

Phylogenetic and microscopic studies in the genus *Lactifluus* (Basidiomycota, Russulales) in West Africa, including the description of four new species

Dao Lamèga Maba^{1,2}, Atsu K. Guelly¹, Nourou S. Yorou^{2,3}, Annemieke Verbeken⁴, and Reinhard Agerer²

¹Département de Botanique et Écologie Végétale, Faculté des Sciences, Université de Lomé, BP 1515, Lomé, Togo; corresponding author e-mail: mlamega@yahoo.com

²Department Biology I, Organismic Biology: Mycology, Ludwig-Maximilians-Universität München, Menzinger Straße 67, 80638 München, Germany

³Faculty of Agronomy, University of Parakou, BP 123, Parakou, Benin

⁴Research Group Mycology, Department of Biology, Ghent University, K.L. Ledeganckstraat 35 B - 9000 Ghent, Belgium

Abstract: Despite the crucial ecological role of lactarioid taxa (*Lactifluus*, *Lactarius*) as common ectomycorrhiza formers in tropical African seasonal forests, their current diversity is not yet adequately assessed. During the last few years, numerous lactarioid specimens have been sampled in various ecosystems from Togo (West Africa). We generated 48 ITS sequences and aligned them against lactarioid taxa from other tropical African ecozones (Guineo-Congolese evergreen forests, Zambezi miombo). A Maximum Likelihood phylogenetic tree was inferred from a dataset of 109 sequences. The phylogenetic placement of the specimens, combined with morpho-anatomical data, supported the description of four new species from Togo within the monophyletic genus *Lactifluus*: within subgen. *Lactifluus* (*L. flavellus*), subgen. *Russulopsis* (*L. longibasidius* and *L. pectinatus*), and subgen. *Edules* (*L. melleus*). This demonstrates that the current species richness of the genus is considerably higher than hitherto estimated for African species and, in addition, a need to redefine the subgenera and sections within it.

Key words:

Cryptic species
Distribution
Lactifluus
Lactarius
Molecular phylogeny
Taxonomy

Article info: Submitted: 22 September 2014; Accepted: 9 March 2015; Published: 24 March 2015.

INTRODUCTION

Although more attention is now being paid to tropical fungi, the species richness of tropical macrofungi remains unclear. The tropical African mycobiota (including the West African) remains under-collected (Rivière *et al.* 2007, Maba *et al.* 2013, 2014). Continued scientific collecting is therefore essential to raise our knowledge of tropical macrofungi, not least in poorly collected African ecosystems.

Russulaceae are among the commonest ectomycorrhizal macrofungi in West African forest ecosystems (Verbeken & Buyck 2001, van Rooij *et al.* 2003, Rivière *et al.* 2007, Verbeken & Walley 2010, Bâ *et al.* 2012, Maba *et al.* 2013, 2014, Sanon *et al.* 2014). Recent progress in molecular investigations within *Russulaceae* has not only led to the separation of the monophyletic genera *Lactifluus* and *Lactarius*, but also indicated the necessity of including West African taxa for any meaningful assessment of the diversity within this group (Verbeken *et al.* 2011). Still, recent mycological investigations using West African specimens have yielded new *Lactifluus* and *Lactarius* species (van Rooij *et al.* 2003, van de Putte *et al.* 2009, Maba *et al.* 2013, 2014). Similarly, previous studies have supported the high species diversity within *Lactifluus* in the region, compared to *Lactarius*, and indicated that many previously undescribed

species might be expected in the region (Buyck *et al.* 2008, Stubbe *et al.* 2010, Verbeken *et al.* 2011, Maba *et al.* 2013, 2014).

Here, we combine morpho-anatomical studies and molecular phylogenetic analyses of rDNA ITS sequences from recent collections from Togo, and describe four new species within *Lactifluus*.

MATERIAL AND METHODS

Specimens sampling

Specimens were collected from 2011 to 2013 in caesalpinoid- and phyllantoid-dominated woodlands of the Fazao-Malfakassa National Park and in the western forest area of Togo. Sampling methods and morphological data recording, as well as specimen handling and conservation, were as described in Maba *et al.* (2013, 2014). The colour data were recorded from fresh material using Kornerup & Wanscher (1978).

Microscopy and scanning electron microscopy

Microscopic studies followed the protocol in Maba *et al.* (2013, 2014), and SEM micrographs were prepared as detailed in Maba *et al.* (2013). Preliminary identification of

© 2015 International Mycological Association

You are free to share - to copy, distribute and transmit the work, under the following conditions:

Attribution: You must attribute the work in the manner specified by the author or licensor (but not in any way that suggests that they endorse you or your use of the work).

Non-commercial: You may not use this work for commercial purposes.

No derivative works: You may not alter, transform, or build upon this work.

For any reuse or distribution, you must make clear to others the license terms of this work, which can be found at <http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode>. Any of the above conditions can be waived if you get permission from the copyright holder. Nothing in this license impairs or restricts the author's moral rights.

specimens was made using the results of investigations in similar ecosystems (van Rooij *et al.* 2003). Additionally, we used the monograph of Verbeken & Walley (2010) on tropical African *Lactarius s. l.* species.

DNA Extraction, sequencing and PCR amplification

DNA was retrieved from dried specimens following the protocol used by Maba *et al.* (2013, 2014). The nrDNA ITS (including the ITS1, ITS2 and 5.8S regions) was amplified using the fungal specific primer ITS1-F in combination with the basidiomycete specific primer ITS4-B (Gardes & Bruns 1993). The 48 new ITS sequences obtained, including seven from the newly described species (specimens MD108, MD140, MD141, MD156, MD157, MD393, and MD397) have been deposited in the European Nucleotide Archive (ENA).

Sequence editing, analyses, and molecular phylogenetic inference

We first checked and downloaded the most similar sequences of fully identified taxa (up to species) and unidentified (up to genus) using BLASTN (Altschul *et al.* 1997) in public sequence databases (NCBI, ENA, and UNITE). Sequences with a minimum of 90 % of similarity to each of our sequences were considered and downloaded. To maximize the alignment, preference was given to tropical Africa sequences (already compiled in Maba *et al.* 2013, 2014). Then, closely related sequences from elsewhere were used to further populate the dataset.

The raw sequences were assembled and edited using BioEdit v. 7.2.5 (Hall 2005, update 12 Nov. 2013). Our final ITS dataset (Table 1) comprised 107 *Russulaceae* sequences including 81 of *Lactifluus*, 14 of *Lactarius*, five of *Multifurca*, and seven of *Russula*, along with two out-group sequences (one *Gloeocystidiellum* and one *Hericium*, both *Russulales*). A multiple alignment was performed using the online version of MAFFT v. 7.130b (Kato & Toh 2008, update 27 Sept. 2013), by applying the “best accurate” option for the alignment. The resulting alignment was corrected manually by removing ambiguously aligned regions as well as mismatched and common empty columns. Our final sequence dataset was composed of 109 rDNA ITS sequences and had a total length of 700 bp.

The Maximum Likelihood (ML) tree was inferred in MEGA v. 6 (Tamura *et al.* 2013) by applying the General Time Reversible (GTR + G + I) (Nei and Kumar 2000) nucleotide substitution model. The rate variation among sites was modeled with a Gamma distribution, shape parameter = 6. The Subtree-Pruning-Regrafting Extensive (SPR level 5) with a very strong branch swap filter was applied as the ML heuristic method for Tree Inference Option. The initial ML tree was obtained automatically by NJ/BioNJ; and the phylogeny was tested using the bootstrap method with 1000 replicates (Felsenstein 1985).

RESULTS

ITS sequence analyses

The inferred phylogenetic tree (Fig. 1) had distinct and supported clades representing the accepted subgenera within

Lactifluus (I to VIII), as well as other genera in *Russulales* (groups A to E). Within clade A, the first well supported (99 %) clade (I) constitutes *Lactifluus* subgen. *Lactariopsis*, with 13 sequences of nine taxa. Clade II encompasses sequences of unidentified samples from Togo (LK392607) and Benin (LK392604, LK392605, and LM999911). The sequences belonging to *Lactifluus* subgen. *Russulopsis* form clade III, which includes six of our newly generated sequences, five of which are from Togo and one from Guinea (LK392608). This last subgeneric clade (III) is phylogenetically supported within *Lactifluus* by a 60 % bootstrap value. The fourth clade (IV) represents *Lactifluus* subgen. *Edules* with 11 sequences corresponding to nine taxa. This clade is supported by 77 % bootstrap value. *Lactifluus* subgen. *Lactifluus* encompasses clades V (21 taxa) and VI (three taxa) and is the largest, with 36 sequences (including 17 of ours) representing 24 taxa. Clades V and VI are supported by 91 and 53 % bootstrap values, respectively. The *Lactifluus* subgenera *Gerardii* and *Piperati* (until now unknown from African ecosystems) are represented by clades VII and VIII respectively. All six subgenera (*Lactariopsis*, *Russulopsis*, *Edules*, *Lactifluus*, *Gerardii* and *Piperati*) included in our analysis, are supported monophyletically and together form the genus *Lactifluus*.

Multifurca, with five samples, is a monophyletic group of its own (62 % bootstrap support) and constitutes clade B. The genus formed a sister clade to *Lactifluus* (clade A) and *Lactarius* (clade C). Clade C encompassed 14 *Lactarius* sequences, monophyletically well-supported by a 98 % bootstrap value. Sequences of *Russula* (five samples, clade D), and of *Lactifluus* and *Lactarius*, as well as those of *Multifurca*, formed a well supported (100 %) monophyletic group, the *Russulaceae* clade. In this analysis, *Russula* deviates and forms a separate monophyletic clade with 97 % bootstrap support. The sister clade (composed of a *Gloeocystidiellum* and a *Hericium* species, both *Russulales*) to all *Russulaceae* sequences formed the outgroup (clade E) in this analysis.

The sequences of MD157 (LK392597) and MD108 (LK392598) belong to the same species (99 % of bootstrap support) and are placed within *Lactifluus* subgen. *Edules*, within a well supported (98 %) clade together with three unnamed species. The phylogenetic placement of both specimens in this subgenus is supported by morpho-anatomical features (see below).

Within the *Lactifluus* subgen. *Russulopsis* clade, both sequences of MD141 (HG426473) and MD156 (LK392596) cluster as a single species (99 %), and together form a sister (100 % of bootstrap value) to MD140 (LK392599). Together, the subclade formed by the above mentioned sequences and the one containing *Lactifluus longipes* (two samples), and an unnamed one (MD224 = LK392608), clustered as a monophyletic clade (61 % of bootstrap) that corresponds to *Lactifluus* subgen. *Russulopsis*. Similarly, the sequences of MD393 (LK392594) and MD397 (LK392595) appear as sister taxa (100 % support value) of the same species. They nested within *Lactifluus* subgen. *Lactifluus* clade with 96 % of bootstrap support and as sister to several named species and un-named collections.

The combination of morpho-anatomical features (see descriptions below) from each sample and the molecular

Table 1. ITS sequences generated in this study.

Species	Collection numbers	Countries	ENA Acc. No
<i>Lactifluus annulatoangustifolius</i>	MD145	Togo	HG426475
<i>Lactifluus densifolius</i>	C2362	Togo	HG917385
<i>Lactifluus edulis</i>	C2168	Togo	HG917384
<i>Lactifluus emergens</i>	DPM04	Togo	HG426467
<i>Lactifluus flammans</i>	MD124	Togo	HG426471
<i>Lactifluus flavellus</i>	MD393	Togo	LK392594
<i>Lactifluus flavellus</i>	MD397	Togo	LK392595
<i>Lactifluus fazaoensis</i>	MD152	Togo	HG426477
<i>Lactifluus foetens</i>	MD150	Togo	HG917381
<i>Lactifluus foetens</i>	MD359	Burkina Faso	LK392603
<i>Lactifluus gymnocarpus</i>	MD125	Togo	HG426472
<i>Lactifluus gymnocarpoides</i>	MD301	Benin	LK392601
<i>Lactifluus gymnocarpoides</i>	MD318	Benin	LK392600
<i>Lactifluus heimii</i>	C2018	Togo	LK392612
<i>Lactifluus longibasidius</i>	MD141	Togo	HG426473
<i>Lactifluus longibasidius</i>	MD156	Togo	LK392596
<i>Lactifluus longipes</i>	ADK4315	Togo	HG917383
<i>Lactifluus longipes</i>	C2445	Togo	HG917391
<i>Lactifluus luteopus</i>	MD102	Togo	LK392602
<i>Lactifluus luteopus</i>	AV94-463	Burundi	LK392611
<i>Lactifluus aff. medusae</i>	MD142	Togo	HG426474
<i>Lactifluus melleus</i>	MD108	Togo	LK392598
<i>Lactifluus melleus</i>	MD157	Togo	LK392597
<i>Lactifluus nonpiscis</i>	MD101	Togo	HG426468
<i>Lactifluus pectinatus</i>	MD140	Togo	LK392599
<i>Lactifluus rubiginosus</i>	MD389	Togo	HG917386
<i>Lactifluus sudanicus</i>	MD105	Togo	HG426469
<i>Lactifluus sudanicus</i>	MD148	Togo	HG426476
<i>Lactifluus</i> sp.	C2349	Togo	HG426478
<i>Lactifluus</i> sp.	MD123	Togo	HG426470
<i>Lactifluus</i> sp.	MD304	Benin	LK392604
<i>Lactifluus</i> sp.	MD317	Benin	LK392605
<i>Lactifluus</i> sp.	MD131	Togo	LK392606
<i>Lactifluus</i> sp.	MD154	Togo	LK392607
<i>Lactifluus</i> sp.	MD224	Guinea	LK392608
<i>Lactifluus</i> sp.	MD355	Burkina Faso	LK392609
<i>Lactifluus</i> sp.	MD234	Guinea	LK392610
<i>Lactifluus</i> sp.	MD160	Togo	LK931501
<i>Lactifluus</i> sp.	C1819	Togo	LM999910
<i>Lactifluus</i> sp.	MD326	Togo	LM999911
<i>Lactifluus</i> sp.	C2157	Togo	HG426466
<i>Lactifluus</i> sp.	MD355B	Burkina Faso	LN651269
<i>Lactarius kabansus</i>	AV99-179	Zimbabwe	HG917390
	MD132	Togo	HG917376
<i>Lactarius miniatescens</i>	MD151	Togo	HG917374
<i>Lactarius subballophaeus</i>	MD100	Togo	HG917372
<i>Lactarius tenellus</i>	MD149	Togo	HG917373

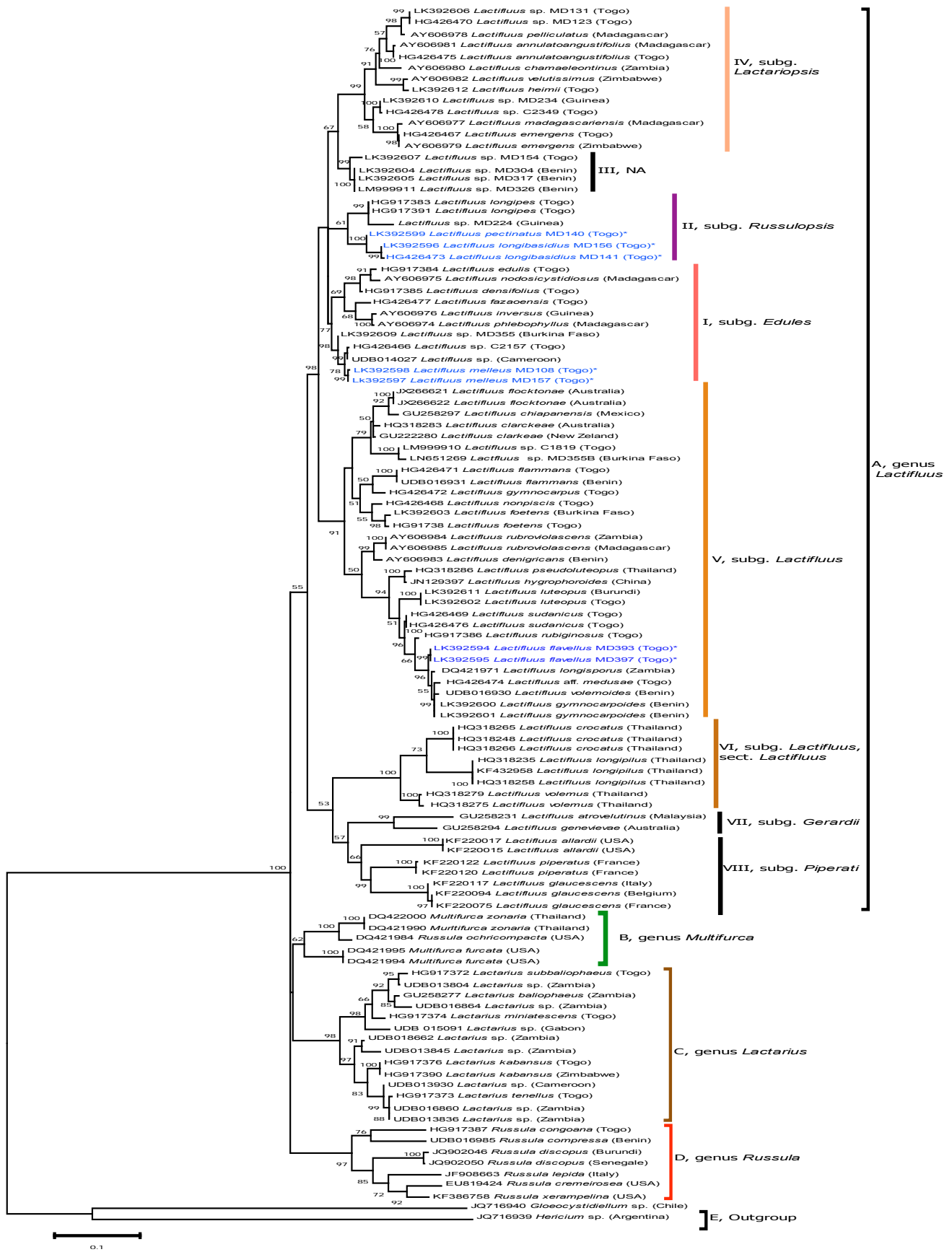


Fig. 1. Maximum Likelihood (ML) tree showing the placement of the four newly described species (*L. flavellus*, *L. longibasidius*, *L. melleus* and *L. pectinatus*) within the *Russulaceae*. Bootstrap values higher than 50 % are shown above/below the branches.

phylogenetic analyses, support the description of four new species: *Lactifluus melleus* (MD157 and MD108), *L. longibasidium* (MD156 and MD141), *L. pectinatus* (MD140), and *L. flavellus* (MD393 and MD397).

TAXONOMY

Lactifluus flavellus Maba & Guelly, sp. nov.

Mycobank MB808850

(Figs 2–4)

Etymology: Refers to the yellowish, small and slender basidiome.

Diagnosis: *Pileus* concave then plano-convex to depressed; pellis dry, entirely and strongly striate; pastel yellow to light yellow, or yellowish white. Lamellae adnate, broadly decurrent to subdecurrent, widely spaced, unequal, regular. *Stipe* very long stipe, the longest known within African lactarioids. Context white, fleshy, thin in the pileus but thick in the stipe; latex white and unchanging; taste spicy. *Basidiospores* with ridged amyloid ornamentation, ridges mostly interconnected. **Anatomy:** marginal cells of lamellae irregular, cylindrical to subclavate, septate and thin-walled; pleurocystidia densely abundant and conspicuously emergent, subcylindrical to narrowly fusiform; pileipellis palisade-like, with a suprapellis composed of cylindrical to subcylindrical cells; stipitipellis hymenoderm-like to subcellular.

Type: Togo: Plateaux region, Prefecture of Wawa, Bèna Eglekoutsè, 7°31' 6.6" N 0°54'7.41" E, on soil in gallery forest dominated by *Uapaca guineensis*, 17 July 2013, *Dao Maba*



Fig. 2. *Lactifluus flavellus* (MD393) basidiome. **A.** Lamellae and the pileus. **B.** Pileus and stipe detail. **C.** Stipe. Bars = 10 mm.

MD393 (TOGO – holotype). ENA accession no. LK392594.

Description: *Pileus* 15–50 mm diam, concave when young then plano-convex when older, slightly depressed in the centre; pellis entirely and strongly striate even in young basidiomes, very slightly smooth in the centre, dry, pastel yellow to light yellow (3A4-5) when young, yellowish white to pastel yellow when older (3A3-4 to 3A5). *Margin* strongly incurved in young



Fig. 3. *Lactifluus flavellus* (MD393) light microscopy. **A.** Pleuropseudocystidia. **B.** Pileipellis. **C.** Basidia. **D.** Basidiospores. **E.** Marginal cells. **F.** Pleurocystidia. Bars = 10 µm.

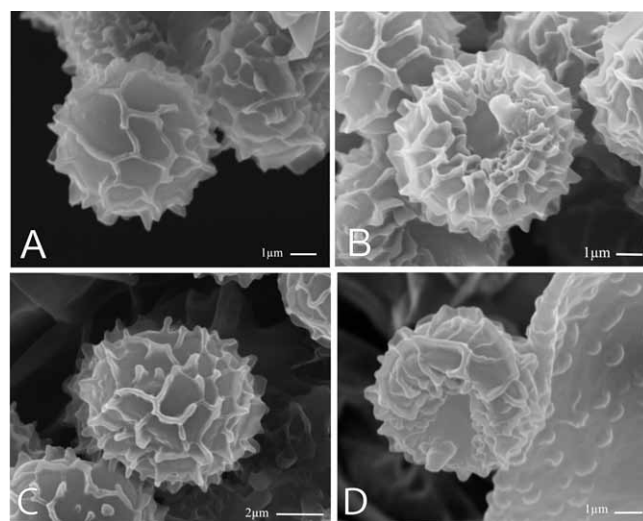


Fig. 4. *Lactifluus flavellus* (MD393) SEM of basidiospores. **A, C.** Dorsal view. **B, D.** Lateral and detail showing the plage.

specimens, straight and involuted and strongly striate in old specimen up to 2/3 from the edges to the centre. *Lamellae* adnate, broadly decurrent to subdecurrent, widely spaced, unequal, regular with 3 lamellulae between 2 lamellae ($L+1 = 4-6/\text{cm}$), very brittle, yellowish white to pale yellow (3A2-3); edge entire. *Stipe* 40–95 × 7–12 mm, smooth, soft, central, cylindrical and slightly tapering downwards, medulla solid, concolorous to pileus, but paler at the base. *Context* white, fleshy, thin in the pileus while thick in the stipe, brittle. *Latex* not abundant, white and unchanging, taste slightly spicy.

Basidiospores (Figs 3D, 4A–D) globose, subglobose to ellipsoid (7.0–)7.5–8.5(–9.0) × (6.5–)7.0–7.5(–8) μm ($Q = (1.08-1.15-1.25-1.30(-1.35))$; $n = 75$), amyloid ornamentation of ridges to 0.5 μm in height, mostly connected, forming almost a complete reticulum; amyloid spot present in a distinct plage. *Basidia* (Fig. 3C) 4-spored, 50–60 × 18.5–11 μm , subclavate; sterigmata 4–6 × 1.5–2 μm . *Lamella edge* sterile. *Marginal cells* (Fig. 3E) 25–50 × 5–6(–7) μm , irregular, cylindrical to subclavate, sometimes tapering downwards, septate; thin-walled. *Hymenophoral trama* cellular, composed of sphaerocytes and numerous laticiferous hyphae. *Pleurocystidia* (Fig. 3F) dense, abundant, 55–80(–85) × 6–11 μm , emergent to 30 μm above the hymenium, subcylindrical to narrowly fusiform, sometimes septate. *Pleuropseudocystidia* (Fig. 3A) not abundant, 5–10(–11) μm diam, subcylindrical, rarely tortuous, sometimes bifurcate, tapering upwards, with a slightly moniliform to mucronate apex; thin-walled, with pale brown contents; incrustations and oleiferic drops present in the hymenium. *Pileipellis* (Fig. 3B) palisade-like, terminal elements of suprapellis 25–70(–85) × 3–4(–6) μm , composed of cylindrical to subcylindrical cells, scarce thick-walled terminal hyphae present; subpellis composed of irregularly spherical and isodiametric cells. *Stipitipellis* hymenoderm-like to subcellular, terminal elements subcylindrical to subclavate, thin-walled, rarely bifurcate. *Clamps* absent.

Distribution: Known only from Bèna Eglekoutsè, Togo.

Notes: *Lactifluus flavellus* (MD393 and MD397) clusters within the clade containing sequences of *L. longisporus* (DQ421971 from Zimbabwe), *L. aff. medusae* (HG426474 from Togo), and *L. gymnocarpoides* (LK392600 and LK392601 both from Benin), from which it differs morphologically and anatomically (Verbenken & Walley 2010). *Lactifluus gymnocarpoides* has both a lampropalisade structure as the pileipellis and basidiospores that are ellipsoid to strongly elongate.

Additional specimen examined: **Togo**: Plateaux region, Prefecture of Wawa, Bèna Eglekoutsè, 7°31'6.18" N 0°54'7" E, on soil, gallery forest dominated by *Uapaca guineensis*, 17 July 2013, *Dao Maba* MD397 (TOGO). ENA accession no. LK392595.

***Lactifluus longibasidius* Maba & Verbeke, sp. nov.**
Mycobank MB808851
(Figs 5–7)

Etymology: After the shape and the size of the basidia.



Fig. 5. *Lactifluus longibasidius* (MD156) basidiome. **A.** Pileus. **B.** Margin and stipe. **C.** Lamellae. **D.** General view (pileus, lamellae, and stipe). Bars = 10 mm.

Diagnosis: *Pileus* irregular, asymmetric, plano-concave to plano-convex, eccentric, even reniform; pellis dry, shiny, wrinkled, strongly rugulose, slightly velvety; reddish orange, darkest in the centre, light orange to dark orange. *Lamellae* adnate, decurrent to subdecurrent, moderately distant, irregular and unequal. Recognized by the particularly long basidia, sometimes tortuous, abundant and long pleurocystidia which are emergent, subcylindrical to cylindrical, sometimes tapering upwards, capitate or conical, thin-walled; marginal cells of lamellae subcylindrical to cylindrical, sometimes branched and septate; pileipellis lamprotrichoderm-like, with a suprapellis composed of subcylindrical to fusiform cells, with a distinctly tapering apex, septate and rather bifurcate, mixed with very slender and thick-walled cells; stipitipellis a trichoderm, composed of wide, irregular, septate, bifurcate, and very slender cells.

Type: **Togo**: Central region: Prefecture of Tchoudjo, Fazao-Malfakassa National Park, 8°42'21" N 0°46'22" E, on soil in woodland dominated by *Uapaca togoensis* and *Isoblerlinia doka*, 19 June 2011, *Dao Maba* MD156 (TOGO – holotype; GENT – isotype). ENA accession no. LK392596.

Description: *Pileus* 25–55 mm diam, very irregular in shape, sometimes asymmetric, concave when young, plano-concave to plano-convex and depressed when old, eccentric and even reniform; pellis dry, shiny, wrinkled, strongly rugulose and slightly velvety, slightly striate near the margin when older, reddish orange (7B7-8) in the centre, darkest in the centre on young basidiomes, light orange to dark orange (5A5-8) near the margin, old specimens paler near the margin. *Margin* incurved to enrolled. *Lamellae* adnate, decurrent to subdecurrent, moderately distant, irregular, unequal ($L+1 = 5-7/\text{cm}$), pastel yellow, light yellow (3A4-5) to orange-yellow (4A4-5). *Stipe* 18–35 × 10–15 mm, central to eccentric, cylindrical, sometimes tapering downwards, dry, firm, smooth, light yellow (4A4-6). *Context* of pileus orange

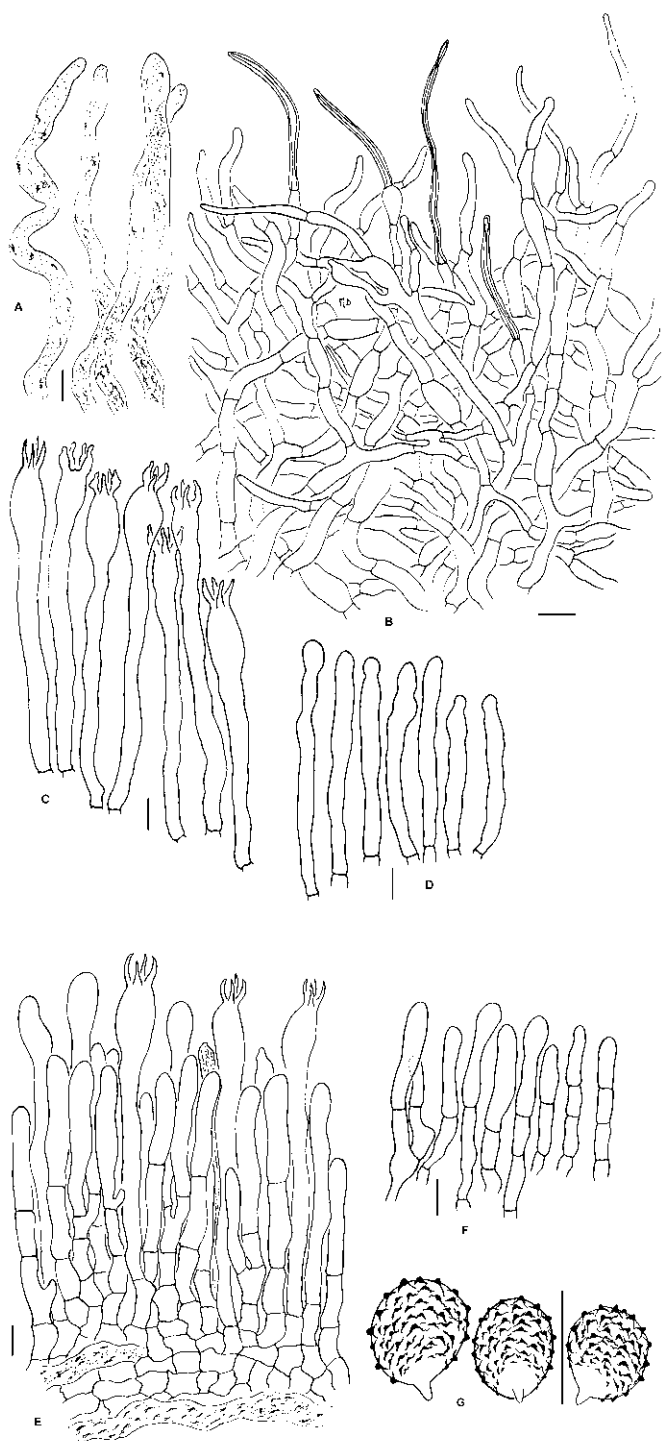


Fig. 6. *Lactifluus longibasidius* (MD156) light microscopy. **A.** Pleuropseudocystidia. **B.** Pileipellis. **C.** Basidia. **D.** Pleurocystidia. **E.** Hymenium. **F.** Marginal cells. **G.** Basidiospores. Bars = 10 μm .

yellowish to whitish, very thin at the margin; stipe firm and whitish. *Latex* copious, milky white and unchanging; taste not special.

Basidiospores (Figs 6G, 7A–D) globose to subglobose, sometimes ellipsoid, $7.5\text{--}8.5\text{--}9.0 \times 6.5\text{--}7\text{--}7.5$ ($Q = 1\text{--}1.15\text{--}1.29$; $n = 82$), ornamentation of distinguishable amyloid warts ($0.5 \mu\text{m}$ high), finely and partially interconnected; no amyloid spot present in the plage. **Basidia** (Fig. 6C)

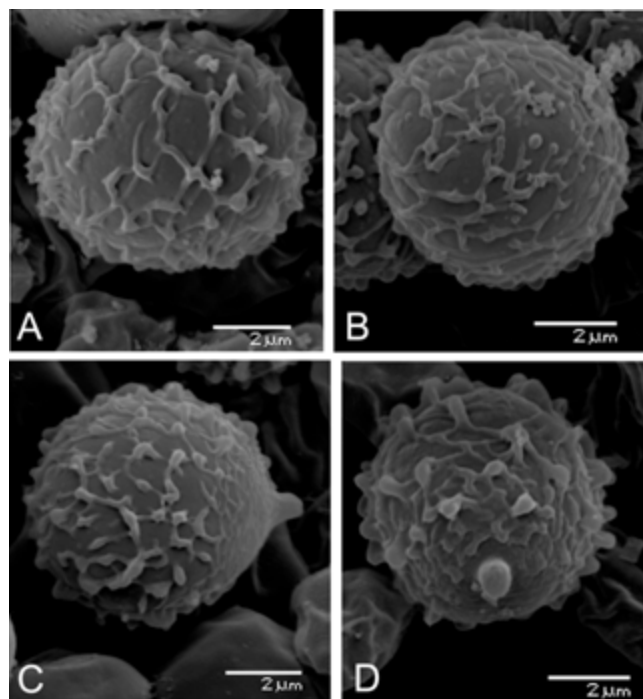


Fig. 7. *Lactifluus longibasidius* (MD156) SEM of basidiospores. **A.** Dorsal to lateral view. **B.** Proximal profile view. **C.** Lateral front view. **D.** Proximal to dorsal view.

4-spored, long and slender, $70\text{--}95\text{--}120(\text{--}130) \times 7\text{--}8(\text{--}9) \mu\text{m}$, sometimes tortuous with sterigmata $6\text{--}8(\text{--}9) \times 2\text{--}3 \mu\text{m}$. *Lamella* edge sterile. *Marginal cells* (Fig. 6F) $30\text{--}66(\text{--}72) \times 3\text{--}5 \mu\text{m}$, subcylindrical to cylindrical, sometimes branched; septate. *Hymenophoral trama* composed of a mixture of sphaerocytes, numerous laticiferous and filamentous hyphae. *Pleurocystidia* (Fig. 6D) $75\text{--}80 \times 6\text{--}7\text{--}7.5 \mu\text{m}$, abundant, emergent, thin-walled, subcylindrical to cylindrical, sometimes tapering upwards, capitate or conical. *Pleuropseudocystidia* (Fig. 6A) abundant, $5\text{--}7(\text{--}8) \mu\text{m}$ diam, not always emergent, with brown needle-like contents. *Pileipellis* (Fig. 6B) lamprotrichoderm-like, terminal elements $2\text{--}3(\text{--}5) \mu\text{m}$ wide, subcylindrical to fusiform, with distinctly tapering apex, septate and rather bifurcate, slender, thick-walled elements present. *Stipitipellis* a trichoderm with terminal elements $2\text{--}3(\text{--}4) \mu\text{m}$ wide, irregular, septate, bifurcate, longer than in the pileipellis. *Clamps* absent.

Distribution: Known only from Fazao-Malfakassa National Park, Togo.

Notes: *Lactifluus longibasidius* (MD141 and MD156) fits phylogenetically within the *Lactifluus* subgen. *Russulopsis* clade, but has some microscopic features that recall *Lactifluus* subgen. *Lactariopsis* sect. *Chamaeleontini* (Verbeke & Walley 2010).

Additional specimen examined: **Togo:** Central region: Prefecture of Tchoudjo, Fazao-Malfakassa National Park, $8^{\circ}42'21'' \text{N } 0^{\circ}46'22'' \text{E}$, on soil in woodland dominated by *Uapaca togoensis* and *Isoberlinia doka*, 19 June 2011, *Dao Maba* MD141 (TOGO). ENA accession no. HG426473.

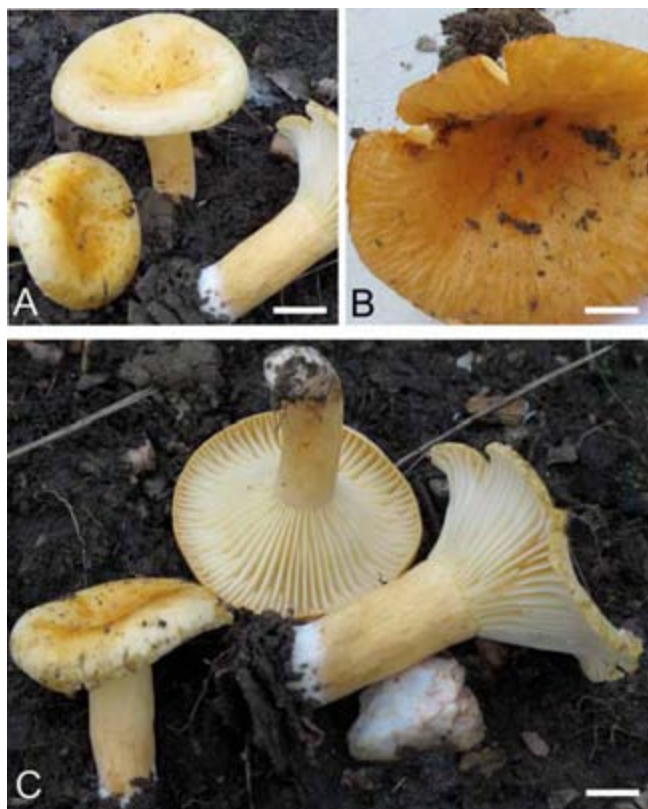


Fig. 8. *Lactifluus melleus* (MD157) basidiome. A. Pileus and stipe. B. Pileus. C. Lamellae and stipe. Bars = 10 mm.

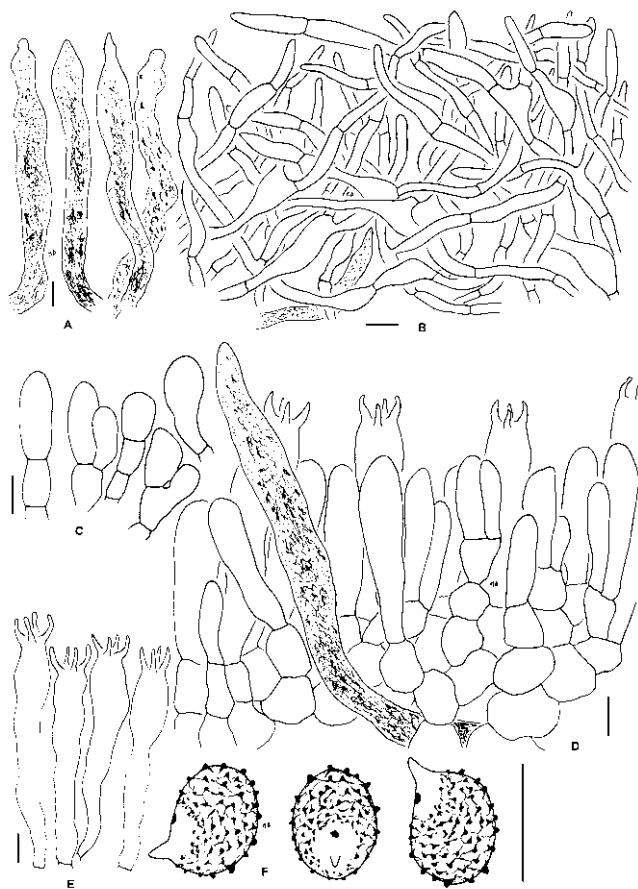


Fig. 9. *Lactifluus melleus* (MD157) light microscopy. A. Pleuropseudocystidia. B. Pileipellis. C. Marginal cells. D. Hymenium. E. Basidia. F. Basidiospores. Bars = 10 µm.

Lactifluus melleus Maba, sp. nov.

Mycobank MB808852

(Figs 8–10)

Etymology: The epithet recalls the honey-coloured basidiome.

Diagnosis: *Pileus* concave then plano-concave, strongly depressed in the centre, sometimes subinfundibuliform; pellis velvety and soft, smooth when freshly harvested, striate when dehydrated. Recognized by the dry, honey-coloured, light yellow to deep yellow or warm yellow pileus, slightly more pronounced in the centre. *Lamellae* adnate, broadly subdecurrent to decurrent, moderately spaced, unequal, irregular; context of pileus brittle, slightly thick in the centre, fleshy, white in the pileus as well as in the stipe; latex scarce, white, and unchanging. *Basidiospores* with well developed amyloid warts connected by fine lines; pleurocystidia absent; an ixocutis to a trichoderm pileipellis with a suprapellis composed of irregular, cylindrical to subclavate cells, septate, interwoven; dermatocystidia with a moniliform to mucronate apex; marginal cells of lamellae irregular, cylindrical to subclavate, thin-walled.

Type: **Togo**: Central region: Prefecture of Tchaoudjo, Fazao-Malfakassa National Park, 8°30' 56"N 0°54'44.1"E, on soil in woodland dominated by *Uapaca togoensis*, *Isobertinia doka* and *I. tomentosa*, 19 June 2011, *Dao Maba* MD157 (TOGO – holotype; GENT –isotype). ENA accession no. LK392597.

Description: *Pileus* 40–60 mm diam, concave when young then plano-concave, strongly depressed in the centre,

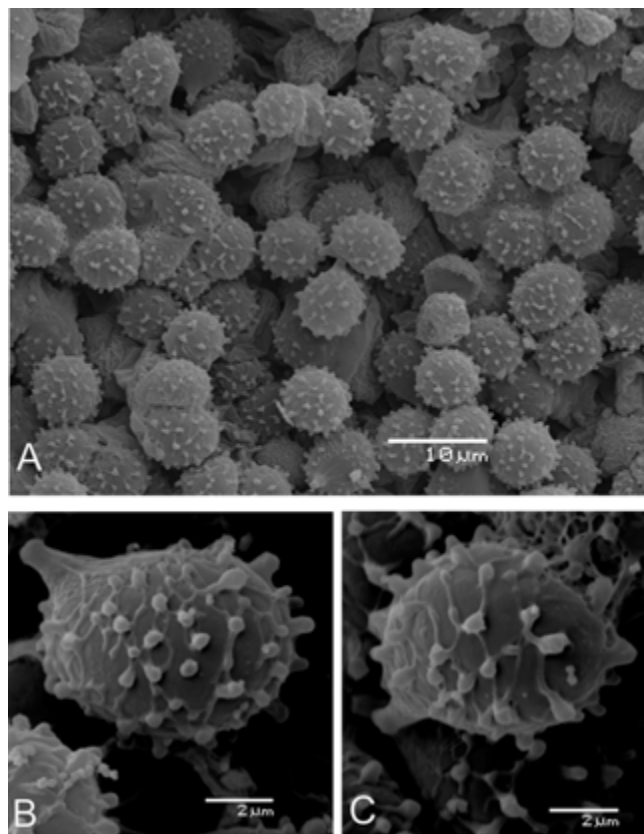


Fig. 10. *Lactifluus melleus* (MD157) SEM of basidiospores. A. Overview. B. Lateral view. C. Lateral/dorsal view.

sometimes subinfundibuliform when old, pellis velvety and soft, appearing smooth in freshly collected specimens, but in dehydrated samples striate to 1/3 from the margin; dry, honey-coloured, light yellow to deep yellow or warm yellow, slightly more pronounced in the centre (4A4-8). *Margin* straight in young specimens, grooved and striate in old specimens to 1/3 from the margin to the centre, slightly thick, striate and involuted. *Lamellae* adnate, broadly subdecurrent to decurrent, moderately spaced, unequal, irregular with 1(-3) lamellulae between two lamellae ($L+1 = 4-6/\text{cm}$), very brittle; yellowish white to light yellow (4A2-4). *Stipe* 30-35 × 12-15 mm, smooth, soft, central, cylindrical and tapering downwards, firm, solid, concolorous with the pileus. *Context* of pileus brittle, slightly thickened in the centre, fleshy, white in the pileus as well as in the stipe. *Latex* not abundant, white and unchanging, taste not observed.

Basidiospores (Figs 9E, 10A-C) subglobose to ellipsoid, rarely elongate (8.0-9.5-9.0-9.5(-10.0) × 7.0-7.5-8.0 μm ($Q = (1.08-1.15-1.25-1.30(-1.34))$; $n = 80$), ornamentation of well developed amyloid warts, to 0.5 μm high, connected by fine lines, sometimes almost with a complete reticulum; amyloid spot present in a distinct place. *Basidia* (Fig. 9E) 4-spored, 50-65(-70) × 10-12 μm, subclavate, sterigmata 4-7 × 2 μm. *Lamella edge* sterile. *Marginal cells* (Fig. 9C) 32-50 × 5-6(-7) μm, irregular, cylindrical to subclavate, thin-walled. *Hymenophoral trama* mostly cellular, composed of sphaerocytes and laticiferous hyphae. *Pleurocystidia* absent. *Pleuropseudocystidia* (Fig. 9A) very abundant, 11-16(-20) μm diam, cylindrical, rarely tortuous, projecting to 40-50 μm above the hymenium, sometimes tapering upwards, moniliform to mucronate, thin-walled, contents brown. *Pileipellis* (Fig. 9B) an ixocutis to a trichoderm, suprapellis of irregular, cylindrical to subclavate cells, septate, interwoven; subpellis of irregular sphaerical, isodiametric cells, 5-15 μm diam; dermatocystidia not very abundant, sometimes with a moniliform to mucronate apex. *Stipitipellis* an ixocutis to a cutis, suprapellis composed of irregular, thin-walled cells, septate and sometimes branched, densely interwoven; subpellis composed of a mixture of isodiametric cells and laticiferous hyphae. *Clamps* absent.

Distribution: Known only from Fazao-Malfakassa National Park, Togo.

Notes: *Lactifluus melleus* (MD108 and MD157) nested phylogenetically within *Lactifluus* subgen. *Edules*, but it has some microscopic features (not all), as mentioned above, that recall *Lactifluus* subgen. *Lactariopsis* sect. *Chamaeleontini* and also *Lactifluus* subgen. *Russulopsis* (Verbeken & Walley 2010).

Additional specimen examined: **Togo**: Central region: Prefecture of Tchoudjo, Fazao-Malfakassa National Park, 08°42'23" N 046°27", on soil in woodland dominated by *Uapaca togoensis*, *Isobertinia doka* and *I. tomentosa*, 19 June 2011, *Dao Maba* MD108 (TOGO, GENT). ENA accession no. LK392598.

Lactifluus pectinatus Maba & Yorou, sp. nov.

MycoBank MB808853

(Figs 11-13)

Etymology: Recalling the pectinate shape of the pileus.

Diagnosis: *Pileus* concave, plano-concave to depressed; pellis dry, tomentose, striate near the margin, sticky, shortly sulcate and broadly pectinate near the margin; pale yellow to light yellowish and light orange to deep orange. *Lamellae* adnate, broadly subdecurrent to decurrent, spaced to distant; context yellowish, fragile, very brittle and thin near the margin, slightly thicker in the centre; latex abundant, white, unchanging. *Basidiospores* with amyloid warts ornamentation interconnected at the base and forming a complete reticulum. *Pleurocystidia* abundant, emergent, cylindrical to subcylindrical, fusiform, sometimes septate, thin-walled. Also recognized by an ixotrichopalisade-like pileipellis with a suprapellis composed of subcylindrical to subclavate cells, mixed with numerous swollen and subglobose to globose cells; marginal cells of the lamellae cylindrical to fusiform, septate.

Type: **Togo**: Central region: Prefecture of Tchoudjo, Fazao-Malfakassa National Park, 8°42'27"N 0°45'13"E, on soil in woodland dominated by *Isobertinia doka* and *Uapaca togoensis*, 19 June 2011, *Dao Maba* MD140 (TOGO - holotype; GHENT - isotype). ENA accession no. LK392599.

Description: *Pileus* 25-75 mm diam, concave when young, later plano-concave and slightly depressed in the centre when older; dry, tomentose, striate to 1/3 from the margin, slightly sticky, shortly sulcate and broadly pectinate near the margin, smooth in the centre, pale yellow to light yellowish (3A4-5) and light orange to deep orange (5A6-8). *Margin* strongly crenulated, strongly involuted when young, becoming incurved to slightly straight when old. *Lamellae* adnate, broadly subdecurrent to decurrent, spaced to very distant ($L+1 = 4-5/\text{cm}$), unequal, irregular, yellowish to pale yellow (3A4-5). *Stipe* 23-30 × 8-12 mm, central, cylindrical, tapering downwards, dry, smooth and concolorous with the pileus. *Context* of pileus fragile, very brittle and thin near the margin, slightly thicker in the centre; stipe firm and yellowish. *Latex* abundant; white, unchanging.

Basidiospores (Figs 12C, 13A-C) globose to subglobose, sometimes ellipsoid, (6.5-7.5-8.5-9.5(-10) × (6.0-6.5-7-7.5(-8) μm ($Q = 1.03-1.12-1.16$; $n = 75$), ornamentation of distinctly amyloid warts, to 0.5 μm high, interconnected at the base and forming a complete reticulum, no amyloid spot present in the place. *Basidia* (Fig. 12F) 54-62 × 8-10 μm, subclavate to clavate, 4-spored, sterigmata 8-11 × 2-3 μm. *Pleurocystidia* (Fig. 12E) rather abundant, 55-65 × 6-8 μm, emergent, cylindrical to subcylindrical, slightly fusiform, sometimes septate, thin-walled. *Pseudopleurocystidia* (Fig. 12B) abundant, 3-8 μm diam, very irregular, fusiform to tortuous, moniliform, capitate, with irregular, brown contents. *Lamella edge* sterile. *Marginal cells* (Fig. 12D) 20-45 × 3-5 μm, cylindrical to fusiform, septate. *Hymenophoral trama* composed of numerous sphaerocytes mixed with laticifers. *Pileipellis* (Fig. 12B) ixotrichopalisade-like, terminal elements



Fig. 11. *Lactifluus pectinatus* (MD140) basidiome. A. Pileus. B. Lamellae and stipe. Bars = 10 mm.

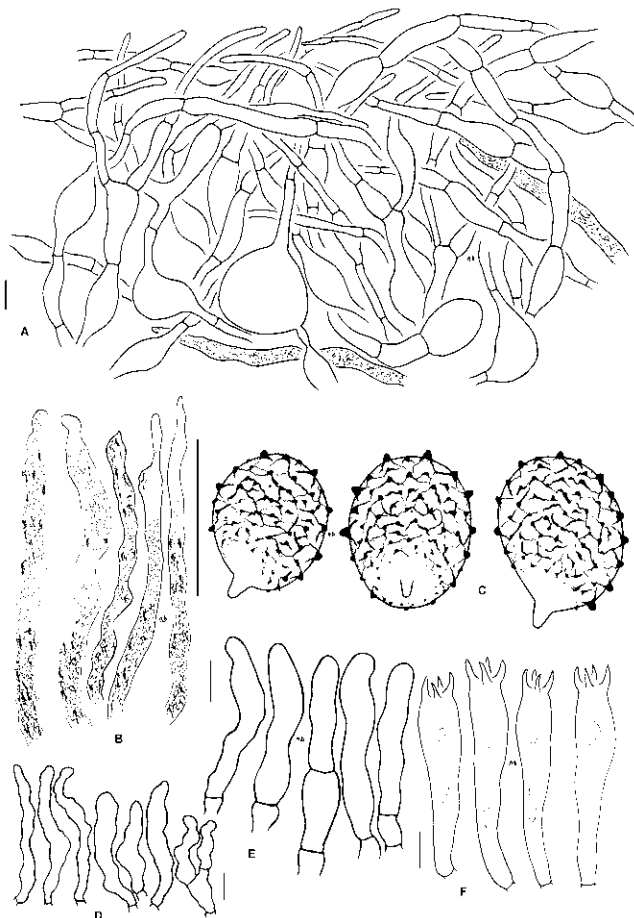


Fig. 12. *Lactifluus pectinatus* (MD140) light microscopy. A. Pileipellis. B. Pleuropseudocystidia. C. Basidiospores. D. Marginal cells. E. Pleurocystidia. F. Basidia. Bars = 10 µm.

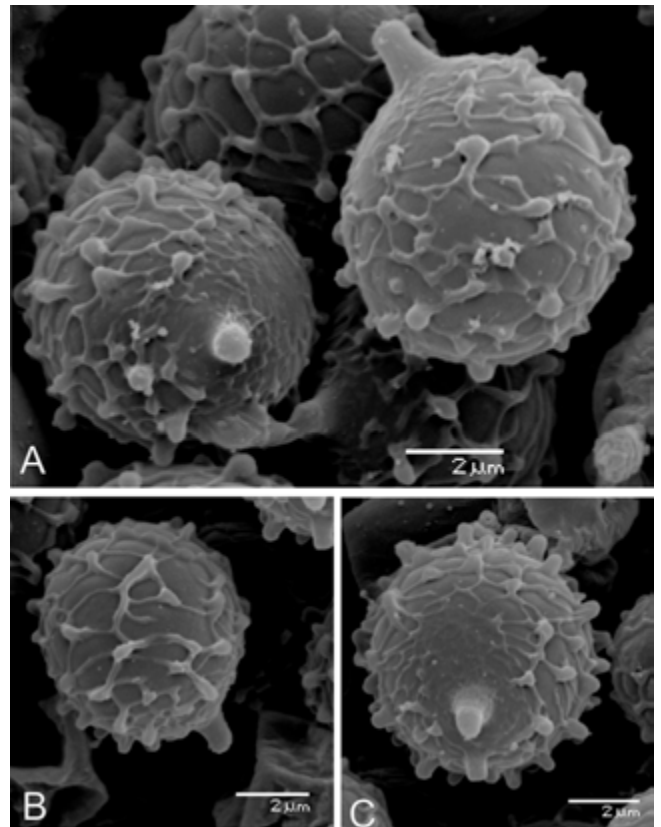


Fig. 13. *Lactifluus pectinatus* (MD140) SEM of basidiospores. A. Proximal view (left), lateral view (right). B. Dorsal view. C. Proximal view. Bars = 2 µm.

subcylindrical to subclavate, mixed with numerous swollen and subglobose to globose elements, thick-walled elements scarce. *Stipitipellis* a palisade, terminal elements 20–35 × 3–4(–5) µm, cylindrical, fusiform; subpellis composed of isodiametrical cells. *Clamps* absent.

Distribution: Known only from Fazao-Malfakassa National Park, Togo.

Notes: *Lactifluus pectinatus* (MD140), as well as *L. longibasidium*, is supported phylogenetically within the *Lactifluus* subgen. *Russulopsis* clade, but, as noted above, they present some microscopic features that recall *Lactifluus* subgen. *Lactariopsis* sect. *Chamaeleontini* (Verbeke & Walley 2010).

DISCUSSION

Lactifluus longibasidium and *L. pectinatus*, are morpho-anatomically clearly different from previously described species, and have microscopic features that confirm both their separateness and phylogenetic positions (Fig. 1). In addition to the presence of pleurocystidia in both species (although with different shapes and sizes, see Figs 6 and 12), they have basidiospores with a well-developed amyloid ornamentation, composed of distinguishable obtuse, finely and partially interconnected warts; they have no amyloid spot

in the plage. These features characterize some known species of *Lactifluus* subgen. *Russulopsis*, as well as *L.* subgen. *Lactariopsis* sect. *Chamaeleontini* (Verbeken & Walley 2010). However, *L. longibasidius*, with two representative collections, differs considerably from *L. pectinatus*, as well as from the known *Lactifluus* species in these groups, in the shape and size of the pleurocystidia (to 130 µm) when present, and the marginal cells (to 72 µm). The species is recognizable by the surprisingly long basidia (to 135 µm) that usually emerge to 50 µm above the hymenium. These features have never been reported for any known *Lactifluus* species from tropical Africa (van Rooij *et al.* 2003, Buyck *et al.* 2007, Verbeken & Walley 2010). In addition, both *L. pectinatus* and *L. longibasidius* have no dermatocystidia, such as those observed in *L. ruvubuensis* and *L. longipes*.

Lactifluus melleus, represented by two samples, is phylogenetically well supported within *Lactifluus* subgen. *Edules*. It has a velvety and soft pellis, appearing smooth in fresh specimens, but is striate in dehydrated samples; moderately spaced, unequal, irregular, and subdecurent to decurrent lamellae; and an ixocutis to trichoderm pileipellis, composed of cylindrical to subclavate cells. These characters separate it from the previously named *L. edules*, *L. aureifolius*, and *L. densifolius*. *Lactifluus melleus* has basidiospore ornamentation and a pileipellis that fits within *Lactifluus* subgen. *Lactariopsis* sect. *Chamaeleontini*. It has thick-walled hair-shaped elements in the pileipellis, pleurocystidia are absent, and the basidiospore ornamentation is to 0.5 µm. It also agrees with *Lactifluus* subgen. *Russulopsis* in having basidiospores with obtuse amyloid warts to 0.5 µm, the presence of dermatocystidia, and a pileipellis with diverticulate and subclavate cells (Verbeken & Walley 2010).

Lactifluus flavellus conforms to *Lactifluus* subgen. *Lactifluus*. Comparative microscopical studies with the closely related *L. uapacae*, show that the new species has some distinct anatomical characters. It has a very long stipe (to 95 mm), the longest yet reported within tropical African lactarioid species, and a strongly striate pileus, even in young basidiomes. In addition, *L. flavellus* has some microscopic features, including pleurocystidia, that are not present in *L. uapacae*. *Lactifluus flavellus* has a palisade-like pileipellis and a hymenoderm-like stipitipellis while these are, respectively, a lampropalisade and a lamprotrichopalissade in *L. uapacae*. The hymenium of *L. flavellus* is crowded with distinctly emergent pleurocystidia to 85 µm, whereas these are absent from *L. uapacae*, and incrustations are present in the hymenium as well as in the pileipellis of *L. flavellus*, but absent in *L. uapacae*.

Considering the morpho-anatomical analyses, *L. flavellus* has a combination of features (see above) that fit in *Lactifluus* subgen. *Lactifluus*, a placement confirmed by the phylogenetic analysis. *Lactifluus melleus* is supported within the *Lactifluus* subgen. *Edules* clade, while *L. longibasidius* and *L. pectinatus* are supported phylogenetically within the *Lactifluus* subgen. *Russulopsis* clade. However, within the genus *Lactifluus*, the species including *L. rufomarginatus*, previously classified in *Lactifluus* sect. *Russulopsidae*, *L. cocosmus* and *L. aurantiifolius*, remained unclassified in any of the accepted subgenera (Verbeken *et al.* 2011, 2012, Stubbe *et al.* 2012). Similarly, *L. brachystegiae*, previously

classified in sect. *Chamaeleontini*, is currently placed in subgen. *Russulopsis* sect. *Russulopsidae*.

This study confirms the high species diversity of *Lactifluus* in tropical West Africa and points to the patchy and/or paucity, of information on lactarioids from West African ecosystems. Apart from the species described in this paper and some other previous publications (van Rooij *et al.* 2003, van de Putte *et al.* 2009, Maba *et al.* 2013, 2014), numerous samples from the same area are still waiting to be assessed. Our results support the monophyly of *Lactifluus* within *Russulaceae*. From the present study and from Maba *et al.* (2013, 2014), we also conclude that a new delimitation at subgenus and section level is needed within *Lactifluus*, to take into account the combinations of morphological and microscopical characters displayed in the recently described species. We consider that mycological inventories throughout tropical African ecosystems, and particularly in West Africa, remain crucial for a real assessment of the extent of tropical African mycodiversity, and will thereby help to highlight the evolutionary traits within milkcaps.

ACKNOWLEDGEMENTS

This research was supported by funds from the International Foundation for Sciences (IFS, grant D/5178-1), and the German Academic Exchange Service (DAAD, grant A/11/72562). We thank Eva Facher from the Ludwig Maximilians-Universität (München), for her guidance and help in the scanning electron microscopy.

REFERENCES

- Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, *et al.* (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25**: 3389–3402.
- Bà AM, Duponnois R, Moyersoen B, Abdala G, Diédhiou AG (2012) Ectomycorrhizal symbiosis of tropical African trees. *Mycorrhiza* **22**: 1–29.
- 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.
- Buyck B, Hofstetter V, Verbeken A, Walley R (2010) Proposal to conserve *Lactarius* nom. cons. (*Basidiomycota*) with a conserved type. *Mycotaxon* **111**: 504–508.
- de Crop E, Nuytinck J, van de Putte K, Lecomte M, Eberhardt U, Verbeken A (2013) *Lactifluus piperatus* (*Russulales*, *Basidiomycota*) and allied species in Western Europe and a preliminary overview of the group worldwide. *Mycological Progress* **13**: 493–511.
- Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**: 783–791.
- Gardes M, Bruns TD (1993) ITS primers with enhanced specificity for basidiomycetes application to the identification of mycorrhizas and rusts. *Molecular Ecology* **2**: 113–118.
- Hall T (2005) *BioEdit: biological sequence alignment editor for Win95/98/NT/2K/XP*. Version 7.2.5. Carlsbad, CA: Ibis

- Therapeutic; <http://www.mbio.ncsu.edu/bioedit/bioedit.html>
- Katoh K, Toh H (2008) Recent developments in the MAFFT multiple sequence alignment program. *Brief Bioinform* **9**: 286–298.
- Kornerup A, Wanscher JH (1978) *Methuen Handbook of Colour*. London: Methuen.
- Maba DL, Guelly AK, Yorou NS, Verbeken A, Agerer R (2013) Two New *Lactifluus* species (*Basidiomycota, Russulales*) from Fazao Malfakassa National Park (Togo, West Africa), *Mycological Progress* **13**: 513–524.
- Maba DL, Guelly AK, Yorou NS, De Kesel A, Verbeken A, Agerer R (2014) The genus *Lactarius* s. str. (*Basidiomycota, Russulales*) in Togo (West Africa): phylogeny and a new species described. *IMA Fungus* **5**: 39–49.
- Nei M, Kumar S. (2000). *Molecular Evolution and Phylogenetics*. New York: Oxford University Press.
- Rivière T, Diedhiou AG, Diabate M, Senthilarasu G, Hatarajan K, et al. (2007) Genetic diversity of ectomycorrhizal basidiomycetes from African and Indian tropical rain forests. *Mycorrhiza* **17**: 415–428.
- Sanon E, Guissou KM-L, Yorou NS, Buyck B (2014) Le genre *Russula* au Burkina Faso (Afrique de l'Ouest): quelques espèces nouvelles de couleur brunâtre. *Cryptogamie, Mycologie* **35**: 377–397.
- Stubbe D, Nuytinck J, Verbeken A (2010) Critical assessment of the *Lactarius gerardii* species complex (*Russulales*). *Fungal Biology* **114**: 271–283.
- Stubbe D, Wang X-H, Verbeken A (2012) New combinations in *Lactifluus*. 2. *L.* subgen. *Gerardii*. *Mycotaxon* **119**: 483–485.
- Tamura K, Nei M, Kumar S (2004) Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proceedings of the National Academy of Sciences, USA* **101**: 11030–11035.
- Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. *Molecular Biology and Evolution* **30**: 2725–2729.
- van de Putte K, de Kesel A, Nuytinck J, Verbeken A (2009) A new *Lactarius* species from Togo with an isolated phylogenetic position. *Cryptogamie, Mycologia* **30**: 39–44.
- van de Putte K, Nuytinck J, Stubbe D, Le HT, Verbeken A (2010) *Lactarius volemus sensu lato* (*Russulales*) from northern Thailand: morphological and phylogenetic species concepts explored. *Fungal Diversity* **45**: 99–130.
- van Rooij P, De Kesel A & Verbeken A (2003) Studies in tropical African *Lactarius* species (*Russulales, Basidiomycota*) 11. Records from Benin. *Nova Hedwigia* **77**: 221–251.
- Verbeken A, Buyck B (2001) Diversity and ecology of tropical ectomycorrhizal fungi in Africa. In: *Tropical Mycology* (Watling R, Frankland JC, Ainsworth AM, Isaac S, Robinson G, eds): 11–24. Wallingford: CABI Publishing.
- Verbeken A, Walley R (2010) *Monograph of Lactarius in Tropical Africa*. [Fungus Flora of Tropical Africa, vol. 2.] Bruxelles: National Botanic Garden of Belgium.
- Verbeken A, Nuytinck J, Buyck B (2011) New combinations in *Lactifluus*. 1. *Lactifluus* subgen. *Edules*, *Lactariopsis*, and *Russulopsis*. *Mycotaxon* **118**: 447–453.
- Verbeken A, van de Putte K, de Crop E (2012) New combinations in *Lactifluus*. 3. *Lactifluus* subgen. *Lactifluus* and subgenera *Piperati*. *Mycotaxon* **120**: 443–450.