

Please cite this article in press as: Stubbe D et al., Critical assessment of the *Lactarius gerardii* species complex (Russulales), Fungal Biology (2010), doi:10.1016/j.funbio.2010.01.008

Critical assessment of the *Lactarius gerardii* species complex (*Russulales*)

Dirk Stubbe*, Jorinde Nuytinck, Annemieke Verbeken

Research Group Mycology, Department of Biology, Ghent University, K.L. Ledeganckstraat
35, 9000 Gent, Belgium

Dirk.Stubbe@UGent.be, Jorinde.Nuytinck@UGent.be, Mieke.Verbeken@UGent.be

* Corresponding author. Tel.: +32 92 64 50 69, fax: +32 92 64 53 34. E-mail address:

Dirk.Stubbe@UGent.be

Running title

The *Lactarius gerardii* species complex

Abstract

This paper investigates species delimitation within the *Lactarius gerardii* species complex and explores its taxonomic and geographical extent. A combined molecular phylogeny based on ITS, LSU and *rpb2* gene sequences is constructed and morphological characters are evaluated. While *L. gerardii* was originally described from North America, it has later been reported from all-over Asia. Therefore a worldwide sampling range was aimed at, including species exhibiting morphological affinities with *L. gerardii*. The phylogenetic analyses indicate that intercontinental conspecificity in *L. gerardii* is absent. Thirty strongly supported

clades are retrieved of which 18 are morphologically identifiable species. The group is elevated to *Lactarius* subg. *Gerardii* stat. nov. It includes, apart from *L. gerardii* s.l., *L. atrovelutinus*, *L. bicolor*, *L. ochrogalactus*, *L. petersenii*, *L. reticulatovenosus*, *L. sepiaceus*, *L. subgerardii* and *L. wirrabara*, as well as the pleurotoid *L. uyedae*. The paraphyletic nature of the genus *Lactarius* is confirmed. *Lactarius* subg. *Gerardii* appears not affiliated with *L.* subg. *Plinthogalus* and this can be substantiated morphologically. No representatives are known from Europe, Africa or South America. The high frequency of intercontinental sister relationships observed between America, Asia and the Australian region, suggests multiple migration and speciation events have occurred across continents.

keywords

biogeography; phylogeography; cryptic species; ectomycorrhiza; *Lactifluus*.

Introduction

Lactarius, also known as the genus of the milkcaps, exhibits the characteristic feature of exuding a latex-like substance when injured. The genus has a subcosmopolitan distribution and is one of the main ectomycorrhizal symbionts in many vegetations ranging from the dipterocarp rainforests in South-east Asia over the Mediterranean *Cistus* vegetations to the boreal, coniferous forests. Recent work of Buyck *et al.* (2008) demonstrated that the genera *Russula* and *Lactarius* are paraphyletic. This resulted in the creation of the new and morphologically distinct genus *Multifurca*, accommodating *M. furcatus* (formerly assigned to *Lactarius*) and most species of former *R.* subsect. *Ochricompactae* (Buyck *et al.* 2008). While the remainder of *Russula* appears monophyletic, the results of Buyck *et al.* (2008) also indicate that *Lactarius* should be further split up into two separate genera, with one genus containing *L.* subgenera *Russularia*, *Piperites* and *Plinthogalus* and the other containing *L.* subgenera *Lactarius*, *Lactifluus* and *Lactariopsis* and *L.* sect. *Edules* (Buyck *et al.* 2008). A formal proposal is currently submitted to conserve the name *Lactarius* for the first genus and to use the name *Lactifluus* for the latter (Buyck *et al.* 2010). Here we maintain the use of *Lactarius* to refer to all milkcaps, except *M. furcatus*.

Lactarius gerardii Peck 1873 was described from eastern North America where it is regarded as a rather common species. Because of the velutinous aspect and brown colour of the cap, the lack of macrocystidia in the hymenium and a reticulate spore ornamentation, it fits well in *L.* subg. *Plinthogalus* (Hesler & Smith 1979). Its white and widely spaced lamellae contrasting with the dark brown cap and stipe and the absence of any discolorations of the context or latex have led to the reputation of being an easily recognizable species. While *L. gerardii* is not known to occur in western North America, the species has been reported all-over Asia (Das & Sharma 2005; Imazeki *et al.* 1988; Le *et al.* 2007; Lee *et al.* 2002; Wang *et al.* 2004; Wu &

Mueller 1997). Specimens identified as *L. aff. gerardii* have been collected from Costa Rica to Newfoundland and from Japan to the western slopes of the Himalaya in India. Earlier, we have included Thai and American specimens in preliminary analyses to test the intercontinental conspecificity of *L. gerardii* (Le *et al.* 2007). The results showed that American and Thai specimens should be considered as different species. This was in line with the findings of Nuytinck *et al.* (2007), where the absence of Asian/American conspecificity could be demonstrated for *L. sect. Deliciosi*. However, our results also indicated that the Thai '*L. gerardii*' consisted of several species. For a better appreciation of this species complex, we expanded our sampling range to a global scale and also tested species with a morphological resemblance to *L. gerardii* for their putative kinship.

The *L. gerardii* complex is an interesting case study for several reasons. *Lactarius gerardii* s.l. is considered as a common and easily recognizable species with a wide-spread, intercontinental distribution – in certain areas it even has a commercial value as an edible mushroom (Wang *et al.* 2004). Some morphological variability has been noticed in Asian specimens, but this has not been assessed molecularly (Le *et al.* 2007; Wang 2008). Recent molecular studies of Thai and Malaysian species of *L. subg. Plinthogalus* suggested *L. gerardii* should be removed from *L. subg. Plinthogalus* (Le *et al.* 2007; Stubbe *et al.* 2008), while the recent rearrangements within the *Russulaceae* (Buyck *et al.* 2008, 2010), called for a re-evaluation of the *L. gerardii* complex at the genus level.

The main goals of this paper are: (1) to determine the extent of the species complex both taxonomically and biogeographically by constructing a worldwide phylogeny of *L. gerardii* s.l.; (2) to reconsider its taxonomical position in light of the new findings in the *Russulaceae*; (3) to confront phylogenetic species delimitation with morphological characters.

Taxonomic history of *Lactarius gerardii*

The description of *Lactarius gerardii* was based on three collections made by W.R. Gerard, in Poughkeepsie, Albany and Croghan (New York) of which the first collection was later designated as lectotype (Smith & Hesler 1962). Smith & Hesler (1962) created *L.* series *Gerardii*, joining those species of *L.* subg. *Plinthogalus* with a truly white spore deposit: *L. subrubescens*, *L. similissimus*, *L. fagicola*, *L. murrillianus* and *L. gerardii*. The first two species differ from *L. gerardii* by turning reddish when injured, while *L. fagicola* turns brown when injured. *Lactarius murrillianus* is unchanging but has narrow and crowded lamellae as opposed to *L. gerardii*. Originally, Smith & Hesler (1962) distinguished *L. similissimus* from *L. subrubescens* based on its narrower caulocystidia and more ellipsoid spores, but they later accepted these differences as variability within one species (Hesler & Smith 1979). Hesler & Smith (1979) synonymized *L. similissimus* with *L. subrubescens* and relegated it, as they did with *L. fagicola*, to a varietal level of *L. gerardii*, resulting in the currently accepted varieties *L. gerardii* var. *subrubescens* and *L. gerardii* var. *fagicola*. The taxon *L.* series *Gerardii* was no longer acknowledged in Hesler & Smith (1979). Instead the authors introduced stirps *Gerardii*, based on the same criterion of a white spore deposit. Although the species were not listed, this implied the inclusion of *L. gerardii*, *L. atro-olivaceus*, *L. louisii*, *L. petersenii*, *L. pseudogerardii* and *L. subgerardii* (Hesler & Smith 1979). *Lactarius murrillianus* was made a variety of *L. subisabellinus*, a species with a buff spore print, and was assigned to stirps *Eburneus* (Hesler & Smith 1979).

Materials and methods

Sampling

In order to achieve the best possible idea of the limits and extent of the species involved in the *Lactarius gerardii* complex, we aimed to use specimens from a wide geographical sampling range and to include all species of stirps *Gerardii* as well as those species whose morphology suggests a putative affinity with *L. gerardii*. The detailed list of our target species – all are members of *L.* subg. *Plinthogalus* – for this study was as follows : *L. gerardii* var. *gerardii*, *L. gerardii* var. *subrubescens*, *L. gerardii* var. *fagicola*, *L. subgerardii*, *L. petersenii*, *L. atroolivaceus*, *L. pseudogerardii*, *L. louisii*, *L. xanthodorheus*, *L. subtomentosus*, *L. subisabellinus* var. *murrillianus*, *L. chiapanensis*, *L. ochrogalactus*, *L. bicolor*, *L. reticulatovenosus*, *L. atrovelutinus*, *L. venosus*, *L. wirrabara* and *L. sepiaceus* (Grgurinovic 1997; Hesler & Smith 1979; McNabb 1971; Montoya *et al.* 1996; Stubbe *et al.* 2008; Verbeken & Horak 1999b; Verbeken & Horak 2002; Wang *et al.* 2006; Wang 2007). Phylogenetic research of the genus *Lactarius* as a whole, suggested an affinity of the pleurotoid *L. uedae* with *L. gerardii* (J.N., unpubl.). Therefore, *L. uedae* and other possibly related, pleurotoid species, *L. panuoides*, *L. multiceps* and *L. brunellus* (Henkel *et al.* 2000; Miller *et al.* 2002) were also considered as target group species. *Multifurca furcata*, *M. ochricompacta* and 14 non-target group *Lactarius* species were added to ascertain the position of *L. gerardii* in one of the three major clades containing milkcaps (Buyck *et al.* 2008): *L. angiocarpus*, *L. baliophaeus*, *L. cyanescens*, *L. lignyotus*, *L. montoyae*, *L. picinus*, *L. romagnesii* (*L.* subg. *Plinthogalus*); *L. volemus*, *L. clarkeae* (*L.* subg. *Lactifluus*); *L. leucophaeus*, *L. glaucescens*, *L. piperatus* (*L.* subg. *Lactarius*); *L. camphoratus* (*L.* subg. *Russularia*); *L. pubescens* (*L.* subg. *Piperites*) (Das & Sharma 2004; Eberhardt & Verbeken 2004; Heilmann-Clausen *et al.* 1998.; Lalli & Pacioni 1992; Stubbe *et al.* 2007; Verbeken 2001a; Verbeken & Horak 1999a). Furthermore, three *Russula* species were included (*R.*

nigricans, *R. persicina* and *R. camarophylla*) and *Auriscalpium vulgare* was used to root the trees.

DNA extraction, PCR, sequencing and nucleotide alignments

DNA was extracted from dried material using the PrepMan® Ultra Sample Reagent kit (Applied Biosystems Inc, Foster City, CA, U.S.A.). Extracts were purified with JetQuick General Clean-up columns (Genomed, Löhne, Germany). If DNA amplification proved unsuccessful, either the extraction protocol as described in Nuytinck *et al.* (2003) was used or a modified version, omitting the CTAB procedure and replacing it by an incubation with proteinase K for 18 hours at 45° C. For several specimens older than 10 years, DNA was extracted with the Genra Puregene Tissue kit (Qiagen Benelux B.V., Venlo, The Netherlands).

Protocols for PCR amplification and for sequencing follow Le *et al.* (2007). Three nuclear loci were amplified and sequenced: (1) the ITS region of the nuc rDNA, comprising ITS1, ITS2 and 5.8S, using primers ITS1-F and ITS4 (White *et al.* 1990), when necessary also using intermediate primers ITS2 and ITS3; (2) part of the LSU nuc rDNA, using primers LR0R and LR5 (R. Vilgalys lab '<http://www.biology.duke.edu/fungi/mycolab/primers.htm>'); (3) the region between conserved domains 6 and 7 of the second largest subunit of the RNA polymerase II (*rpb2*), using primers bRPB2-6f and fRPB2-7cR (Liu *et al.* 1999; Matheny 2005). For specimens of which *rpb2* amplification failed, intermediate primers have been constructed: i1f-RPB2 (5'-GCA RAA RAA GCA CGT DMG GTG GC-3') (forward primer) and i2r-RPB2 (5'-ACV GTT TCC TCT TCT TCR GCR TC-3') (reverse primer).

For all three loci, both forward and reverse sequences were obtained to resolve undetermined sites as much as possible. Sequences were assembled and edited with the Sequencher™ 4.8

software (Gene Codes Corporation, Ann Arbor, MI, U.S.A.). Alignments were performed with the program MAFFT v6 (Kato & Toh 2008) with setting L-INS-i for ITS and setting FFT-NS-i for LSU and *rpb2*, and manually edited in BioEdit v7.0.9.0 (Hall 1999). The ITS alignment acquired from MAFFT was first subjected to the online program Gblocks v0.91b (Castresana 2000; Castresana lab '<http://molevol.cmima.csic.es/castresana/Gblocks.html>') (settings: 'allow smaller final blocks', 'allow gaps within blocks') to delimit most of the ambiguously aligned segments. The combined alignment was then visually screened for additional, ambiguously aligned regions that needed to be excluded from further analyses. The only intron of the *rpb2* marker was excluded entirely. The alignments are made available on the Russulales News website (Russulales News '<http://www.mtsn.tn.it/russulales-news>') or can be obtained from the first author.

Phylogenetic analyses

The dataset was subdivided into 7 partitions: ITS1, 5.8S, ITS2, LSU, *rpb2* first codon positions (*rpb2*-1), *rpb2* second codon positions (*rpb2*-2) and *rpb2* third codon positions (*rpb2*-3). To test compatibility of the combined nuc rDNA markers with the *rpb2* marker, a ML Rapid Bootstrapping algorithm was implemented on both datasets for 500 replicates in the program RAxML v7.0.3, using the GTRMIX model with parameters optimized for each partition (Stamatakis 2006b; Stamatakis *et al.* 2008). The program Compat.py (Kauff & Lutzoni 2002; Compat.py '<http://www.lutzonilab.net/pages/download.shtml>') was then used to detect conflicting clades with at least 70% BS support. The ML analysis of the combined dataset was executed with RAxML applying the Rapid Bootstrapping algorithm as described above, followed by a ML tree search. Five partitioning strategies were tested: (1) ITS1, 5.8S, ITS2, LSU, *rpb2*-1, *rpb2*-2, *rpb2*-3; (2) ITS1+ITS2, 5.8S+LSU, *rpb2*-1, *rpb2*-2, *rpb2*-3; (3)

ITS1+ITS2, 5.8S, LSU, *rpb2-1+2*, *rpb2-3*; (4) ITS1, 5.8S, ITS2, LSU, *rpb2-1*, *rpb2-2+3*; (5) ITS1+ITS2, 5.8S+LSU, *rpb2-1*, *rpb2-2+3*. Bayesian Inference analysis (BI) was executed in MrBayes v3.1.2 (Ronquist & Huelsenbeck 2003) applying the GTR+I+G model of character evolution. The BI was executed twice: once with maximized partitioning (strategy (1)) and once with 5 partitions (strategy (3)). For both analyses, two independent runs with four chains each were run for 10M generations, sampling every 100th tree. The BI output was evaluated with Tracer (Tracer v1.4 '<http://beast.bio.ed.ac.uk/Tracer>'), convergence and stationarity of the two runs were checked and a burn-in value determined. A majority rule consensus tree was built after discarding the trees from the burn-in.

Morphological analyses

Macromorphological data of specimens are based on notes of the collectors. Specimens collected by the authors were described in daylight conditions. Colour changes of context and latex were observed up to 90 minutes after injury of the fruit body.

Microscopic features were studied on dried material. Terminology is adopted from Vellinga (1988) and, especially for pellis structures, from Verbeken (1998a). Pellis structures and hymenial elements were observed either in Congo-red in L4 or in Congo-red in ammonia and in a 10 % aqueous potassium hydroxide solution. If necessary, slides were shortly heated above a flame to improve colour impregnation and rehydration. Spores were illustrated and described as observed in Melzer's reagent. Drawings of the spores were made with a drawing tube at 6000 × magnification, pellis structures were drawn at 1600 × magnification.

Identification of specimens was checked again after preliminary results of the molecular analyses and specimen names were corrected if necessary (Table 1). Type material was studied from the following species: *L. gerardii* var. *gerardii* (lectotype NYS 1341, NYS), *L.*

gerardii var. *subrubescens* (Hesler 22326, TENN), *L. gerardii* var. *fagicola* (Smith 63693, MICH), *L. subgerardii* (Hesler 30321, TENN), *L. ochrogalactus* (E. Nagasawa 80-102, TMI), *L. wirrabara* (AD 9974, AD), *L. sepiaceus* (PDD 26384, PDD), *L. bicolor* (E.M. Burkill 73, K), *L. uyedae* (T. Uyeda, F), *L. reticulativenosus* (isotype Horak ZT 6472, GENT), *L. venosus* (isotype, Horak ZT 73-181, GENT).

Results

Sampling

Sequences of all target species could be included in the molecular analyses, except for *L. brunellus*, *L. louisii*, *L. multiceps*, *L. pseudogerardii*, *L. subisabellinus* var. *murrillianus*, *L. subtomentosus*, *L. venosus* and *L. xanthhydrorheus*. Either no specimen was obtained or no useful DNA could be extracted, presumably due to the age of the specimens. The analyses include 92 specimens of target species, collected from Guyana, Costa Rica, the U.S.A., Canada, Japan, South Korea, China, Thailand, Nepal, India, Sri Lanka, Malaysia, Indonesia, Australia and New Zealand (Table 1). No specimens are known from Europe or Africa (including Madagascar) that show a putative affinity with *L. gerardii* or any of the other target species.

Molecular analyses

We produced 90 new sequences for the ITS region varying between 578 and 708 base pairs, 95 new LSU sequences varying between 804 and 906 base pairs, and 100 new *rpb2* sequences

varying between 326 and 827 base pairs (Table 1). The complete dataset (including non-target and outgroup species) involves 114 specimens, and contains 108 ITS, 110 LSU and 111 *rpb2* sequences. Missing or highly incomplete sequences and sequences obtained from GenBank are indicated in Table 1. After exclusion of the ambiguously aligned regions, the concatenated alignment that is used for further analyses consists of 1925 characters (1-381: ITS, 382-1226: LSU, 1227-1925: *rpb2*).

Compat.py detected only two minor instances of conflicting clades between nuc rDNA and *rpb2* BS analysis. The first one involves the internal relations of 3 Malaysian specimens (*R.W.26708*, *S.S.L.1098*, *N.S.Y.1357*), the second one indicates *Lactarius camphoratus* and *L. pubescens* form a monophyletic group in the *rpb2* analysis but not in the nuc rDNA analysis. The five partitioning strategies tested for the ML analysis of the concatenated dataset, produced highly similar trees but strategies (1)-(3) gave a tree with a better likelihood value and clades with higher BS values than (4) and (5). Evaluation of the BI analysis output with Tracer showed that after 10M generations, the effective sample size of all parameters had amounted to at least 100 when strategy (3) was used. This was not the case with maximized partitioning and also, the PP values were slightly worse. Burn-in was set at 10 %. We will continue with the results and discussion based on ML and BI analyses using partitioning strategy (3) (ITS1+ITS2, 5.8S, LSU, *rpb2*-1+2, *rpb2*-3).

The ML and BI analyses result in nearly identical phylograms. The four major *Russulaceae* clades, as found by Buyck *et al.* (2008), are retrieved and the paraphyly of *Lactarius* is confirmed (Fig 1 A). The *Russula* clade, *Multifurca* clade and ‘*Lactarius* 1’ clade comprising *L. camphoratus*, *L. pubescens* and the species of *L.* subg. *Plinthogalus*, all have strong support in both ML and BI analyses. The remaining *Lactarius* species cluster together in a clade designated as ‘*Lactarius* 2’. *Lactarius* subg. *Plinthogalus* is a strongly supported clade within ‘*Lactarius* 1’. All target group species, except *L. chiapanensis* and *L. panuoides*, cluster

together in one strongly supported clade, that we refer to as the ‘*Gerardii* clade’, which belongs in ‘*Lactarius* 2’. Our results confirm the exclusion of *L. gerardii* from *L.* subg. *Plinthogalus*.

Phylogeny of the ‘*Gerardii* clade’

Within the ‘*Gerardii* clade’ we find 30 terminal branches and strongly supported, monophyletic clades that correspond to 30 different species. Eighteen species are morphologically distinct (clades 2, 10-12, 14-15, 17, 20-30). The other species lack any distinctive, morphological features and in many cases exhibit considerable morphological and genetic variability. The North American, Asian and Australian species do not form monophyletic clades according to continent.

Clade 1 is a North American clade containing a.o. two specimens with a colour change in the context: a specimen identified as *Lactarius atro-olivaceus* (*D.E. Desjardin* 3630) which stains brownish lavender, and a specimen identified as *L. gerardii* var. *subrubescens* (*R.E. Halling* 6918). For some specimens of *A. Verbeken* brown spots were observed, attributed to insect damage. Spore ornamentation is variable but other micromorphologic features are consistent with the studied lectotype of *L. gerardii* var. *gerardii*. Specimen *A.E. Franco-Molano* 27-06-1989, the only specimen collected from New York, the same state as the lectotype, is on a separate branch in this clade.

Clade 2 represents a Thai species with unchanging context, rather wide-meshed spore ornamentation (Fig 2 A), short terminal elements in the pileipellis and similar marginal cells (on the lamella edge) with brown pigmentation.

In clades 3-6, spore morphology is not uniform and genetic variability is high. For the specimens of North American clade 4, the same observations can be made as for clade 1, from which they cannot be distinguished morphologically.

Clades 7-8 are two very similar species with densely reticulate spore ornamentation. Malaysian species 7 has slightly higher and acute ornamentations. No detailed macroscopical descriptions were provided for these specimens.

In clade 9, morphology is variable and specimens cannot be distinguished from those of clade 6.

Clades 10, 11 and 14 (*L. bicolor*) are similar species with strikingly coloured lamella edges and low-ridged spores. Species 10 differs from *L. bicolor* (Fig 1 D) by its long and slender marginal cells and terminal elements. The Chinese species 11 is set apart by its larger spores.

Clades 12 and 15 are two Sri Lankan species. Species 12 has characteristic spores bearing a reticulum of interconnected, acute spines (Fig 2 F). Species 15 (Fig 1 E, Fig 2 H) has dense and brown staining lamellae, and spores with firm ridges.

Clade 16 consists of specimen *K. Das* 4062. It had been identified, together with *K. Das* 4073 (clade 6) as *L. gerardii* var. *subrubescens* (Das & Sharma 2004). The two species have similar morphologies.

Clade 17 corresponds with *L. atrovelutinus*, a species characterized by the short, terminal elements in the pileipellis, the small spores and a pinkening colour reaction (Fig 1 C).

Clades 13 and 18 correspond with *L. cfr. wirrabara*, originally described from Australia by Grgurinovic (1997), and *L. cfr. sepiaceus*, originally described from New Zealand by McNabb (1971), respectively. The micromorphologic features of these two species are very similar and do not allow to distinguish between them. All specimens are morphologically consistent with the type of *L. wirrabara* and a paratype of *L. sepiaceus* (PDD 31236, PDD) respectively, but the holotype of *L. sepiaceus* has mostly spores with a lower and denser reticulum.

Macroscopically, *L. sepiaceus* should be the darkest of the two species, being more blackish brown, and its context should turn rapidly pink in contact with ferrous sulphate. Grgurinovic tested this for *L. wirrabara* but only on dried material, which gave no reaction. She provided no comparison of the two species. We designated clade 18 as *L. cfr. sepiaceus* since all specimens, except one, had been identified as *L. sepiaceus* and because the detailed description of the New Zealand specimen (*P. Leonard* 40509), mentioning the rapid pink reaction with ferrous sulphate, matches exactly the description of the species.

Clade 19 is a Malaysian species, similar to the Thai species 2, but the spores are slightly more densely reticulate and have many, minute, isolated warts between the ridges.

Clade 20 is labeled *L. cfr. gerardii* var. *fagicola*. The, often large, spores are similar to those of the type specimen, but the ridges are more acute (Fig 2 J). A brownish colour change of the context was observed in *D.E. Desjardin* 3564 but absent in *J. Nuytinck* 07-029.

Clade 21 represents *L. subgerardii*. This species is recognized by its small stature and the spores ornamented by a mixture of ridges and interconnected, acute warts (Fig 2 K).

Clade 22 consists of the type of *L. reticulatovenosus*, a small, Indonesian species with spores bearing irregular ridges (Fig 2 L).

Clade 23 is a Malaysian specimen exhibiting a pinkening of the context. It differs from *L. gerardii* var. *subrubescens* and other species by a very rudimentary layer of globose cells in the pileipellis, small spores (av. $6.8 \times 5.7 \mu\text{m}$) and the absence of distinct, marginal cells.

Clade 24 is a Costa Rican specimen collected in a high altitude (2600 m) *Quercus* forest. It is distinct from any North American collection because of its small basidiocarps with conspicuous, brown lamella edges and its particularly acute spore ornamentation.

Clades 25, 26 and 27 together form a monophyletic group of white, pleurotoid species, containing *L. uyedae*, described from Japan. The three species can be told apart by the shape

of their macrocystidia. The Thai species (clade 25) also has smaller, and differently ornamented spores (Fig 2 M).

Clades 28, 29 and 30 are three species with spore ornamentation composed of interconnected warts and with latex that is brown (*L. petersenii* and *L. ochrogalactus*) or becomes brown (*L. aff. ochrogalactus*). The North American *L. petersenii* is further characterized by its dense lamellae (Fig 1 F) and low spore ornamentation (Fig 2 N). In both the Japanese *L. ochrogalactus* and the Australian/New Zealand *L. aff. ochrogalactus* the context stains reddish pink and macrocystidia are present, but only *L. ochrogalactus* has thick-walled terminal elements. The macroscopic characteristics of *L. aff. ochrogalactus* correspond to Fuhrer's concept of *L. wirrabara* (Fuhrer 2005). However, his concept cannot be considered correct, since the pinkening of the context and the brown discoloration of the latex are not consistent with the original description of *L. wirrabara* and the spores of *L. aff. ochrogalactus* (Fig 2 O) are strikingly different from those of the type of *L. wirrabara*.

Diagnostic characters

Species of the 'Gerardii clade' can be morphologically recognized, based on five important characters: the white spore print, the reticulate spore ornamentation not higher than 2 μm (Fig 2), the palisade structure in the pileipellis with globose cells in the subpellis (Fig 3), the lack of macrocystidia and the general habitus of a brown pileus and stipe contrasting with the white, mostly distant, lamellae (Fig 1 B-F). Some exceptions exist, like *L. ochrogalactus*, which has macrocystidia, and *L. uyedae* which is a white, pleurotoid species and also has macrocystidia in its hymenium.

Characters of diagnostic value within the 'Gerardii clade' are:

Spore ornamentation – In most cases, accurate observation of the spore characteristics, and especially spore ornamentation, may allow identification of the different species. The reticulum can differ in density, its ridges can vary in thickness, height and regularity and warts can be differently shaped. Exceptions include *L. cfr. sepiaceus* and *L. cfr. wirrabara* (Fig 2 E, I), and *L. bicolor* and *L. aff. bicolor* (Fig 2 G) which cannot be distinguished based on spore characteristics.

Lamellar density – While most species of the ‘*Gerardii* clade’ have distantly spaced lamellae when mature, this is not the case for *L. petersenii* and the Sri Lankan species 15 (Fig 1 E-F).

Lamella edge –The lamella edge may be concolorous with the pileus. This sometimes facilitates species recognition, especially when very conspicuous as in *L. bicolor* (Fig 1 D) and the species from clades 10, 11 and 24. In the Thai species of clade 9 and in *L. sepiaceus* this feature appears to be variable, being present in some basidiocarps and absent in others. However, in most clades, a coloured lamella edge is consistently absent. None of the North American species in the ‘*Gerardii* clade’ exhibit coloured lamella edges.

Colour and colour changes of latex and contex – These are important in identifying *L. atrovelutinus*, *L. petersenii*, *L. ochrogalactus*, *L. aff. ochrogalactus* and the species 15. Colour changes of the context appear not consistent in clades 1, 4, 6 and 20, where only for some specimens a colour change has been noted by the collectors.

Pellis structures and marginal cells – All species have a clear palisade (Fig 3 A) in the pileipellis, except for *L. cfr. sepiaceus* and *L. cfr. wirrabara* which have a trichopalisade. Terminal elements of the pileipellis may differ in shape and length between species. When the lamella edge is concolorous with the pileus, the form of the terminal elements is often repeated in the marginal cells. Terminal elements and marginal cells allow to discriminate between *L. bicolor* and species 10 and between Thai species 2 and the other Thai species.

Macrocystidia and *thick-walled elements* – Thick-walled pellis elements (cell walls min. 1 µm thick) and macrocystidia that are clearly distinct from developing basidia are rare in the ‘*Gerardii* clade’. They are present in *L. (aff.) ochrogalactus* (clades 29-30) and in the three, pleurotoid species *L. (aff.) uyedae* (clades 25-27). The shape of the macrocystidia is species-specific in these pleurotoid species.

Taxonomy

Lactarius* subg. *Gerardii (A.H. Sm. & Hesler) Stubbe **stat. nov.**

Basionym: *Lactarius* series *Gerardii* A.H. Sm. & Hesler, *Brittonia* 14: 378, 1962

Type: *Lactarius gerardii* Peck, *Bull. Buffalo Soc. Nat. Sci.* 1: 57, 1873 (as *L. ‘gerardii’*)

Species: *L. gerardii*, *L. subgerardii*, *L. petersenii*, *L. ochrogalactus*, *L. sepiaceus*, *L. wirrabara*, *L. reticulatovenosus*, *L. uyedae*, *L. bicolor*, *L. atrovelutinus*.

Description: Brown and velutinous pileus and stipe with whitish, mostly distant lamellae. Sometimes small, white, pleurotoid species. White spore print. Spores reticulately ornamented with ridges or interconnected warts, not higher than 2 µm. Macrocystidia mostly lacking. Pileipellis usually a well-developed palisade.

Discussion

If the proposal to conserve the name *Lactarius* for what we referred to here as ‘*Lactarius* 1’, and to apply the name *Lactifluus*, for what we referred to here as ‘*Lactarius* 2’, gets accepted, then *Lactarius* subg. *Gerardii* will be transferred to *Lactifluus*.

Publication of the here newly discovered species is currently in preparation.

The actual number of species belonging to *L.* subg. *Gerardii* is likely to be higher than we can demonstrate here. The phylogeny here presented suggests 30 species but not all putative members have been included in the analyses. The following species show morphological affinities, but still need to be tested molecularly: *L. brunellus*, *L. louisii*, *L. multiceps*, *L. pseudogerardii*, *L. subisabellinus* var. *murillianus*, *L. subtomentosus*, *L. venosus* and *L. xanthodorheus*. Further sampling might reveal additional new species.

Ectomycorrhizal hosts that have been recorded for species of *L.* subg. *Gerardii* are *Fagaceae* (*Fagus*, *Quercus*, *Lithocarpus*, *Castanopsis*), *Nothofagaceae* (*Nothofagus*), *Dipterocarpaceae* (*Shorea*, *Dipterocarpus*), *Myrtaceae* (*Eucalyptus*) and conifers (*Pinus*, *Tsuga*, *Abies*). The subgenus inhabits forest biotopes, varying from deciduous, coniferous and mixed temperate forests to tropical montane forests and lowland rainforests. The currently known distribution covers Central America, eastern North America, eastern Asia, Southern China, the Himalayan region, Sri Lanka, South-east Asia, and the Australian region.

Lactarius subg. *Plinthogalus* and *L.* subg. *Gerardii* share the brown colours, the velutinous aspect, the reticulate spore ornamentation and the lack of macrocystidia. In *L.* subg. *Plinthogalus* spore print colours are never pure white, like in *L.* subg. *Gerardii*, but vary from cream to buff, and are among the darkest in the genus. The reticulate spore ornamentation in *L.* subg. *Gerardii* is often quite regular and ridges are not as venose and never winged or zebroid, as they can be in *L.* subg. *Plinthogalus*. Critical observation of the pileipellis is important to distinguish the two taxa (Fig 3). In *L.* subg. *Gerardii* the pileipellis is typically a palisade with a subpellis of multiple layers of globose cells (Fig 3 A). Especially the subpellis is different in *L.* subg. *Plinthogalus*, where, instead of distinct globose cells, it is composed of strongly inflated hyphal elements, that in most cases allow one to follow how individual elements are linked to one another. These hyphal elements are not so much globose, but rather pear-shaped and irregular in form (Fig 3 B).

One of the most notable problems that has come forth in this study of *L.* subg. *Gerardii*, is the position of the type species in the phylogeny. The type most likely belongs to either clade 1 or clade 4, but these clades cannot be distinguished from each other morphologically, nor could any useful DNA be extracted from the lectotype to ascertain its position molecularly. Besides that, North American specimens identified as *L. gerardii* var. *subrubescens*, *L. gerardii* var. *fagicola* and *L. atro-olivaceus* group together with specimens without a colour reaction, complicating interpretation of these colour reactions. A comprehensive study using detailed descriptions of newly collected North American specimens, might resolve these matters.

At least four species of *L.* subg. *Gerardii* exist in the Australian region: *L.* aff. *uyedae*, *L.* aff. *ochrogalactus*, *L. sepiaceus* and *L. wirrabara*. These last two species remain very difficult to tell apart but are not as closely related as their similar morphologies would suggest. The reaction of ferrous sulphate on fresh specimens of *L. wirrabara* should be tested. In the original descriptions, *L. wirrabara* was found near *Eucalyptus* and *L. sepiaceus* near *Nothofagus*. However, several of the Australian specimens of *L.* cfr. *sepiaceus* were also found with *Eucalyptus*. An ectomycorrhizal study could be useful to confirm the host tree. More New Zealand samples need to be checked to know whether *L.* cfr. *wirrabara* can also be found on the archipelago.

Lactarius chiapanensis, described from Mexico, was considered a member of *L.* subg. *Plinthogalus*, resembling *L. petersenii* and *L. pseudogerardii* (Montoya *et al.* 1996). It now is clear that the species belongs neither to *L.* subg. *Plinthogalus*, nor to *L.* subg. *Gerardii*. It appears closely related to the Australian *L. clarkeae* which has an orange and coarsely tomentose cap. Microscopically this makes sense, since their faintly ornamented spores and subcapitate macrocystidia are practically identical. Moreover, these are features that are atypical for *L.* subg. *Gerardii* or *L.* subg. *Plinthogalus*.

The pleurotoid habitus of *L. panuoides* and *L. uyedae*, was developed independently from one another. In spite of this, we were unable to detect any morphological feature that can be related to their different phylogenetic position. The phylogenetic positions of *L. brunellus* and *L. multiceps* still need to be determined molecularly. It is curious to notice that a white spore print and thick-walled terminal elements appear present in all known, pleurotoid *Lactarius* species (Henkel *et al.* 2000; Miller *et al.* 2002; Redhead & Norvell 1993; Verbeken 1998b).

Biogeography

One of the most striking observations that can be made from the phylogeny of *L.* subg. *Gerardii* is the multitude of intercontinental sister relationships between species from America, Asia and the Australian region. The species in this subgenus do not cluster together according to continent, and sympatric lineages are often distantly related. These results suggest that allopatric speciation, which is the most common speciation mechanism (Kohn 2005), is likely to be one of the major mechanisms responsible for the species diversity in *L.* subg. *Gerardii*. Migration was possible via land bridges in Beringia and in the Malesian region that have existed intermittently throughout the Tertiary and Quaternary. In the Tertiary, the Beringian land connections sustained broad-leaved, and later-on, coniferous forests (Graham 1993), which could have supported an ectomycorrhizal mycoflora. Even during the Pleistocene, Beringia experienced prolonged ice-free periods with shrub-tundra environments (Elias *et al.* 1997). In the Malesian region, a combination of land bridges and island hopping made migrations possible between Asia and Australia, starting from the mid-Miocene (Cox & Moore 2006; Hewitt 2000). This would situate the first Australasian distributions earliest around 15 million years ago. Considering the limited genetic variability in *L. cfr. sepiaceus* and in *L. aff. ochrogalactus*, their Australian/New Zealand distribution appears to be recent

and therefore most likely caused by long distance dispersal (LDD). LDD events between Australia and New Zealand have been demonstrated in *Lentinula* (Hibbett 2001) and in the ectomycorrhizal genus *Pisolithus* (Moyersoen *et al.* 2003). In fact, one cannot rule out LDD as an alternative explanation for the entire amphi-Pacific distribution. However if LDD would indeed be so prevalent, it would be difficult to explain the absence of *L.* subg. *Gerardii* species in Europe, western North America and, as far as we know, in South America and Africa. A possible explanation for the lack of European species could be the mass extinctions during the Quaternary glaciations, and the absence in western North America might be related to the uplift of the Rocky Mountains during the Tertiary (Cox & Moore 2006). The African continent was isolated during most of the Paleogene, until approximately 19 million years ago, when it collided with Europe (Cox & Moore 2006), but a more recent barrier for colonization might have been the desertification of the Sahara and the Arabian Peninsula during the Pleistocene (Burgoyne *et al.* 2005; Cox & Moore 2006). The possibility remains that related African species have simply not yet been discovered, given the relatively low exploration rate of particular biotopes on this continent. We have no knowledge of any South American collections with a putative affinity with *L. gerardii*. The one Costa Rican specimen included in the analyses, comes from high elevation *Quercus* forest and is no reference for the potential presence of *L.* subg. *Gerardii* in lowland tropical rainforests of South America. Still, a lower abundance of *Russulaceae* has been observed on this continent (Buyck and Ovrebo 2002; Verbeken 2001b).

Phylogeographic studies often try to find out whether or not the phylogeny is regionally structured (*e.g.* Old World versus New World clades, northern versus southern hemisphere) and whether or not species with a disjunct or intercontinental distribution truly exist. For *L.* subg. *Gerardii* we can say that it contains little geographical structure since many lineages have spread across two or three continents. The close relationship between eastern North

American and Australasian species is evident but species with an intercontinental distribution have not been found. If we look at other phylogeographic studies involving ectomycorrhizal fungi, we find that close, intercontinental relationships are not uncommon, while disjunct populations of the same species are rather rare. Our results are comparable with those of Zhang *et al.* (2004) who demonstrated that Asian species of *Amanita* are often closely related to North American species, but they cannot not be confirmed as species with disjunct distributions. In the *Amanita muscaria* complex, there are several North American and Eurasian clades, but they do not cluster according to their geography (Geml *et al.* 2008). A study of matsutake and its close allies (Chapela & Garbelotto 2004) revealed the existence of a circumboreal species and indicated that eastern North American and Asian species are closer related with one another than eastern with western North American species. Mueller *et al.* (2001) focussed on the relationship between look-a-like species of *Suillus* from China and North America. They concluded that in most cases these species are not as closely related as the morphological similarities might suggest. However, their results also detected several species with an intercontinental distribution. The study of the *Hysterangiales* by Hosaka *et al.* (2008) is on a larger scale, and although it uncovered multiple intercontinental sister relations, the phylogeny is structured in southern hemisphere clades, a northern hemisphere clade and a mixed clade. Matheny *et al.* (2009) performed a large-scale study of the *Inocybaceae* which demonstrates that its phylogeography is mainly the result of ancient vicariance events and subsequent radiations. Despite some dispersal events, most *Inocybaceae* species cluster together with species from the same region ('North temperate', 'South temperate', 'paleotropics' or 'neotropics'). The phylogeography of *L.* subg. *Gerardii* is not inconsistent with the results of the above mentioned studies and if phylogeographical studies in the *Russulales* further progress, the subgenus will certainly be considered a 'mixed clade'. Still,

the multitude of intercontinental sister relationships *L. subg. Gerardii*, in combination with its non-random distribution of on the globe remains intriguing.

Acknowledgements

The first author is supported by a scholarship of the Institute for the Promotion of Innovation through Science and Technology (*IWT-Vlaanderen*). We would like to express our sincere gratitude to all persons and herbaria who graciously provided specimens for this research: Kanad Das (BSD), Dennis Desjardin (SFSU), Lee Su See (FRIM), Roy Watling (E), David P. Lewis, Xiang-Hua Wang (HKAS), Eji Nagasawa (TMI), Roy Halling (NY), Christen Mortensen, Gregory Mueller (CHIC), Josephine Milne (MEL) and Leticia Montoya. We would especially like to thank Huyen Le Than, Genevieve Gates and Patrick Leonard for their contributions. We thank the reviewers for their suggestions to improve the manuscript.

REFERENCES

- Burgoyne PM, van Wyk AE, Anderson JM, Schrire BD, 2005. Phanerozoic evolution of plants on the African plate. *Journal of African Earth Sciences* **43**: 13-52.
- Buyck B, Hofstetter V, Verbeken A, Walley R, 2010. Proposal to conserve *Lactarius* nom. cons. (Basidiomycota) with a conserved type. *Taxon*: in press.
- Buyck B, Ovrebo CL, 2002. New and interesting *Russula* species from Panamá. *Mycologia* **94**: 888-901.

- Buyck B, Hofstetter V, Eberhardt U, Verbeken A, Kauff F, 2008. Walking the thin line between *Russula* and *Lactarius*: the dilemma of *Russula* subsect. *Ochricompactae*. *Fungal Diversity* **28**: 15-40.
- Castresana J, 2000. Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Molecular Biology and Evolution* **17**: 540-552.
- Chapela IH, Garbelotto M, 2004. Phylogeography and evolution in matsutake and close allies inferred by analyses of its sequences and AFLPs. *Mycologia* **96**: 730-741.
- Cox CB, Moore PD, 2006. *Biogeography. An Ecological and Evolutionary Approach*, 7th edn. Blackwell Publishing, Malden, Oxford, Victoria.
- Das K, Sharma JR, 2004. *Lactarius* in Kumaon Himalaya 2: New and interesting species of subgenus *Plinthogali*. *Mycotaxon* **89**: 289-296.
- Das K, Sharma JR, 2005. *Russulaceae of Kumaon Himalaya*. Govt. of India, Botanical Survey of India, Ministry of Environment & Forests, Kolkata
- Eberhardt U, Verbeken A, 2004. Sequestrate *Lactarius* species from tropical Africa: *L. angiocarpus* sp. nov. and *L. dolichocaulis* comb. nov. *Mycological Research* **108**: 1042-1052.
- Elias SA, Short SK, Birks HH, 1997. Late Wisconsin environment of the Bering Land Bridge. *Palaeogeography, Palaeoclimatology, Palaeoecology* **136**: 293-308.
- Fuhrer B, 2005. *A field guide to Australian fungi*. Blooming Books Pty Ltd, Melbourne.
- Graham A, 1993. History of the vegetation: Cretaceous (Maastrichtian)–Tertiary. In: Flora of North America Editorial Committee (eds), *Flora of North America. Vol. 1*. New York, Oxford, pp. 57-70.
- Geml J, Tulloss RE, Laursen GA, Sazanova NA, Taylor DL, 2008. Evidence for strong inter- and intracontinental phylogeographic structure in *Amanita muscaria*, a wind-dispersed ectomycorrhizal basidiomycete. *Molecular Phylogenetics and Evolution* **48**: 694-701.

- Grgurinovic CA, 1997. *Larger Fungi of South Australia*. The Botanic Gardens of Adelaide and State Herbarium and The Flora and Fauna of South Australia Handbooks Committee, Adelaide.
- Hall TA, 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41**: 95-98.
- Heilmann-Clausen J, Verbeken A, Vesterholt J, 1998. *The genus Lactarius. Fungi of Northern Europe, vol. 2*. Svampetryk, Mundelstrup.
- Henkel TW, Aime MC, Miller SL, 2000. Systematics of pleurotoid Russulaceae from Guyana and Japan, with notes on their ectomycorrhizal status. *Mycologia* **92**: 1119-1132.
- Hesler LR, Smith AH, 1979. *North American species of Lactarius*. Univ. Michigan Press, Ann Arbor.
- Hewitt G, 2000. The genetic legacy of the Quaternary ice ages. *Nature* **405**: 907-913.
- Hibbett DS, 2001. Shiitake mushrooms and molecular clocks: historical biogeography of *Lentinula*. *Journal of Biogeography* **28**: 231-241.
- Hosaka K, Castellano MA, Spatafora JW, 2008. Biogeography of *Hysterangiales* (*Phallomycetidae*, *Basidiomycota*). *Mycological Research* **112**: 448-462.
- Imazeki R, Otari Y, Hongo T, 1988. *Nihon-no-Kinoko. Fungi of Japan*. Yama-kei Publishers Co., Ltd., Tokyo .
- Katoh K, Toh H, 2008. Recent developments in the MAFFT multiple sequence alignment program. *Briefings in bioinformatics* **9**: 286-298.
- Kauff F, Lutzoni M, 2002. Phylogeny of the Gyalectales and Ostropales (Ascomycota, Fungi): among and within order relationships based on nuclear ribosomal RNA small and large subunits. *Molecular Phylogenetics and Evolution* **25**: 138-156.
- Kohn LM, 2005. Mechanisms of fungal speciation. *Annual Review of Phytopathology* **43**: 12.1-12.30.

- Lalli G, Pacioni G, 1992. *Lactarius* sect. *Lactifluus* and allied species. *Mycotaxon* **44**: 155-195.
- Le HT, Stubbe D, Verbeken A, Nuytinck J, Lumyong S, Desjardin DE, 2007. *Lactarius* in Northern Thailand: 2. *Lactarius* subgenus *Plinthogali*. *Fungal Diversity* **27**: 61-94.
- Lee SS, Watling R, Sikin YN, 2002. Ectomycorrhizal basidiomata fruiting in lowland rain forests of Peninsular Malaysia. *Bois et Forêts des Tropiques* **274**: 33-43.
- Liu YJ, Wehlen S, Hall BD, 1999. Phylogenetic relationships among ascomycetes: evidence from an RNA polymerase II subunit. *Molecular Biology and Evolution* **16**:1799-1808.
- Matheny PB, 2005. Improving phylogenetic inference of mushrooms with RPB1 and RPB2 nucleotide sequences (*Inocybe*; Agaricales). *Molecular Phylogenetics and Evolution* **35**:1-20.
- Matheny PB, Aime MC, Bougher NL, Buyck B, Desjardin DE, Horak E, Kropp BR, Lodge DJ, Soyong K, Trappe JM, Hibbett DS, 2009. Out of the Palaeotropics. Historical biogeography and diversification of the cosmopolitan ectomycorrhizal mushroom family Inocybaceae. *Journal of Biogeography* **36**: 577-592.
- McNabb RFR, 1971. The Russulaceae of New Zealand. 1. *Lactarius* DC ex S.F. Gray. *New Zealand Journal of Botany* **9**: 46-66.
- Miller SL, Aime MC, Henkel TW, 2002. *Russulaceae* of the Pakaraima Mountains of Guyana. I. New species of pleurotoid *Lactarius*. *Mycologia* **94**: 545-553.
- Montoya L, Band-Muñoz VM, Guzmán G, 1996. New and interesting species of *Lactarius* from Mexico including scanning electron microscope observations. *Mycotaxon* **57**: 412-424.
- Mueller GM, Wu QW, Huang YQ, Guo SY, Aldana-Gomez R, Vilgalys R, 2001. Assessing biogeographic relationships between North American and Chinese macrofungi. *Journal of Biogeography* **28**: 271-281.

- Nuytinck J, Verbeken A, Miller S, 2007. Worldwide phylogeny of *Lactarius* section *Deliciosi* inferred from ITS and glyceraldehyde-3-phosphate dehydrogenase gene sequences. *Mycologia* **99**: 820-832.
- Nuytinck J, Verbeken A, Delarue S, Walley R, 2003. Systematics of European sequestrate lactarioid Russulaceae with spiny spore ornamentation. *Belgian Journal of Botany* **136**:145-153.
- Redhead SA, Norvell LL, 1993. Notes on *Bondarzewia*, *Heterobasidion* and *Pleurogala*. *Mycotaxon* **48**: 371-380.
- Ronquist F, Huelsenbeck JP, 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**: 1572-1574.
- Smith AH, Hesler LR, 1962. Studies on *Lactarius* - III. The North American species of section *Plinthogali*. *Brittonia* **14**: 369-440.
- Stamatakis A, 2006a. Phylogenetic models of rate heterogeneity: a high performance computing perspective. In: *Proceedings of IPDPS 2006*. Rhodos, p. 8.
- Stamatakis A, 2006b. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* **22**: 2688-2690.
- Stamatakis A, Hoover P, Rougemont J, 2008. A rapid bootstrap algorithm for the RAxML Web Servers. *Systematic Biology* **57**: 758-771.
- Stubbe D, Verbeken A, Watling R, 2007. Blue-staining species of *Lactarius* subgenus *Plinthogali* in Malaysia. *Belgian Journal of Botany* **140**: 197-212.
- Stubbe D, Nuytinck J, Verbeken A, 2008. *Lactarius* subgenus *Plinthogalus* of Malaysia. *Fungal Diversity* **32**: 125-156.
- Vellinga EC, 1988. Glossary. In: Bas C, Kuyper TW, Noordeloos ME and Vellinga EC (eds) *Flora Agaricina Neerlandica. Vol. 1*. AA Balkema, Rotterdam, pp. 54-64.

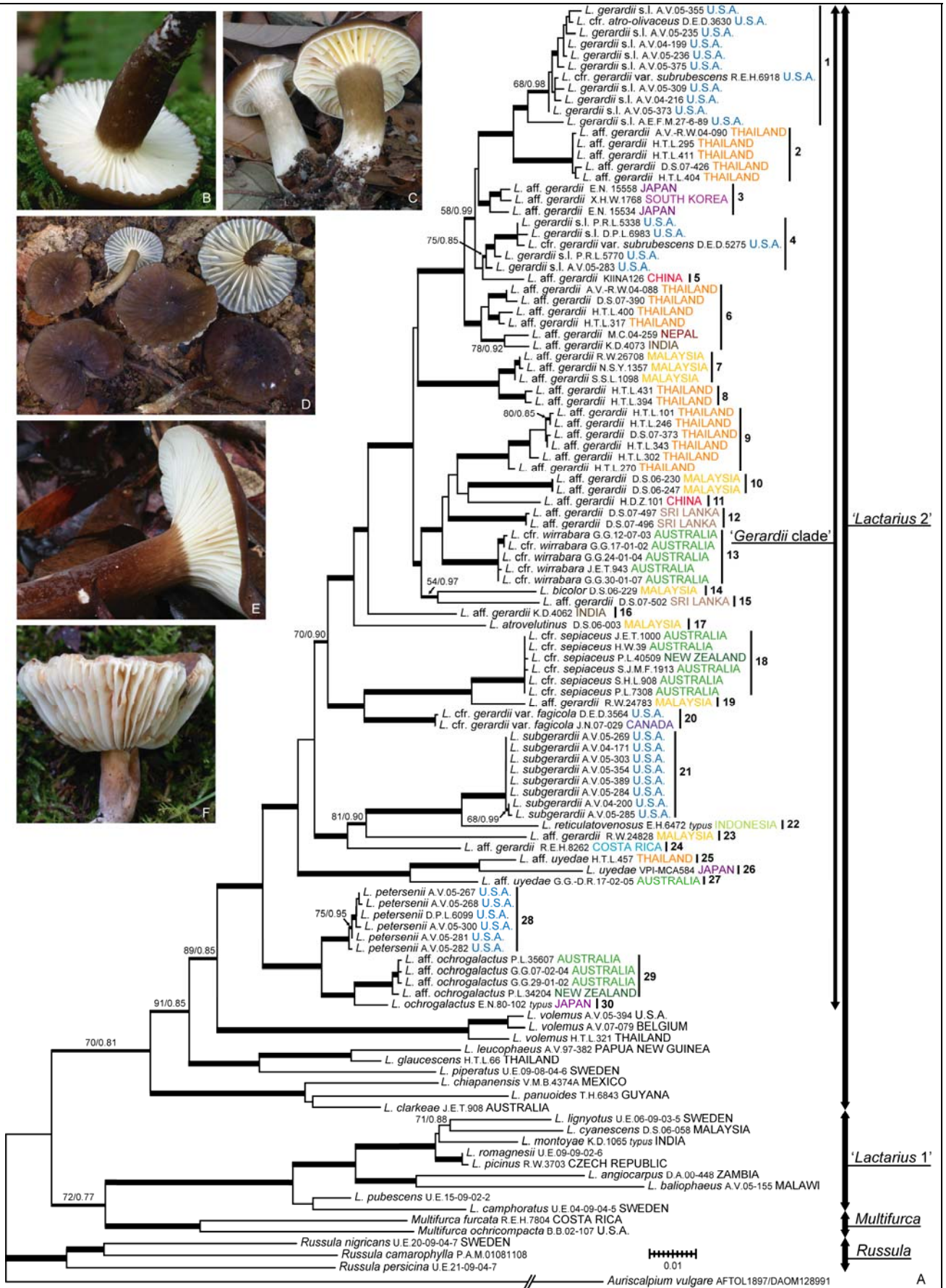
- Verbeken A, 1998a. Studies in tropical African *Lactarius* species. 5. A synopsis of the subgenus *Lactifluus* (Burl.) Hesler and A.H. Sm. Emend. *Mycotaxon* **66**: 363-386.
- Verbeken A, 1998b. Studies in tropical African *Lactarius* species. 6. A synopsis of the subgenus *Lactariopsis* (Henn.) R. Heim emend. *Mycologia* **66**: 387-418.
- Verbeken A, 2001a. Studies in tropical African *Lactarius* species. 10. Infrageneric classification. *Mycotaxon* **77**: 435-444.
- Verbeken A, 2001b. Worldwide systematics of *Lactarius*: a state of the art. *Micologia e Vegetazione Mediterranea* **16**: 71-88.
- Verbeken A, Horak E, 1999a. *Lactarius* (Basidiomycota) in Papua New Guinea. 1. Species of tropical lowland habitats. *Australian Systematic Botany* **12**: 767-779.
- Verbeken A, Horak E, 1999b. *Lactarius* (Basidiomycota) in Papua New Guinea. 2. Species in tropical-montane rainforests. *Australian Systematic Botany* **13**: 649-707.
- Verbeken A, Horak E, 2002 ['2001']. *Agaricales* of Indonesia. 3. New records of the genus *Lactarius* (Basidiomycota, Russulales) from Java. *Sydowia* **53**: 261-289.
- Wang XH, 2007. Type studies of *Lactarius* species published from China. *Mycologia* **99**: 253-268.
- Wang XH, Hashiya M, Verbeken A, 2006. *Lactarius ochrogalactus*, a new species of the genus *Lactarius* (Russulaceae, Russulales) with yellowish-brown latex. *Mycoscience* **47**: 232-234.
- Wang XH, Liu P, Yu F, 2004. *Color atlas of wild commercial mushrooms in Yunnan*. Yunnan Science and Technology Press, Kunming
- Wang XH, 2008. The genus *Lactarius* in southwestern China: taxonomy, ontogeny and floristic biogeography. Unpubl. Ph.D. dissertation, Kunming.
- White TJ, Bruns T, Lee SS, Taylor J, 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ

(eds), *PCR protocols: a guide to methods and applications*. Academic Press, New York, pp. 315-322.

Wu Q, Mueller GM, 1997. Biogeographic relationships between the macrofungi of temperate eastern Asia and eastern North America. *Canadian Journal of Botany* **75**: 2108-2116.

Wu QX, Mueller GM, Lutzoni FM, Huang YQ, Guo SY, 2000. Phylogenetic and biogeographic relationships of eastern Asian and eastern North American disjunct *Suillus* species (Fungi) as inferred from nuclear ribosomal RNA ITS sequences. *Molecular Phylogenetics and Evolution* **17**: 37-47.

Zhang LF, Yang JB, Yang, ZL, 2004. Molecular phylogeny of eastern Asian species of *Amanita* (Agaricales, Basidiomycota): taxonomic and biogeographic implications. *Fungal Diversity* **17**: 219-238.



A

Fig 1 – (A) The best ML tree based on the combined analysis of ITS, LSU and *rpb2* sequences, resulting from a 500 replicates Rapid Bootstrapping algorithm and a ML tree search in RAxML. Thickest branches are supported with min. 70 % BS support and 0.95 posterior probability (PP). Branches supported by either the ML or the BI analysis are semi-thickened and support values are given. Numbered clades are discussed in the text. (B) *Lactarius gerardii* s.l. from the U.S.A., clade 1, A. Verbeken 05-309, (C) *L. atrovelutinus* from Malaysia, with pinkening latex, clade 17, D. Stubbe 06-003 (photograph: R. Walley), (D) *L. bicolor* from Malaysia with distinctly coloured lamella edges, clade 14, D. Stubbe 06-229, (E) *L. aff. gerardii* from Sri Lanka with dense lamellae, clade 15, D. Stubbe 07-502, (F) *L. petersenii* from the U.S.A. with brown latex, clade 28, A. Verbeken 05-268.

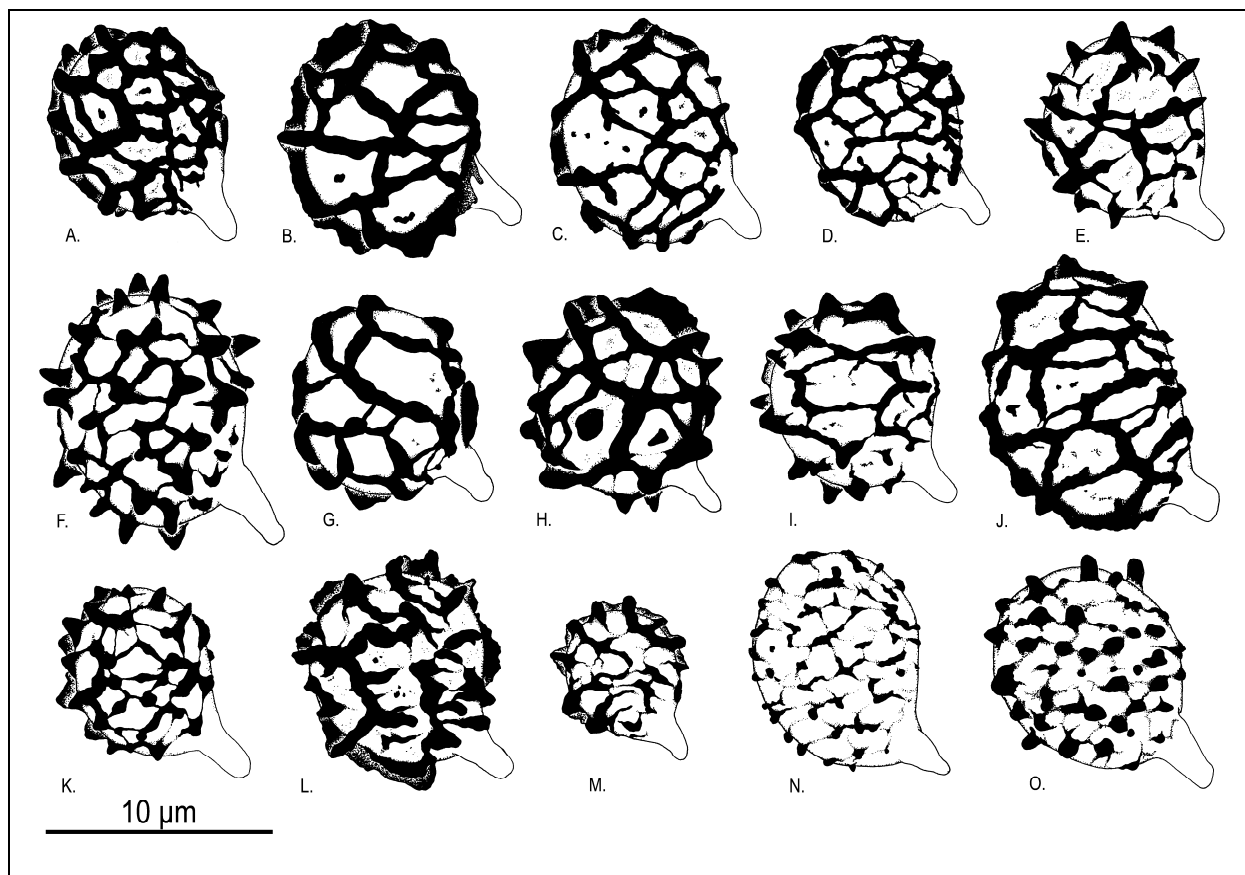


Fig 2 – Examples of the different spore morphologies: (A) *Lactarius gerardii* s.l., U.S.A., clade1, A. Verbeken 04-199, (B) *L. aff. gerardii*, Thailand, clade 2, D. Stubbe 07-426, (C) *L. aff. gerardii*, South Korea, clade 3, X.H. Wang 1768, (D) *L. aff. gerardii*, Thailand, clade 8, H.T. Le 394, (E) *L. cfr. wirrabara*, Australia, clade 13, J.E. Tonkin 943, (F) *L. aff. gerardii*, Sri Lanka, clade 12, D. Stubbe 07-496, (G) *L. aff. gerardii*, Malaysia, clade 10, D. Stubbe 06-230, (H) *L. aff. gerardii*, Sri Lanka, clade 15, D. Stubbe 07-502, (I) *L. cfr. sepiaceus*, Australia, clade 18, S.J. McMullan-Fisher 1913, (J) *L. cfr. gerardii* var. *fagicola*, Canada, clade 20, J. Nuytinck 07-029, (K) *L. subgerardii*, U.S.A., clade 21, A. Verbeken 04-200, (L) *L. reticulatovenosus*, Indonesia, clade 22, *typus*, (M) *L. aff. uyedae*, Thailand, clade 25, H.T. Le 457, (N) *L. petersenii*, U.S.A., clade 28, A. Verbeken 05-300, (O) *L. aff. ochrogalactus*, Australia, clade 29, P. Leonard 35607.

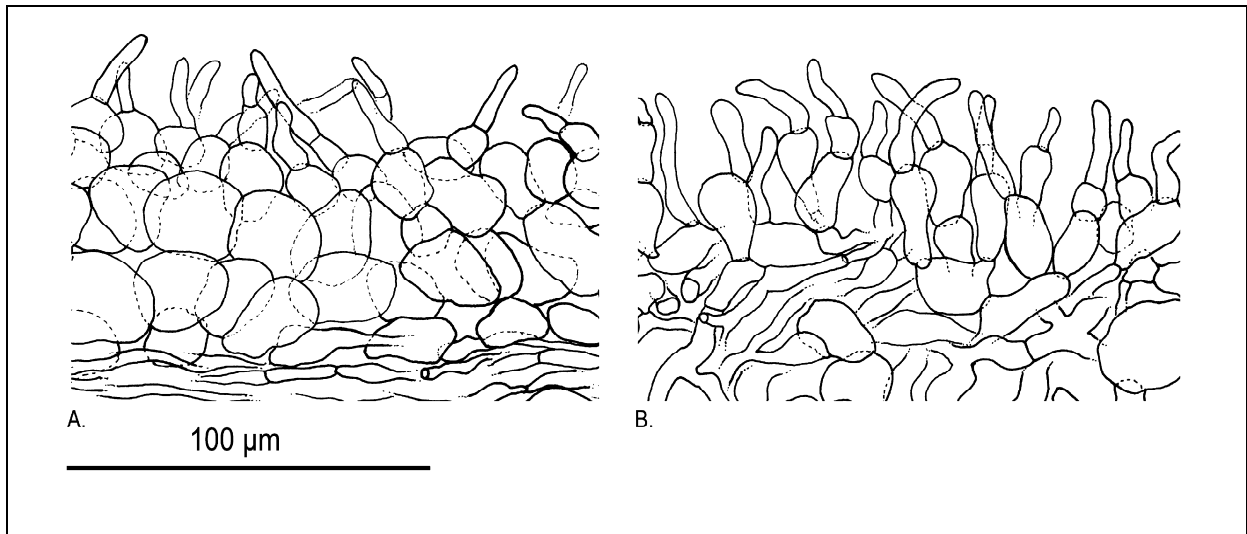


Fig 3 – Comparison of the typical pileipellis found in *Lactarius* subg. *Gerardii* and the one found in *L.* subg. *Plinthogalus*: (A) *L.* aff. *gerardii*, Thailand, *H.T. Le* 295, (B) *L. picinus* Fr., Czech Republic, *R. Walley*n 3703.

Table 1 – Specimens and GenBank accession numbers of DNA sequences used in the molecular analyses. Accession numbers of newly produced sequences are indicated in bold.

Species	Original identification	Voucher	Location	ITS GenBank accession no.	LSU GenBank accession no.	<i>rpb2</i> GenBank accession no.
Lactarius						
<i>L. atrovelutinus</i>	<i>L. aff. gerardii</i>	<i>D.Stubbe</i> 06-003 (GENT)	Selangor, Malaysia	GU258231	GU265588	GU258325
<i>L. bicolor</i>	<i>L. aff. gerardii</i>	<i>D.Stubbe</i> 06-229 (GENT)	Negeri Sembilan, Malaysia	GU258221	GU265577	GU258313
<i>L. chiapanensis</i>	<i>L. chiapanensis</i>	<i>V.M.Bandala</i> 4374A (GENT)	Chiapas, Mexico	GU258297	GU265580	GU258316
<i>L. aff. gerardii</i>	<i>L. aff. gerardii</i>	<i>D.Stubbe</i> 06-230 (GENT)	Negeri Sembilan, Malaysia	GU258222	GU265578	GU258314
<i>L. aff. gerardii</i>	<i>L. aff. gerardii</i>	<i>D.Stubbe</i> 06-247 (GENT)	Negeri Sembilan, Malaysia	GU258223	GU265579	GU258315
<i>L. aff. gerardii</i>	<i>L. subrubescens</i>	<i>R.Watling</i> 24828 (E)	Negeri Sembilan, Malaysia	GU258275	GU265636	GU258374
<i>L. aff. gerardii</i>	<i>L. gerardii</i>	<i>R.Watling</i> 24783 (E)	Negeri Sembilan, Malaysia	-	GU265637	GU258375
<i>L. aff. gerardii</i>	<i>L. sect. Plinthogali</i>	<i>R.Watling</i> 26708 (E)	Negeri Sembilan, Malaysia	-	GU265590	GU258327
<i>L. aff. gerardii</i>	<i>Lactarius</i> sp.	<i>S.N.Yahya</i> , FRIM1357 (FRIM)	Selangor, Malaysia	GU258233	GU265591	GU258328
<i>L. aff. gerardii</i>	<i>Lactarius</i> sp.	<i>S.S.Lee</i> , FRIM1098 (FRIM)	Selangor, Malaysia	GU258232	GU265589	GU258326
<i>L. aff. gerardii</i>	<i>L. gerardii</i>	<i>E.Nagasawa</i> , TMI15558 (TMI)	Aomori, Japan	GU258230	GU265587	GU258324
<i>L. aff. gerardii</i>	<i>L. gerardii</i>	<i>E.Nagasawa</i> , TMI15534 (TMI)	Tottori, Japan	GU258229	GU265586	GU258323
<i>L. aff. gerardii</i>	<i>L. gerardii</i>	<i>X.H.Wang</i> 1768 (HKAS)	South Korea	GU258225	GU265583	GU258319
<i>L. aff. gerardii</i>	<i>Lactarius</i> sp.	<i>KIINA</i> 126 (GENT)	Hunan, China	GU258227	GU265584	GU258321
<i>L. aff. gerardii</i>	<i>L. gerardii</i>	<i>H.D.Zheng</i> 101 (HKAS)	Yunnan, China	GU258226	-	GU258320
<i>L. aff. gerardii</i>	<i>L. aff. gerardii</i>	<i>H.T.Le</i> 295 (GENT)	Chiang Mai, Thailand	EF560687	GU265600	GU258337
<i>L. aff. gerardii</i>	<i>L. aff. gerardii</i>	<i>D.Stubbe/R.Walleyn</i> 07-426 (GENT)	Chiang Mai, Thailand	GU258241	GU265602	GU258339
<i>L. aff. gerardii</i>	<i>L. aff. gerardii</i>	<i>A.Verbeke/R.Walleyn</i> 04-090 (GENT)	Chiang Mai, Thailand	GU258244	GU265605	GU258342
<i>L. aff. gerardii</i>	<i>L. aff. gerardii</i>	<i>H.T.Le</i> 404 (GENT)	Chiang Mai, Thailand	GU258245	GU265606	GU258343
<i>L. aff. gerardii</i>	<i>L. aff. gerardii</i>	<i>H.T.Le</i> 411 (GENT)	Chiang Mai, Thailand	GU258250	GU265611	GU258348
<i>L. aff. gerardii</i>	<i>L. aff. gerardii</i>	<i>A.Verbeke/R.Walleyn</i> 04-088 (GENT)	Chiang Mai, Thailand	GU258239	GU265597	GU258334
<i>L. aff. gerardii</i>	<i>L. aff. gerardii</i>	<i>D.Stubbe</i> 07-390 (GENT)	Chiang Mai, Thailand	GU258252	GU265613	GU258350
<i>L. aff. gerardii</i>	<i>L. aff. gerardii</i>	<i>H.T.Le</i> 317 (GENT)	Chiang Mai, Thailand	GU258243	GU265604	GU258341
<i>L. aff. gerardii</i>	<i>L. aff. gerardii</i>	<i>H.T.Le</i> 400 (GENT)	Chiang Mai, Thailand	GU258246	GU265607	GU258344
<i>L. aff. gerardii</i>	<i>L. aff. gerardii</i>	<i>H.T.Le</i> 394 (GENT)	Chiang Mai, Thailand	GU258249	GU265610	GU258347
<i>L. aff. gerardii</i>	<i>L. aff. gerardii</i>	<i>H.T.Le</i> 431 (GENT)	Chiang Mai, Thailand	GU258251	GU265612	GU258349
<i>L. aff. gerardii</i>	<i>L. aff. gerardii</i>	<i>H.T.Le</i> 270 (GENT)	Chiang Rai, Thailand	EF560685	GU265598	GU258335
<i>L. aff. gerardii</i>	<i>L. aff. gerardii</i>	<i>H.T.Le</i> 343 (GENT)	Chiang Mai, Thailand	GU258240	GU265599	GU258336
<i>L. aff. gerardii</i>	<i>L. aff. gerardii</i>	<i>H.T.Le</i> 246 (GENT)	Chiang Mai, Thailand	EF560686	GU265601	GU258338
<i>L. aff. gerardii</i>	<i>L. aff. gerardii</i>	<i>D.Stubbe/R.Walleyn</i> 07-373 (GENT)	Chiang Mai, Thailand	GU258242	GU265603	GU258340
<i>L. aff. gerardii</i>	<i>L. aff. gerardii</i>	<i>H.T.Le</i> 101 (GENT)	Chiang Mai, Thailand	GU258247	GU265608	GU258345
<i>L. aff. gerardii</i>	<i>L. aff. gerardii</i>	<i>H.T.Le</i> 302 (GENT)	Chiang Mai, Thailand	GU258248	GU265609	GU258346
<i>L. aff. gerardii</i>	<i>L. gerardii</i>	<i>M.Christensen</i> 04-259 (GENT)	Nepal	GU258234	GU265592	GU258329
<i>L. aff. gerardii</i>	<i>L. aff. gerardii</i>	<i>D.Stubbe</i> 07-496 (GENT)	Sri Lanka	GU258236	GU265594	GU258331
<i>L. aff. gerardii</i>	<i>L. aff. gerardii</i>	<i>D.Stubbe</i> 07-497 (GENT)	Sri Lanka	GU258237	GU265595	GU258332
<i>L. aff. gerardii</i>	<i>L. aff. gerardii</i>	<i>D.Stubbe</i> 07-502 (GENT)	Sri Lanka	GU258238	GU265596	GU258333
<i>L. aff. gerardii</i>	<i>L. gerardii</i> var. <i>subrubescens</i>	<i>K.Das</i> 4073 (BSD)	Uttaranchal, India	GU258274	-	GU258373(incompl.)
<i>L. aff. gerardii</i>	<i>L. gerardii</i> var. <i>subrubescens</i>	<i>K.Das</i> 4062 (BSD)	Uttaranchal, India	GU258228	GU265585	GU258322
<i>L. gerardii</i> s.l.	<i>L. gerardii</i>	<i>A.Verbeke</i> 05-355 (GENT)	North Carolina, U.S.A.	GU258253	GU265615	GU258352
<i>L. gerardii</i> s.l.	<i>L. gerardii</i>	<i>A.E.Franco-Molano</i> 27-6-89 (NY)	New York, U.S.A.	-	-	GU258377
<i>L. gerardii</i> s.l.	<i>L. gerardii</i>	<i>A.Verbeke</i> 05-283 (GENT)	Tennessee, U.S.A.	GU258259	GU265621	GU258358
<i>L. gerardii</i> s.l.	<i>L. gerardii</i>	<i>P.R.Leacock</i> 5338 (F)	Indiana, U.S.A.	GU258261	GU265623	GU258360
<i>L. ochrogalactus</i>	<i>L. ochrogalactus</i>	<i>E.Nagasawa</i> 80-102 = <i>typus</i> (TMI)	Tottori, Japan	GU258280	-	GU258381
<i>L. aff. ochrogalactus</i>	<i>L. wirrabara</i>	<i>G.Gates</i> 29-01-02	Tasmania, Australia	GU258304	GU265664	GU258404

<i>L. aff. ochrogalactus</i>	<i>Lactarius</i> sp.	<i>P.Leonard</i> 35607	Queensland, Australia	GU258295	GU265658	GU258398
<i>L. aff. ochrogalactus</i>	<i>L. sepiaceus</i>	<i>P.Leonard</i> 34204	New Zealand	GU258288	GU265651	GU258391
<i>L. aff. ochrogalactus</i>	<i>L. wirrabara</i>	<i>G.Gates</i> 07-02-04	Tasmania, Australia	GU258308	GU265668	GU258408
<i>L. panuoides</i>		<i>T. Henkel</i> 6843 (DUKE)	Guyana	-	AF218566	-
<i>L. petersenii</i>	<i>L. cfr. petersenii</i>	<i>A. Verbeken</i> 05-267 (GENT)	North Carolina, U.S.A.	GU258282	GU265643	GU258383
<i>L. petersenii</i>	<i>L. petersenii</i>	<i>D.P.Lewis</i> 6099	Texas, U.S.A.	-	GU265647	GU258387
<i>L. reticulatovenosus</i>	<i>L. reticulatovenosus</i>	<i>E.Horak</i> 6472 = <i>typus</i> (GENT)	Java, Indonesia	GU258286	GU265649	GU258389
<i>L. cfr. sepiaceus</i>	<i>L. sepiaceus</i>	<i>H.Weatherhead</i> 39 (MEL)	Victoria, Australia	GU258289	GU265652	GU258392
<i>L. cfr. sepiaceus</i>	<i>L. sepiaceus</i> ?'	<i>S.H.Lewis</i> 908 (MEL)	New South Wales, Australia	GU258290	GU265653	GU258393
<i>L. cfr. sepiaceus</i>	<i>L. sepiaceus</i>	<i>J.E.Tonkin</i> 1000 (MEL)	Victoria, Australia	GU258292	GU265655	GU258395
<i>L. cfr. sepiaceus</i>	<i>L. sepiaceus</i>	<i>S.J.McMullan-Fisher</i> 1913 (MEL)	Tasmania, Australia	GU258293	GU265656	GU258396
<i>L. cfr. sepiaceus</i>	<i>L. wirrabara</i>	<i>P.Leonard</i> 7308	Queensland, Australia	GU258309	GU265669	GU258409
<i>L. cfr. sepiaceus</i>	<i>L. sepiaceus</i>	<i>P.Leonard</i> 40509	South Island, New Zealand	GU258287	GU265650	GU258390
<i>L. subgerardii</i>	<i>L. gerardii</i>	<i>A. Verbeken</i> 05-269 (GENT)	North Carolina, U.S.A.	GU258263	GU265625	GU258362
<i>L. subgerardii</i>	<i>L. gerardii</i>	<i>A. Verbeken</i> 04-200 (GENT)	Tennessee, U.S.A.	GU258266	GU265628	GU258365
<i>L. uyedae</i>		<i>MCA</i> 584 (VPI)	Japan	-	AF218562	-
<i>L. aff. uyedae</i>	<i>Lactarius</i> sp.	<i>G.Gates/D.Ratkowsky</i> 17-02-05	Tasmania, Australia	GU258294	GU265657	GU258397
<i>L. aff. uyedae</i>	<i>L. cfr. uyedae</i>	<i>H.T.Le</i> 457 (GENT)	Chiang Mai, Thailand	GU258296	GU265659	GU258399
<i>L. cfr. wirrabara</i>	<i>L. sepiaceus</i>	<i>J.E.Tonkin</i> 943 (MEL)	Victoria, Australia	GU258291	GU265654	GU258394
<i>L. cfr. wirrabara</i>	<i>L. wirrabara</i>	<i>G.Gates/D.Ratkowsky</i> 17-01-2002	Tasmania, Australia	GU258305	GU265665	GU258405
<i>L. cfr. wirrabara</i>	<i>L. wirrabara</i>	<i>G.Gates/D.Ratkowsky</i> 12-07-2003	Tasmania, Australia	GU258306	GU265666	GU258406
<i>L. cfr. wirrabara</i>	<i>L. wirrabara</i>	<i>G.Gates/D.Ratkowsky</i> 24-01-2004	Tasmania, Australia	GU258307	GU265667	GU258407
<i>L. cfr. wirrabara</i>	<i>L. wirrabara</i>	<i>G.Gates</i> 30-01-2007	Tasmania, Australia	GU258303	GU265663	GU258403
<i>L. cfr. wirrabara</i>	<i>L. wirrabara</i>	<i>P. Leonard</i> 10409	Queensland, Australia	XXX	XXX	XXX
<i>L. cfr. wirrabara</i>	<i>L. subg. Plinthogali</i>	<i>R.E.Halling</i> 6800	Queensland, Australia	XXX	XXX	XXX
<i>L. baliophaeus</i>		<i>A. Verbeken</i> 05-155 (GENT)	Malawi	GU258277	GU265576	GU258312
<i>L. camphoratus</i>		<i>UE</i> 04.09.2004-5 (UPS)	Sweden		DQ422009	DQ421933
<i>L. clarkeae</i>		<i>JET</i> 980 (MEL)	Australia		EU019924	-
<i>L. glaucescens</i>		<i>H.T.Le</i> 66 (GENT)	Thailand	GU258298	GU265639	GU258378
<i>L. leucophaeus</i>		<i>A. Verbeken</i> 97-382 (GENT)	Papua New Guinea	GU258299	GU265640	GU258379
<i>L. lignyotus</i>		<i>U.Eberhardt</i> 06.09.2003-5 (UPS)	Sweden	DQ421993	DQ421993	DQ421926
<i>L. montoyae</i>		<i>K. Das</i> 1065 = <i>typus</i> (BSD)	India	EF560673	GU265641	GU258380
<i>L. picinus</i>		<i>R.Walley</i> 3703 (GENT)	Czech Republic	GU258279	GU265648	GU258388
<i>L. piperatus</i>		<i>U.Eberhardt</i> 09.08.2004-6 (UPS)	Sweden		DQ422035	DQ421937
<i>L. pubescens</i>		<i>U.Eberhardt</i> 15.09.2002-2 (UPS)			DQ421996	DQ421929
<i>L. volemus</i>		<i>A. Verbeken</i> 05-394 (GENT)	U.S.A	GU258300	GU265660	GU258400
<i>L. volemus</i>		<i>A. Verbeken</i> 07-079 (GENT)	Belgium	GU258301	GU265661	GU258401
<i>L. volemus</i>		<i>H.T.Le</i> 321 (GENT)	Thailand	GU258302	GU265662	GU258402
<i>L. deceptivus</i>		<i>A. Verbeken</i> 04-181 (GENT)	U.S.A.		DQ422020	DQ421935
<i>L. edulis</i>		<i>A. Verbeken</i> 99-041 (GENT)	Zimbabwe	AY606973	DQ421977	DQ421916
<i>L. emergens</i>		<i>A. Verbeken</i> 99-005 (GENT)	Zimbabwe	AY606979	-	DQ421919
<i>L. longisporus</i>		<i>A. Verbeken</i> 99-197 (GENT) /	Zimbabwe		DQ421971 (AV)	DQ421910 (BB)
		<i>B.Buyck</i> 00-1519 (PC)	Madagascar			
<i>L.madagascariensis</i>		<i>B.Buyck</i> 99-409 (PC)	Madagascar	AY606977	DQ421975	DQ421914
<i>L. pelliculatus</i>		<i>B.Buyck</i> 00-1335 (PC)	Madagascar	AY606978	DQ421974	DQ421913
<i>L. phlebophyllus</i>		<i>B.Buyck</i> 00-1388 (PC)	Madagascar	AY606974	DQ421979	DQ421918
<i>L. rubroviolascens</i>		<i>B.Buyck</i> 97-266 (PC)	Madagascar	-	DQ421972	DQ421911
<i>L. vellereus</i>		<i>U.Eberhardt</i> 20.09.2004-22 (UPS)	Sweden		DQ422034	DQ421936
<i>L. velutissimus</i>		<i>A. Verbeken</i> 99-185 (GENT)	Zimbabwe	AY606982	DQ421973	DQ421912
<i>L. volemus</i>		<i>U.Eberhardt</i> 09.08.2002-2 (UPS)	Sweden		DQ422008	DQ421932
Multifurca						
<i>M. furcata</i>		<i>RH7804</i> (NY)	Costa Rica		DQ421994	DQ421927
<i>M. ochricompacta</i>		<i>BB02.107</i> (PC)	U.S.A.		DQ421984	DQ421940
Russula						
<i>R. camarophylla</i>		<i>PAM</i> 01081108 (PC)			DQ421982	DQ421938
<i>R. nigricans</i>		<i>UE</i> 20.09.2004-07 (UPS)	Sweden		DQ422010	DQ421952

<i>R. persicina</i>	UE 21.09.2003 (UPS)	DQ422019	DQ421960	
<i>Auriscalpium</i>				
<i>A. vulgare</i>	AFTOL1897/DAOM128994	DQ911613	DQ911314	AY218472
