432984	KR025735
Lactarrius falcatus	KVP08-038	Thailand	KF133262	KR025731
Lactarius austrozonarius	FH12-007	Thailand	KF432965	KR025747
Lactarius trivialis	KVP10-005	Russia	KT165317	
Lactarius hatsudake	JN2011-065	Vietnam	KF432967	KR025732
Lactarius scrobiculatus	JN01-058	Slovakia	KF432968	
Lactarius torminosus	JN11-086	Greece	KR025613	KR025733
Lactarius purpureus	FH12-008	Thailand	KF432966	KR025746
Lactarius pallidus	JN2012-032	Germany	KT881542	KR025744
Lactarius friabilis	FH12-103	Thailand	KF432961	KR025749
Lactarius pterosporus	JN2012-037	Germany	KF432963	KR025748
Lactarius fuliginosus	M.T.Basso 97-24	Sweden	JQ446111	

Appendix 7.2: Sequences used for dataset 1 dating phylogny

Order	**Taxa (Genbank label)**	**LSU**	***rpb2***
Russulales	*Lactarius chrysorrheus*	KF133293	KF133325
Russulales	*Lactarius helvus*	KF133295	KF133327
Russulales	*Lactarius rufus*	JN389007	JN375609
Russulales	*Lactarius quietus*	KF133296	KF133328
Russulales	*Lactarius tabidus*	JN389012	JN375614
Russulales	*Lactarius sphagneti*	KF133300	KF133332
Russulales	*Lactarius subdulcis*	KF133312	KF133344
Russulales	*Lactarius spinosulus*	KF133294	KF133326
Russulales	*Lactarius deliciosus*	KF133305	KF133337
Russulales	*Lactarius torminosus*	KF133314	KF133346
Russulales	*Lactarius fuliginosus*	JQ446179	JQ446239
Russulales	*Lactarius pterosporus*	JN389002	JN375605
Russulales	*Lactarius lignyotus*	AY631898	DQ408128
Russulales	*Russula columbicolor*	JN389003	JN375606
Russulales	*Russula tsokae*	JN389006	JN375608
Russulales	*Russula albonigra*	JF834503	JF834452
Russulales	*Lactifluus glaucescens*	KF220128	KF220222
Russulales	*Lactifuus leucophaeus*	KF220157	KF220243
Russulales	*Lactifluus piperatus*	KF220177	KF220255
Russulales	*Lactifluus bicolor*	GU265577	GU258313
Russulales	*Lactifluus gerardii*	GU265614	GU258351
Russulales	*Lactifluus subgerardii*	GU265625	GU258362
Russulales	*Lactifluus deceptivus*	AY631899	AY803749
Russulales	*Amylostereum laevigatum*	AF287843	HM461109
Russulales	*Echinodontium tinctorium*	AF393056	AY218482
Russulales	*Bondarzewia montana*	DQ234539	AY218474
Russulales	*Stereum hirsutum*	AF393078	AY218520
Boletales	*Strobilomyces floccopus*	AY612824	AY786065
Boletales	*Boletus edulis*	HQ326927	LN714632
Boletales	*Boletus projectellus*	AY684158	AY787218
Boletales	*Calostoma cinnabarinum*	AY645054	AY780939
Boletales	*Suillus bresadolae*	GU187598	GU187810
Boletales	*Suillus pictus*	AY684154	AY786066
Boletales	*Gomphidius roseus*	DQ534669	GU187818
Boletales	*Leucogyrophana lichenicola*	GU187583	GU187789
Boletales	*Serpula lacrymans*	GU187596	GU187809
Boletales	*Serpula himantioides*	AF518648	DQ366283
Boletales	*Tapinella atrotomentosa*	GU187603	GU187813
Boletales	*Pseudomerulius curtisii*	GU187589	GU187796
Agaricales	*Marasmius rotula*	DQ457686	DQ474118
Agaricales	*Marasmius alliaceus*	AY635776	AY786060
Agaricales	*Mycena galericulata*	AY647216	DQ385888
Agaricales	*Mycena aurantiidisca*	DQ470811	DQ474122
Polyporales	*Trametes versicolor*	AY684159	DQ408125
Polyporales	*Pycnoporus sp.*	AY684160	DQ408121
Polyporales	*Polyporus squamosus*	AY629320	DQ408120
Polyporales	*Grifola sordulenta*	AY645050	AY786058
Polyporales	*Fomitopsis pinicola*	AY684164	AY786056
Polyporales	*Climacodon septentrionalis*	AY684165	AY780941
Polyporales	*Spongipellis pachyodon*	AY629322	DQ408123
Gomphales	*Gautieria ottihii*	AF336249	AY218486
Hymenochaetales	*Formitiporia mediterranea*	AY684157	AY803748

Appendix 7.2. Continued.

Order	**Taxa (Genbank label)**	**LSU**	***rpb2***
Hymenochaetales	*Phellinus hartigii*	JX093833	JX093877
Hymenochaetales	*Coltricia perennis*	AJ406472	AY218526

Appendix 7.3: Sequences used for dataset 2 dating phylogny

Order	**Taxa (Genbank label)**	***rpb2***	**ITS**
Russulales	*Lactarius inconspicuus*	KR025677	KF433001
Russulales	*Lactarius tangerinus*	KR025675	KR025626
Russulales	*Lactarius kesiyae*	KR025644	KR025619
Russulales	*Lactarius sublaccarioides*	KR025678	KF432996
Russulales	*Lactarius lachungense*	KR025659	KT165282
Russulales	*Lactarius badiosanguineus*	KR025655	KR025578
Russulales	*Lactarius dombangense*	KR025664	KT165258
Russulales	*Lactarius rubrocinctus*	KR025663	KF432977
Russulales	*Lactarius sphagneti*	KR025658	KF432975
Russulales	*Lactarius sikkimensis*	KR025656	KT165248
Russulales	*Lactariusfulvissimus*	KR025661	KR025576
Russulales	*Lactarius flavigalactus*	KR025639	KT165265
Russulales	*Lactarius lacunarum*	KR025638	KF432982
Russulales	*Lactarius sp.*	KR025641	KT165269
Russulales	*Lactarius omphaliformis*	HQ714851	HQ714719
Russulales	*Lactarius hepaticus*	KR025674	KF432980
Russulales	*Lactarius subdulcis*	KR025673	KR025571
Russulales	*Lactarius atrii*	KR025635	KT165239
Russulales	*Lactarius obscuratus*	KR025629	KF432978
Russulales	*Lactarius herrerae*	KF891379	KF891372
Russulales	*Lactarius cyathuliformis*	HQ714869	HQ714738
Russulales	*Lactarius brunneohepaticus*	HQ714896	HQ714773
Russulales	*Lactarius aurantionitidus*	KR025631	KT165236
Russulales	*Lactarius aurantiacus*	KR025634	KT165235
Russulales	*Lactarius tabidus*	KR025666	KR025582
Russulales	*Lactarius rufus var. parvus*	KR025669	KT165278
Russulales	*Lactarius rubrobrunneus*	KR025719	KF432985
Russulales	*Lactarius fuscomaculatus*	KR025718	KR025603
Russulales	*Lactarius subumbonatus*	KR025721	KF432981
Russulales	*Lactarius pasohensis*	KR025725	KF432987
Russulales	*Lactarius crenulatulus*	KR025723	KJ458980
Russulales	*Lactarius atrobrunneus*	KR025728	KP744442
Russulales	*Lactarius camphoratus*	KR025697	KF432971
Russulales	*Lactarius austrorostratus*	KR025710	KF433012
Russulales	*Lactarius chichuensis*	KR025705	KT881541
Russulales	*Lactarius rubrocorrugatus*	KR025698	KR025590
Russulales	*Lactarius helvus*	KR025713	KT165301
Russulales	*Lactarius vinaceorufescens*	KR025730	KF241542
Russulales	*Lactarius chrysorrheus*	KR025729	KT165290
Russulales	*Lactarius politus*	KR025743	KF433019
Russulales	*Lactarius falcatus*	KR025731	KF133262
Russulales	*Lactarius gracilis*	KR025690	KR025608
Russulales	*Lactarius glabrigracilis*	KR025684	KR025606
Russulales	*Lactarius perparvus*	KR025695	KJ458981
Russulales	*Lactarius laccarioides*	KR025683	KF432991
Russulales	*Lactarius atlanticus*	KR025681	KF432976
Russulales	*Lactarius quietus*	KR025736	KR025623
Russulales	*Lactarius aquosus*	KR025735	KF432984
Russulales	*Lactarius spinosulus*	KF133326	KF133262
Russulales	*Lactarius deliciosus*	KF133337	KF133272
Russulales	*Lactarius torminosus*	KF133346	KF133281

Appendix 7.3. Continued.

Order	Taxa (Genbank label)	***rpb2***	**ITS**
Russulales	*Lactarius fuliginosus*	JQ446239	JQ446110
Russulales	*Lactarius pterosporus*	JN375605	KR025628
Russulales	*Lactarius lignyotus*	DQ408128	DQ221107
Russulales	*Russula albonigra*	JF834452	DQ421997
Russulales	*Lactifluus glaucescens*	KF220222	KF220028
Russulales	*Lactifuus leucophaeus*	KF220243	KF220059
Russulales	*Lactifluus piperatus*	KF220255	KF220081
Russulales	*Lactifluus bicolor*	GU258313	GU258221
Russulales	*Lactifluus gerardii*	GU258351	EF560688
Russulales	*Lactifluus subgerardii*	GU258362	GU258263
Russulales	*Lactifluus deceptivus*	AY803749	AY854089

CURRICULUM VITAE

Komsit Wisitrassameewong

1. Personal details

Address: 54/45 M.6 Bangkrui, Nonthaburi, Thailand, 11130
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Date of birth: 24 April 1982
Nationality: Thai

2. Educational background

BSc in Biotechnology (2001-2004): 2nd class honour, GPA 3.29, Mae Fah Luang University

MSc in Biotechnology (2005-2007): GPA 3.46, King Mongkut's University of Technology Thonburi

3. Language skill

- **Thai:** mother tongue
- **English:** excellent

4. Professional experience

July 2008-October 2009
Researcher at King Mongkut's University of Technology Thonburi

February 2010-October 2010
Researcher at Mae Fah Luang University

November 2010-present
Ph.D. student at Mae Fah Luang University and Ghent University
Subject: Biodiversity and phylogeny of *Lactarius* subgenus *Russularia* in Southeast Asia
Advisor: Prof. Dr. Annemieke Verbeken, Asst. Prof. Dr. Kevin D Hyde, Dr. Jorinde Nuytinck

5. Additional courses and fieldwork

5.1 Training

- Internship on food chemistry at national center for genetic engineering and biotechnology, Thailand: February-May 2004
- Basidiomycetes workshop at mushroom research center, Thailand: June-July 2010
- Russulales workshop 2014, Slovakia: September 2014
- Transferable skill training at Ghent University, Belgium: presentation skill in English, October 2014
- Transferable skill training at Ghent University, Belgium: project management, December 2014
- Transferable skill training at Ghent University, Belgium: meeting skill, February 2015

5.2 Fieldwork

- Vietnam: 2 weeks in Dalat and Hanoi, June 2010
- Thailand: June to September in 2010-2013
- Slovakia: 1 week, September 2014

6. Scientific publication

6.1 A1 publication (internationally peer-reviewed)

- **Wisitrassameewong K.**, Karunarathna S.C., Thongklang N., Zhao R., Callac P., Chukeatirote E., Bahkali A.H., Hyde K.D. (2012). *Agaricus subrufescens*: new to Thailand. Chiang Mai Journal of Science 39(2): 281-291.
- **Wisitrassameewong K.,** Karunarathna S.C., Thongklang N., Zhao R., Callac P., Moukla S., Férandon C., Chukeatirote E., Hyde K.D. (2012). *Agaricus subrufescens*: a review. Saudi Journal of Science 19: 131-146.
- **Wisitrassameewong K.**, Nuytinck J., Hyde K.D., Verbeken A. (2014). *Lactarius* subgenus *Russularia* (Russulaceae) in Southeast Asia: 1. Species with very distant gills. Phytotaxa 158(1): 23-42.

- **Wisitrassameewong K.**, Nuytinck J., Hampe F., Hyde K.D., Verbeken A. 2014. *Lactarius* subgenus *Russularia* (Russulaceae) in South-East Asia: 2. Species with remarkably small basidiocarps. Phytotaxa 188(4): 181-197.
- Verbeken A., Hampe F., **Wisitrassameewong K.**, Hyde K.D., Eberhardt E., Nuytinck J. (2014). A new angiocarpous *Lactarius* species from Thailand. Phytotaxa 181 (3): 163-170.
- **Wisitrassameewong K.**, Nuytinck J., Le H.Y., De Crop E., Hampe F., Hyde K.D., Verbeken A. (2015). *Lactarius* subgenus *Russularia* (Russulaceae) in South-East Asia: 3. new diversity in Thailand and Vietnam. Phytotaxa 207(3): 215-241.

- Liu J.K., Hyde K.D., Jone E.B.G., Ariyawansa H.A., Bhat D.J., Boonmee S., Maharachchikumbura S.S.N., Mckenzie E.H.C., Phookamsak R., Phukhamsakda C., Shenoy B.D., Abdel-Wahab M.A., Buyck B., Chen J., Chethana K.W.T., Singtrinop C., Dai D.Q., Dai Y.C., Daranagama D.A., Dissanayake A.J., Doilom M., D'souza M., Fan X.L., Goonasekara I.D., Hirayama K., Hongsanan S., Jayasiri S.C., Jayawardena R.S., Karunarathna S.C., Li W.J., Mapook A., Norphanphoun C., Pang K.L., Perera R.H., Peršoh D., Pinruan U., Senanayake I.C., Somrithipol S., Suetrong S., Tanaka K., Thambugala K.M., Tian Q., Tibpromma S., Udayanga D., Wijayawardene N.N., Wanasinghe D., **Wisitrassameewong K.**, Zeng X.Y., Abdel-Aziz F., Adamčik S., Bahkali A.H., Boonyuen N., Bulgakov T., Callac P., Chomnunti P., Greiner K., Hashimoto A., Hofstetter V., Kang J.C., Lewis D., Li X.H., Liu X.Z., Liu Z.Y., Matsumura M., Mortimer P.E., Rambold G., Randrianjohany E., Sato G., Sri-Indrasutdhi V., Tian C.M., Verbeken A., Brackel W.V., Wang Y., Wen T.C., Xu J.C., Yan J.Y., Zhao R.L., Camporesi E. (2015). Fungal diversity notes 1-110: taxonomic and phylogenetic contribution to fungal species. Fungal diversity DOI 10.1007/s13225-015-0324-y

6.2 A2 publication (nationally peer-reviewed)

- Chukeatirote E., **Wisitrassameewong K.**, Jongmahasavat J. (2008). In silico PCR-RFLP of Bacillus species: problem-based case of teaching bioinformatics. Kasetsart journal: Natural Science 42: 693-700
- **Wisitrassameewong K.**, Jongmahasavat J., Lumyong S., Chukeatirote E. (2009). Towards understanding in molecular taxonomy using an In silico approach: a case study in lactic acid bacteria. Suranaree journal of science and technology 16(1): 53-62

7. Congresses and symposia

- **Wisitrassameewong K.**, Hyde K.D. (oral presentation). Basidiomycota: distribution and new records from northern Thailand. Southeast Asia conference on education, research, science and technology (SAC-ERST), Thailand, February 2011.
- **Wisitrassameewong K.**, Nuytinck J., Hyde K.D., Verbeken A (oral presentation). *Lactarius* subgenus *Russularia* and its new species in Southeast Asia: 2nd Malaysia-Thailand graduate forum in life sciences, food science and agriculture (MTGF) 2013, Malaysia, December 2013.
- **Wisitrassameewong K.**, Nuytinck J., Hyde K.D., Verbeken A (oral presentation). *Lactarius* subgenus *Russularia* in Southeast Asia: the presence of four newly striking species. International conference on fungal biodiversity and biotechnology, Mae Fah Luang University, Thailand, Jan 2014.

- **Wisitrassameewong K.**, Nuytinck J., Hyde K.D., Verbeken A (poster presentation). Southeast Asia reveals new diversity of *Lactarius* subgenus *Russularia*. The 10th international mycological congress (IMC10), Thailand, August 2014.
- Nuytinck J., **Wisitrassameewong K.**, Eberhardt U., Verbeken A. (oral presentation). Towards a new subgeneric classification in *Lactarius*. Russulales workshop 2014, Slovakia, September 2014.

8. Acquired scholarships

- Bijzonder Onderzoeksfonds Gent University” (BOF), Ghent University

As many more individuals of each species are born
than can possibly survive; and as, consequently,
there is a frequently recurring struggle for existence,
it follows that any being, if it vary however slightly in any
manner profitable to itself, under the complex and
sometimes varying conditions of life, will have a better
chance of surviving, and thus be *naturally selected*.
From the strong principle of inheritance, any selected
variety will tend to propagate its new and modified form.

Charles Darwin