Tales of the unexpected: angiocarpous representatives of the Russulaceae in tropical South East Asia

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Key words
Arcangeliella
gasteroid fungi
hypogeous fungi
Lactarius
Martellia
morphology
phylogeny
Zelleromyces

Abstract Six new sequestrate Lactarius species are described from tropical forests in South East Asia. Extensive macro- and microscopical descriptions and illustrations of the main anatomical features are provided. Similarities with other sequestrate Russulales and their phylogenetic relationships are discussed. The placement of the species within Lactarius and its subgenera is confirmed by a molecular phylogeny based on ITS, LSU and rpb2 markers. A species key of the new taxa, including five other known angiocarpous species from South East Asia reported to exude milk, is given. The diversity of angiocarpous fungi in tropical areas is considered underestimated and driving evolutionary forces towards gasteromycetization are probably more diverse than generally assumed. The discovery of a large diversity of angiocarpous milkcaps on a rather local tropical scale was unexpected, and especially the fact that in Sri Lanka more angiocarpous than agaricoid Lactarius species are known now.

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INTRODUCTION

Sequestrate and angiocarpous basidiomata have developed in several groups of Agaricomycetes. Various plausible selective pressures have been proposed to explain this transformation from agaricoid to gasteroid basidiomata (Miller et al. 2001). It is often assumed that changing environmental conditions led to enclosed basidiome morphology and eventually to the hypogeous gasteroid fruting bodies, offering protection against frost and moisture loss from the hymenium and thus preventing desiccation (Thiers 1984a, Bruns et al. 1989, Bougher & Lebel 2001, Wilson et al. 2011). Arid or seasonally dry climates thus exert a selection pressure towards a sequestrate fruting body, especially in ectomycorrhizal fungi which provide the plants with coloration was recorded as it was exuded from the mushroom, and Petersen (1996, indicated as FK in descriptions). Latex following the colour guide by Kornerup & Wanscher (1978) was used to ascertain their phylogenetic position and full descriptions and illustrations are given.

MATERIALS AND METHODS

The study is based on collections made by Kobeke van de Putte, Annemiek Verbeken and Dirk Stubbe. The studied material is deposited in the Herbarium Universitatis Gandavensis (GENT). An overview of the studied specimens, including information on the collection locality and ecology is given after each species description section.

Morphological study

Descriptions of macromorphological features are based on fresh material. Colours were described in daylight conditions following the colour guide by Konnerup & Wanscher (1978) and Petersen (1996, indicated as FK in descriptions). Latex coloration was recorded as it was exuded from the mushroom,
| Species | Voucher collection | Origin | ITS accession no. | LSU accession no. | rpb2 accession no. |
|---------|--------------------|--------|------------------|------------------|-------------------|
| Amylostereum laevigatum | olrin409/CSB523.84 | Switzerland | AY781246 | AF287843 | AY218469 |
| Arcangelia borziana | | Switzerland | A286204 | A373599 | |
| | | Italy | | JF908775 | |
| A. camphorata | | USA | EU644700 | EU644701 | EU644702 |
| | | USA | EU644700 | EU644701 | EU644702 |
| | | USA | EU834192 | EU846241 | |
| A. crassa | | USA | AY587870 | | |
| A. sp. | | Thailand | FJ454900 | | |
| Auriscalpium vulgare | AFTOL1897/DAOM128994 | Switzerland | DQ911613 | DQ911614 | AY218472 |
| Echinodontium tinctorium | AFTOL455 | | AY854088 | AF393056 | AY218482 |
| Lactarius acris | EU014 (UPS) | Germany | DQ421988 | DQ421988 | DQ421992 |
| L. akahatsu | | VKU2004-141 (GENT) | | | |
| L. atroviridis | AV04-11 (GENT) | USA | EU644700 | EU644701 | EU644702 |
| L. auriolla | | Switzerland | AF286204 | | |
| L. azonites | AV99-010 (GENT) | Switzerland | AF373599 | | |
| L. baliophaeus | | Italy | AY218469 | | |
| L. camphoratus | UE04.09.2004 (UPS) | Sweden | DQ421992 | DQ421992 | DQ421925 |
| L. chromospermus | AV99-174 (GENT) | Switzerland | KF133260 | KF133292 | KF133324 |
| L. chrysorrheus | UE10.09.2002-8 (UPS) | Italy | KF133261 | KF133293 | KF133325 |
| L. citriolens | UE20.09.2004-03 (UPS) | Sweden | DQ422003 | DQ422003 | DQ421931 |
| L. controversus | AV00-117 (GENT) | Italy | KF241544 | | |
| L. cyanescens | DS06-058 (GENT) | Malaysia | GU265581 | GU265581 | GU265317 |
| L. deliciosus | JN2001-046 (GENT) | Slovakia | KF133272 | KF133305 | KF133337 |
| L. falcatus | LTH369 (GENT) | Thailand | GU258378 | GU258378 | GU258317 |
| L. flexuosus | UE06.09.2002-1 (UPS) | Sweden | DQ421992 | DQ421992 | DQ421925 |
| L. formosus | AV00-118 (GENT) | Belgium | KF241547 | | |
| L. fuliginosus | MTB97-24 (GENT) | Italy | KF133261 | KF133293 | KF133325 |
| L. helvus | UE06.09.2003-5 (UPS) | Sweden | DQ421993 | DQ421993 | DQ421926 |
| L. hispidulus | AV99-010 (GENT) | Guinea | GU258378 | GU258378 | GU258317 |
| L. kabansus | AV00-117 (GENT) | Zimbabwe | KF133266 | KF133305 | KF133323 |
| L. lawsonii | UE08.09.2004-1 (UPS) | Italy | KF133267 | KF133299 | KF133331 |
| L. lilacinus | RW3774 (GENT) | Belgium | KF133271 | KF133300 | KF133340 |
| L. luridus | OB11-011 (GENT) | Norway | KF133272 | KF133307 | KF133347 |
| L. mammosus | UE09.09.2004-03 (UPS) | USA | KF133277 | KF133309 | KF133342 |
| L. montoyae | KD1065 (BSHC) | India | KF133278 | KF133310 | KF133343 |
| L. necator | AV01-120 (GENT) | France | KF133279 | KF133311 | KF133344 |
| L. peckii | JN2004-020 (GENT) | USA | KF133279 | KF133307 | KF133342 |
| L. pomiolens | AV07-159 (GENT) | Sri Lanka | KF133280 | KF133307 | KF133342 |
| L. puntarius | LTH382 (CMU, SFSU, GENT) | Thailand | KF133281 | KF133308 | KF133343 |
| L. quieticolor | UE10.09.2004-1 (UPS) | Sweden | DQ421992 | DQ421992 | DQ421925 |
| L. quietus | UE10.09.2004-1 (UPS) | USA | KF133277 | KF133310 | KF133342 |
| L. romagnesii | UE29.09.2002-6 (UPS) | France | KF133279 | KF133307 | KF133342 |
| L. rubriviridis | DED7312 (SFSU) | USA | KF133280 | KF133307 | KF133342 |
| L. saturnisporus sp. nov. | AV07-170 (GENT) | Sri Lanka | KF133280 | KF133307 | KF133342 |
| L. sphagneti | PL2805 (pers. herb. P. Leonard) | UK | KF133280 | KF133307 | KF133342 |
| L. tenellus | DK1398 (BR) | Benin | KF133280 | KF133307 | KF133342 |
| L. thyinos | A.Voitk23-08-2004 (GENT) | Canada | KF133280 | KF133307 | KF133342 |
| L. torminosus | LVL2002-013 (GENT) | Belgium | KF133280 | KF133307 | KF133342 |
| L. trivialis | UE27.08.2002-17a (UPS) | Sweden | DQ421991 | DQ421991 | DQ421924 |
| L. vietus | UE11.19.2004-1 (UPS) | Sweden | KF133280 | KF133307 | KF133342 |
### Table 1 (cont.)

| Species | Voucher collection | Origin | ITS accession no. | LSU accession no. | rpb2 accession no. |
|---------|---------------------|--------|-------------------|-------------------|---------------------|
| L. vinaceaefascens | JN2007-018 (GENT) | Canada | KF341542 | | |
| Lactifluus deceptivus | AV04-181 (GENT) | USA | DQ422020 | DQ422020 | DQ421935 |
| L. edulis | AV99-041 (GENT) | Zimbabwe | DQ421977 | DQ421977 | DQ421916 |
| L. emergens | AV99-055 (GENT) | Zimbabwe | DQ421977 | DQ421977 | DQ421916 |
| L. gerardi | AV95-375 (GENT) | USA | DQ421971 | DQ421971 | DQ421901 (PC) |
| L. longiporus | AV99-107 (GENT)/BB 00.1519 (PC) | Zimbabwe/Madagascar | DQ421976 | DQ421976 | DQ421915 |
| L. nodosicystidiosus | BB97-072 (PC) | Madagascar | DQ421979 | DQ421979 | DQ421918 |
| L. phlebophyllus | BB00-1388 (PC) | Madagascar | DQ421979 | DQ421979 | DQ421918 |
| L. piperatus | UE09.08.2004-6 (UPS) | Sweden | DQ422035 | DQ422035 | DQ421937 |
| L. velleicans | UE20.09.2004-22 (UPS) | Sweden | DQ422034 | DQ422034 | DQ421936 |
| L. velutinissimus | AV99-185 (GENT) | Sweden | DQ421973 | DQ421973 | DQ421912 |
| L. volens | UE09.08.2004-5 (UPS) | Sweden | DQ422008 | DQ422008 | DQ421932 |
| Multifurca furcata | RHT804 (NY) | Costa Rica | DQ421994 | DQ421994 | DQ421927 |
| M. ochricompacta | BB02.107 (PC) | USA | DQ421984 | DQ421984 | DQ421940 |
| M. zonaria | DED7442 (PC) | Thailand | DQ421990 | DQ421990 | DQ421942 |
| Russula aeruginea | AT2003017 (UPS) | Sweden | DQ422029 | DQ422029 | DQ421966 |
| R. albonigra | AT2002064 (UPS) | Sweden | DQ422004 | DQ422004 | DQ421949 |
| R. camphorophylla | PAN01081108 (PC) | France | DQ421982 | DQ421982 | DQ421938 |
| R. earlei | WCRW0-412 (PC) | USA | DQ422025 | DQ422025 | DQ421963 |
| R. emeticus | UE05.10.2003-11 (UPS) | Sweden | DQ422013 | DQ422013 | DQ421954 |
| R. fimula | AT2004142 (UPS) | Sweden | DQ422017 | DQ422017 | DQ421958 |
| R. gracillima | UE23.08.2014-14 (PC) | Sweden | DQ422004 | DQ422004 | DQ421949 |
| R. heterophylla | UE20.08.2004-2 (UPS) | Sweden | DQ422006 | DQ422006 | DQ421951 |
| R. illota | UE26.07.2002-3 (UPS) | Sweden | DQ422024 | DQ422024 | DQ421967 |
| R. lepida | HJ99900 (UPS) | Belgium | DQ422013 | DQ422013 | DQ421954 |
| R. nigricans | UE20.09.2004-7 (UPS) | Sweden | DQ422010 | DQ422010 | DQ421952 |
| R. ochrascopora | GD20.07.2004 (UPS) | Italy | DQ422012 | DQ422012 | DQ421953 |
| R. pararezaurea | BW06.09.2002-16/MF01.10.2003 (UPS) | Sweden | DQ422007 (MF) | DQ422007 (MF) | DQ421945 (BW) |
| R. pectinatoides | AT2001049 (UPS) | Sweden | DQ422026 | DQ422026 | DQ421964 |
| R. perrinae | UE31.09.2003-01 (UPS) | Sweden | DQ422019 | DQ422019 | DQ421960 |
| R. risigallina | UE30.07.2003-08 (UPS) | Sweden | DQ422022 | DQ422022 | DQ421961 |
| R. vesca | AT2002091 (UPS) | Sweden | DQ422018 | DQ422018 | DQ421959 |
| R. virens | AT2001049 (UPS) | Belgium | DQ422014 | DQ422014 | DQ421955 |
| Stereum hirsutum | AFL01492 | USA | DQ453696 | | |
| Zelleromyces gardneri | | USA | DQ422029 | DQ422029 | DQ421966 |
| Z. gienensis | | | | | |
| Z. hispanicus | | | | | |
| Z. sp. | | | | | |

but also from a drop placed on a glass slide held over white paper, and from a drop placed directly on white paper. Pictures of the basidiocarps will be published on the Russulales News website (http://www2.muse.it/russulales-news/).

Micromorphological characters were registered from the dried specimens. Spores were observed in Melzer’s reagent for measurements and drawings; other structures in 2–5 % KOH or Congo-red. For each collection the length and width of at least 20 spores were measured in side view in Melzer’s reagent, excluding the ornamentation. Measurements are given as (MIN × AV × 2 × SD) – AV × AV – [AV × 2 × SD] (MAX), in which AV = lowest mean value for the measured collections, AV = greatest mean value and SD = standard deviation calculation for the measurements of one collection. Q stands for ‘quotient length/width’ and is given as (MINQ) – Q – Q – (MAXQ) in which Q and Q stand for the lowest and the highest, respectively mean value for the measured specimens. In case only one collection was available spore measurements are given as (MINQ) – AV × 2 × SD) – AV – [AV × 2 × SD] (MAX). Micromorphological features were illustrated with the aid of a drawing tube attached to an Olympus CX-41 research compound microscope. For the details of description and terminology of micromorphological features see Verbeken (1998) and Verbeken & Walley (2010).

DNA extraction, PCR amplification and sequencing

Nine gasteroid *Lactarius* collections were used for the molecular analyses. DNA was extracted from dried and fresh fruiting bodies using the methods described by Nuytinck & Verbeken (2003) with slight modifications (van de Putte et al. 2010). Three loci were amplified and sequenced: 1) the internal transcribed spacer region of the nuclear ribosomal DNA (ITS), using primers ITS1-F and ITS4 (White et al. 1990, Gardes & Bruns 1993); 2) a part of the nuclear ribosomal large subunit region (LSU), using primers LROR and LRR (Vilgalys & Hester 1990, Rehner & Samuels 1994); and 3) the region between domains 6 and 7 of the nuclear gene encoding the second largest subunit of RNA polymerase II (*rpb2*), using primers bRPB2-6F and fRPB2-7cR (Liu et al. 1999, Matheny 2005).

Protocols for PCR amplification and sequencing follow Le et al. (2007), sequencing was also conducted with an ABI 3730XL or ABI 3700 by MACROGEN (Amsterdam, The Netherlands). Sequences were assembled and edited with the software Sequencher™ 4.9. The sequences were then aligned and phylogenetic analyses were conducted with the software PhyML 3.0. The analysis was performed with the GTR+G model for the LSU (including 12 data sets), rpb2 and ITS datasets. DNA extraction, PCR and sequencing protocols described by Eberhardt (2002) or Taylor et al. (2006).
Alignment and phylogenetic analyses

Table 1 shows an overview of all specimens and sequences used in the phylogenetic analyses, including GenBank accession numbers. Two alignments were constructed. The first alignment consists of ITS sequences only; it includes all sequestrate Lactarius, Arcangeliella and Zelleromyces sequences available from GenBank, the newly discovered sequestrate collections from Sri Lanka and Thailand, and a broad selection of agaricoid Lactarius taxa; three Multifurca species were used as the outgroup. This alignment is used to confirm that our new taxa are well supported and differ from the sequestrate milk cap species that have been known and sequenced before. The second alignment consists of ITS, LSU and rpb2 sequences, including only specimens for which all three loci are available. The sampling covers a broader selection of Russulaceae: the four genera of Russulaceae (Lactarius, Lactifluus, Multifurca and Russula, see Buyck et al. 2008) are represented. Outgroup Russulales species are Auriscalpium vulgare Gray, Stereum hirsutum (Willd.) Pers., Amylostereum laevigatum (Fr.) Boidin and Echinodontium tintorium (Ellis & Everh.) Ellis & Everh. This second alignment is used to study the phylogenetic placement of the newly described species within Lactarius and its

Fig. 1 ML tree (RAxML) based on ITS sequences. Bootstrap values are indicated if they exceed 50%. Names in orange are the new angiocarpous species described in this paper, names in green are angiocarpous Lactarius species for which ITS sequences are available on GenBank, Arcangeliella sp. FJ454900 was obtained by sequencing plant roots; the fruiting body was not observed, and thus it is unclear whether this sequence is actually from an angiocarpous species. The scale bar represents the number of nucleotide changes per site.
subgenera. Alignments were constructed with the online version of MAFFT v6 (Katoh & Toh 2008), applying the E-INS-I strategy, a very slow method recommended for less than 200 sequences with multiple conserved domains and long gaps. The alignments were manually refined in BioEdit v7.0.9.0 (Hall 1999) and made available in TreeBASE (www.treebase.org, study ID: S14274). For the second alignment, ambiguously aligned positions (mainly within ITS1 and 2) were detected using Gblocks v0.91b (Castresana 2000), specifying less stringent conditions than default in order to keep gapped sites. Apart from the positions identified by Gblocks, the intron region of rpb2 was also deleted from the analyses to avoid the inclusion of ambiguous alignment. Sequence data were partitioned as follows: 1) ITS was partitioned into the ribosomal genes 18S (partial) and 5.8S and the spacer regions ITS1 and ITS2; 2) LSU; and 3) rpb2 was partitioned into codon positions 1, 2, and 3.

Maximum Likelihood (ML) analyses were performed in RAxML v7.0.3 (Stamatakis 2006), combining a ML search with the Rapid Bootstrapping algorithm for 1000 replicates. The model GTR+I+G was estimated for each partition separately. The model was partitioned into codon positions 1, 2 and 3 heated chains were run for 20 million generations on a High Performance Computer (HPC) of the Ghent University. Sample frequency was set at 100. The log probability of the data given the parameter values and effective sample size statistics (ESS) of the runs were examined with Tracer v1.5 (Drummond & Rambaut 2007). To check convergence, 1) the standard deviation of split frequencies across the 2 runs on the desktop computer was assessed; and 2) topologies and posterior probabilities from the 4 runs on the HPC were compared. An appropriate burn-in value was determined visually using Tracer.

Bayesian Inference (BI) analyses were carried out in MrBayes v3.2.0 (Ronquist & Huelsenbeck 2003). The general time-reversible model with rate variation across sites and a proportion of invariable sites (GTR+I+G) was used. Rates and model parameters were unlinked between all partitions. Two analyses were run: 1) an analysis on a desktop computer with 2 runs and 1 chain per run was executed for 20 million generations (Ronquist et al. 2009); and 2) 4 independent, parallel runs of 1 cold and 3 heated chains were run for 20 million generations on a High Performance Computer (HPC) of the Ghent University. Sample frequency was set at 100. The log probability of the data given the parameter values and effective sample size statistics (ESS) of the runs were examined with Tracer v1.5 (Drummond & Rambaut 2007). To check convergence, 1) the standard deviation of split frequencies across the 2 runs on the desktop computer was assessed; and 2) topologies and posterior probabilities from the 4 runs on the HPC were compared. An appropriate burn-in value was determined visually using Tracer.

Fig. 2 ML tree (RAxML) based on ITS, LSU and rpb2 sequences. Bootstrap values and Posterior Probabilities (resulting from Bayesian analysis using the HPC) are indicated if they exceed 50 % or 95 %, respectively (BS/PP). Names in orange are the new angiocarpous species described in this paper. The scale bar represents the number of nucleotide changes per site.
Russularia

All gasteroid milkcaps included in this study belong to the genus Russula and subg. Lactarius and subg. Lactifluus. Although we also consider these species to be clearly distinct from the previously known and sequenced sequestrate milk cap species (indicated in green).

The analysis based on all three sampled loci (ITS, LSU and rpB2) reveals the position of our South East Asian collections within the genus Lactarius. Since there was no conflict among the single loci trees in clades with a bootstrap support of 70 % or higher, a combined analysis was performed. Fig. 2 shows the obtained ML topology with BS values and Bayesian posterior probabilities (PP). ML and both Bayesian phylogenies differ only in the placement of some terminal, non-gasteroid taxa. All 3 analyses show 3 well-delimited genera in the Russulaceae (Lactarius BS 92 % - PP 100 %, Multifurca BS 100 % - PP 100 % and Russula BS 90 % - PP 100 %) but fail to support the monophyly of the genus Lactifluus. Instead, Lactifluus consistently comes out as paraphyletic and basal to the other Russulaceae genera. Lactifluus volemus, Lactifluus piperatus and Lactifluus gerardii are not included in a monophyletic ‘core’-group of Lactifluus (BS 83 % - PP 100 %) represented by Lactifluus subg. Lactifluus p.p. (excluding section Lactifluus), subg. Edules and subg. Lactariopsis.

All gasteroid milkcaps included in this study belong to the genus Lactarius. Lactarius falcatus sp. nov. is member of L. subg. Russularia, while L. saturnisporus sp. nov., L. echinus sp. nov, L. chinellus sp. nov. and L. shoreae sp. nov. belong to L. subg. Plinthogalus. The affinities of L. pomiolens sp. nov. are less clear. It appears as one of the long, basal branches of the genus Lactarius for which no subgeneric subdivisions are available.

Taxonomy

All newly proposed species (L. pomiolens, L. echinus, L. echinellus, L. saturnisporus, L. shoreae and L. falcatus) produce milky exudates or latex. The additional known angiocarpous species from South East Asia reported to exude milk are also included in the species key. These are: Arcangelieilla lactifera (B.C. Zhang & Y.N. Yu) J.M. Vidal, A. densa (R. Heim) Singer & A.H. Sm., Zelleromyces raminispinus (B.C. Zhang & Y.N. Yu) Trappe, T. Lebel & Castellano, Z. sinensis (B.C. Zhang & Y.N. Yu) J.M. Vidal, Arcangeliella lactifera, Z. striatus (Bank MB804182; Fig. 3)

1. Basidia 4-spored, 2-spored basidia sometimes present 6
2. Basidia exclusively 2-spored .......................... 8
3. Spores ornamented with irregular warts to short spines, never more than 1 µm long ......... 4A. densa 7
4. Spores ornamented with spines up to 2.5–3.5 µm high, lacking striations; peridioles a strongly interwoven pali-sade to trichopalisade, without obvious slime layer ........................................ L. shoreae 8
5. Spores on average 12 × 11.5 µm, ornamented with slender and straight spines up to 4 µm long .......... L. echinus 9
6. Spores ornamented with spines up to 2.5 µm long .... 7
7. Basidia only 4-spored; spines straight and slender, not branched ........................................ L. echinellus 9
8. Spores ornamented with spines up to 4 µm long ........ 5A. lactifera 8
9. Spores ornamented with spines that are at most 2 µm long ........................................ M. nanjingensis 10
10. Spores ornamented with irregular and curved spines up to 2 µm long ........................................ L. falcatus 10
11. Spores ornamented with conical to blunt spines up to 1.5 µm long ........................................ L. pomiolens 11
12. Spores ornamented with conical to blunt spines up to 1.5 µm long ........................................ M. nanjingensis 10

1. Lactarius pomiolens Verbeken & Stubbe, sp. nov. — MycoBank MB804182; Fig. 3

Holotype. Sri LANKA, near Sinharaja Forest, trail along river, on sandy wet soil in rainforest with Shorea trapezzolia, Shorea disticta and Dipterocarpus hispidus, 13 Dec. 2007, Verbeke 07-159 (GENT).

Etymology. With the smell of apples.

Basidiocarp 25–45 mm diam, subglobose, rather irregular. Peridium very slightly tomentose, felty, ochraceous to leather- brown (FK13–14), buff to ochraceous, irregularly coloured, with patches. Stipe absent. COLUMella absent. Gleba strongly labyrinthoid, with small loculi, with some, but very few gelatinous veins among them, greyish yellow (4B4), a bit more flesh-coloured, staining dark brown where eaten by insect larvae, firm in youngest ones, more compressible, rubbery in older specimens. Latex rather abundant, white, staining immediately sulphur yellow to greenish yellow (1A5–6) on white paper, slowly changing yellow on the context then apparently disappearing, when isolated turning golden yellow in a 10 % aqueous potassium hydroxide solution, not forming a whitish layer on the gleba when drying. Taste bitter, astringent, not just dry. Smell very sweet, fruity, like apples, Russula fellea-like.

Spores globose to subglobose. 10.5–12.0–13.3 × 10.2–11.5–12.8 µm, n = 20, Q = 1.01–1.04–1.13; ornamentation amylloid, very highly winged; ridges up to 3–4 µm high, seldom branched, rather broad and not completely amylloid but

RESULTS

Phylogeny

Fig. 1 shows the obtained ML topology based on the alignment including only ITS sequences; bootstrap (BS) values are indicated on the branches. The six new sequestrate Lactarius species from South East Asia are indicated in orange and are clearly distinct from the previously known and sequenced sequestrate milk cap species (indicated in green).

Because there was no conflict among the single loci trees in clades with a bootstrap support of 70 % or higher, a combined analysis was performed. Fig. 2 shows the obtained ML topology with BS values and Bayesian posterior probabilities (PP). ML and both Bayesian phylogenies differ only in the placement of some terminal, non-gasteroid taxa. All 3 analyses show 3 well-delimited genera in the Russulaceae (Lactarius BS 92 % - PP 100 %, Multifurca BS 100 % - PP 100 % and Russula BS 90 % - PP 100 %) but fail to support the monophyly of the genus Lactifluus. Instead, Lactifluus consistently comes out as paraphyletic and basal to the other Russulaceae genera. Lactifluus volemus, Lactifluus piperatus and Lactifluus gerardii are not included in a monophyletic ‘core’-group of Lactifluus (BS 83 % - PP 100 %) represented by Lactifluus subg. Lactifluus p.p. (excluding section Lactifluus), subg. Edules and subg. Lactariopsis.

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1. Spores winged, reticulate, usually with high ridges .... 2
2. Spores echinulate, with isolated warts or spines ....... 5
3. Spores distinctly winged, with ornamentation that is clearly exceeding 2 µm high ........ 3
4. Spore ornamentation with ridges up to 3–4 µm high, with distinct transverse striations; peridioles a strongly interwoven trichopalisade, embedded in a narrow and incrusted slime-layer .......................... L. saturnisporus 4
5. Spores ornamentation with spines up to 2.5–3.5 µm high, lacking striations; peridioles a strongly interwoven palisade to trichopalisade, without obvious slime layer .................................. L. shoreae 5
6. Spores ornamented with warts up to 0.5 µm high, lowly ornamented, with small ridges that are never more than 1 µm long .......... A. densa 6
7. Basidia only 4-spored; spines straight and slender, not branched ........................................ L. echinellus 7
8. Spores ornamented with spines up to 4 µm long ........ 5A. lactifera 8
9. Spores ornamented with spines that are at most 2 µm long ........................................ M. nanjingensis 10
10. Spores ornamented with irregular and curved spines up to 2 µm long ........................................ L. falcatus 10
11. Spores ornamented with conical to blunt spines up to 1.5 µm long ........................................ L. pomiolens 11
12. Spores ornamented with conical to blunt spines up to 1.5 µm long ........................................ M. nanjingensis 10
smell of apples seems a striking character, but more records are needed to evaluate the stability of this feature.

2. Lactarius saturnisporus Verbeken & Stubbe, sp. nov. — MycoBank MB804180; Fig. 4

Holotype. SRI LANKA, Kudawa, near Sinharaja Forest Reserve, primary rainforest with Shorea spp., 14 Dec. 2007, Verbeken 07-170 (GENT).

Etymology. Referring to the spores that are so spectacularly winged that they are reminiscent of the planet Saturn and its ring system.

Basidiocarp 15–25 mm diam, 10–15 mm high, subglobe to flattened or irregular, sometimes with minute papilla, slightly rooting. Peridium surface, minutely velutinous, chamois-leather-like, locally smooth or wrinkled, ochraceous cream coloured, sometimes with pinkish and purplish tinges. Stipe absent. Columella absent. Gleba rather soft and compressible, with labyrinthuloid and rounded loculi, 1–3 mm per mm, dull cream coloured to pale or greyish orange (6AB3) sometimes with pinkish and purplish tinges near the margin, faintly staining yellow, ultimately becoming pinkish but drying pale fawn, indistinct reaction with ferrous sulphate. Latex scarce to abundant, whitish hyaline, unchanging or slightly yellowing on the gleba, staining white paper yellow, becoming yellow in a 10 % aqueous potassium hydroxide solution. Taste bitter to astringent, disagreeable but also somewhat acrid. Smell not remarkable or somewhat like citrus fruit.

Spores globose to subglobose, 8.0–8.9–9.7 × 7.6–8.3–9.0 μm, n = 20, Q = 1.01–1.07–1.12; ornamentation amyloid, very highly winged; ridges up to 3–4 μm high with distinct transversely with strongly amyloid tranversal bands; surface with amyloid spots between the ridges; plage not distinct, not amyloid. Basidia 40–55 × 5–13 μm, slender and cylindrical to subclavate, 4-spored, thin-walled, hyaline; sterigmata up to 8 μm long. Pseudocystidia present, irregular, sometimes branching, not emergent, 5–7 μm diam. Cystidia extremely abundant, mostly cylindrical, subclavate or clavate and regularly rounded on top, sometimes fusiform, 25–60 × 10–16 μm, with very dense needle-like and yellowish brown contents, with walls slightly refringent to very slightly thickened. Peridiopellis an ixocutis, composed of intricate, mostly pericline hyphae, 3–5 μm diam, sometimes with small bulges.

Habitat — Rainforest with Shorea sp. and Dipterocarpus sp.

Specimen examined. SRI LANKA, Kudawa, near Sinharaja Forest, trail along river, on sandy wet soil in rainforest with Shorea trapezifolia, Shorea disticta and Dipterocarpus hispidus, 13 Dec. 2007, A. Verbeken GENT AV07-159, holotype.

Notes — The species is outstanding because of its very large (average 12 × 11.5 μm) spores and wings. The distinct

Fig. 3 Lactarius pomiolens (holotypus). a. Basidia; b. pseudocystidia; c. basidiospores; d. cystidia; e. peridiopellis. — Scale bar = 10 μm.

Fig. 4 Lactarius saturnisporus (holotypus). a. Basidiospores; b. basidia; c. cystidia; d. pseudocystidia; e. peridiopellis. — Scale bar = 10 μm.
striped and bifurcating pattern, mostly unbranching, sometimes branched but never forming a reticulum, edges sharp and mostly crenate; surface roughly amyloid and verrucose in between the ridges; plage not distinct, not amyloid. Basidia 4-spored, subcylindrical, 45–60 × 10–12 µm, thin-walled, hyaline or with some oil-drops; sterigmata up to 8 µm long. Cystidia present in the hymenial cavities, rather abundant, variable in shape, some clavate, some slightly utriform, 20–45 × 10–15 µm, with slightly thickened wall, hyaline. Pseudocystidia less abundant, cylindrical to somewhat tortuous, 4–6 µm diam. Peridiopellis a strongly interwoven trichopalisade, embedded in a narrow and incrusted slime-layer, some small globose elements present but rare, terminal elements usually on top of intricate and short hyphae; terminal elements cylindric to subclavate, 20–25(45) × 4–7 µm, some with a prominent needle-like content, thin-walled. hyphae; terminal elements cylindric to subclavate, 20–25(45) × 4–7 µm, some with a prominent needle-like content, thin-walled.

Habitat — Primary tropical forest with Shorea spp.

Notes — The species is easily recognized among most other known angiocarpous Lactarius species because of the extremely high wings in the spore ornamentation. With a height of 3–4 µm on relatively small spores, they are so far known, the highest winged Lactarius spores. They do share this character with *L. pomiolens*, which has a similar high ornamentation up to 4 µm, but remarkably larger spores (see further).

3. *Lactarius shoreae* Stubbe & Verbeken, sp. nov. — Myco-Bank MB804181; Fig. 5

Holotype. *Sri Lanka*, near Shinharaja Forest, primary rainforest with Shorea spp., 13 Dec. 2007, *A. Verbeken* GENT 07-164 (GENT).

Etymology. Referring to the association with the ectomycorrhizal host Shorea spp.

Basidiocarp 15 mm diam, 10 mm high, irregular. *Peridium* irregularly shaped, with bulges and folds; pale yellow (2A3), in some places darker; surface smooth, showing the loculi by transparency. *Stipe* absent. *Columella* absent. *Gleba* with rounded and labyrinthuloid, small loculi, buff, pale yellow (4A3). *Latex* white, rather abundant, but soon after cutting becoming hyaline and disappearing. *Taste* mild, very dry. *Smell* not very remarkable, a bit sweetish and rubber-like.

Spores globose to subglobose, 7.9–9.1 × 7.6–8.7–9.9 µm, n = 20, Q = 1.01–1.04–1.10; ornamentation amyloid, very highly winged; ridges up to 2.5–3.5 µm high, sharp, mostly unbranching, sometimes branched without forming a reticulum; surface roughly amyloid and verrucose in between the ridges; plage not distinct, not amyloid. *Basidia* 4-spored, subcylindric to subclavate, 40–60 × 12–15 µm, thin-walled, hyaline, sometimes with oil-drops; sterigmata up to 7 µm long. *Cystidia* present in the hymenial cavities where they occur dispersed between the basidia but also locally clustered, hyaline, thin-walled (occasionally slightly thick-walled parts are observed), very variable in shape, some fusiform or very narrow, others irregular and somewhat knotty. *Pseudocystidia* rare, cylindrical, 4–6 µm diam. *Peridiopellis* a strongly interwoven palisade to trichopalisade, with very small globose cells present; terminal elements partly anticline, but sometimes adpressed and intricate, cylindrical, 10–20 × 4–6 µm, with thin or slightly thickened walls.

Habitat — Primary forest with *Shorea* spp.

Specimen examined. *Sri Lanka*, Kodawa, near Shinharaja Forest Reserve, primary rainforest with Shorea spp., 14 Dec. 2007, *A. Verbeken* GENT AV07-170, holotype; Kodawa, near Shinharaja Forest Reserve, alongside Pitakele river with mostly *S. trapezifolia* and some *Dipterocarpus hispidus* stands, half buried in the soil near *Shorea* spp., 13 Dec. 2007, *D. Stubbe* GENT DS07-488, DS07-490.

Notes — Like the previous species, *L. saturnisporus*, this species has rather small (< 10 µm) but highly winged spores. It differs with *L. saturnisporus*, however, by the lower wings (2.5–3.5 µm) without striations, and the lack of a slime layer in the peridiopellis.

4. *Lactarius echinellus* Verbeken & Stubbe, sp. nov. — Myco-Bank MB804184; Fig. 6

Holotype. *Sri Lanka*, near Shinharaja Forest, 13 Dec. 2007, *A. Verbeken* GENT AV07-164, holotype.

Notes — Like the previous species, *L. saturnisporus*, this species has rather small (< 10 µm) but highly winged spores. It differs with *L. saturnisporus*, however, by the lower wings (2.5–3.5 µm) without striations, and the lack of a slime layer in the peridiopellis.

Basidiocarp globose to irregularly subglobose and even knotty, somewhat flattened, 15–35 mm diam, often with short rhizomorphs. *Peridium* smooth but mostly with several pleats, sometimes with venose wrinkles, forming a thin layer (< 1 mm thick) around the gleba, somewhat translucent revealing loculoid structure underneath; surface glabrous with chamois-leather-like patches, dry, often pruinose and whitish in pleats and dents, predominantly buff to pale ochraceous, reddish blond to brownish orange (5C4–5), slightly more ochraceous (FK13–14), pale yellow to pale orange locally (4A3–5A3) with some small whitish cracks. *Stipe* absent. *Columella* absent. *Gleba* with very small, round or labyrinthuloid, irregular loculi (± 3 mm), firm, hardly compressible, pale yellow to greyish yellow (4A3–4B4), dark cream coloured, mostly with a pinkish tinge after exposure, pinkish buff to pale orange (5A3) in older specimens. *Latex* white, abundant, thick and sticky, unchanging...
or staining the gleba slightly pinkish, drying soon and leaving a whitish layer on the gleba, not hyaline at all, unchanging with a 10% aqueous potassium hydroxide solution. Smell distinct but variable sweetishly rancid or motor oil, boiled rice. Taste mild, immediately very dry, then mild. Both gleba and peridium unchanging with ferrous sulphate.

**Spores** globose to subglobose, 6.8–7.4–7.8–8.6(8.8) × (5.8)6.0–6.4–6.6–7.1 µm, n = 40, Q = 1.10–1.16–1.19–1.29; apiculus 2–4 µm long; ornamentation echinate, composed of long, isolated spines up to 2.5 µm, rather slender and straight, sometimes slightly curved, rounded on top, not acute. **Basidia** 4-spored, some subclavate, some very long and narrowly cylindrical, but mostly irregularly shaped, 25–40(55) × 8–12 µm, sterigmata up to 5 µm long. **Cystidia** absent. **Pseudocystidia** present, irregular, tortuous to moniliform, 2–4 µm diam. **Lactifers** very abundant in the gleba. **Peridioellae** a loose ixotrichoderm; terminal elements irregularly shaped and branched, with intricate finger-like bulges, 10–20 × 2–10 µm, some locally with thickened wall.

**Notes** — **Arcangelilla lactifera** (basionym: **Gymnomyces lactifer** B.C. Zhang & Y.N. Yu) is a similar species described from China. It is obviously similar to **L. echinus** and **L. echinellus** owing to the spores ornamented with isolated spines. It shares the 2-spored basidia with **L. echinus** but the spores are distinctly smaller: 8–10 µm. Macroscopically the species is also characterized by globose, subglobose to flattened or irregular basidomata without stipe or columnella, a pale peridium and white milky latex. The peridioellae, however, is described to be a layer of repent hyphae.

White milky latex is also present in the Chinese angiocarpous species **Zelleromyces rassinipes** (basionym: **Martellia rassinipes** B.C. Zhang & Y.N. Yu), which differs by the striking spore ornamentation where 2–2.5 µm high spines have double or triple forked tips and the peridioellae which is also a cutis. Another gasteroid **Russulales** representative with spores bearing isolated spines is **Arcangelilla densa** (basionym: **Elasmostyles densus** R. Heim), described from Thailand. The species has a better developed stipe than the ones proposed here, but we doubt whether this is a constant feature as intermediates between sequestrate species with a well-developed stipe and true angiocarpous species without stipe are possible. A more important difference is the peridioellae which is an ixocutis resulting in a viscid peridium which is ochraceous and zonate. Heim (1959) suggests a connection with **Lactarius** species in L. section **Zonarii**. Judging from his drawings, the spines ornamenting the spores are also rather short compared to our Sri Lanka species.

**5. Lactarius echinus** Stubbe & Verbeken, sp. nov. — Myco-Bank MB804183; Fig. 7

*Holotype.* **SRI LANKA,** near Sinharaja Forest, primary rainforest with **Shorea spp.,** 14 Dec. 2007, **Verbeken** sp. nov. — **Etymology.** Latin for sea urchin or hedgehog, referring to the spores that are large, round and distinctly echinate.

**Basidiocarp** globose to subglobose, 10–15 mm diam. **Peridium** light orange to greyish orange (5AB4), smooth, slightly felty, in some places wrinkled, rugulose or strongly rugulose and deeply grooved, slightly pinkening after cutting. **Stipe** absent. **Columella** absent. **Gleba** greyish orange to brownish orange (5BC5), with very labyrinthuloid loculi. **Latex** white, very scarce to rather abundant. **Taste** mild. **Smell** distinctly of **Geranium robertianum,** but in other specimens not remarkable.

**Spores** globose to subglobose, 9.6–11.8–12.0–14.0(14.3) × 9.4–11.2–11.4–13.4 µm, n = 40, Q = 1.01–1.04–1.07–1.15; apiculus up to 5 µm long; ornamentation amyloid, echinate, composed of long, isolated spines; spines up to 4 µm long, rather slender and straight, sometimes slightly curved, rounded on top, not acute. **Basidia** 2-spored, some subclavate, but mostly irregularly shaped, 20–35 × 8–14 µm, sterigmata up to 5 µm long. **Cystidia** absent. **Pseudocystidia** present, tortuous to moniliform, sometimes branched, 2–4 µm diam. **Lactifers** very abundant in the gleba. **Peridioellae** a palissade to trichopalisade, embedded in a thin and strongly incrusted slime-layer; terminal elements usually on a chain of subglobose, small elements or short hyphal parts; terminal elements clavate to irregularly subglobose, 10–17 × 4–14 µm, sometimes with slightly thickened wall.

**Notes** — Within the angiocarpous species with echinate spore ornamentation, **L. echinus** is easily characterized by its very large spores (average 11.8 × 11.2 µm). Such spores are exceptionally large for the genus, but were also observed in **L. pomicolens,** though clearly different because highly winged. In **Lactarius,** the species with the largest spores are species...
with 2-spored basidium (such as *L. acerrimus*). In contrast to *L. pomiolens*, which has strictly 4-spored basidia, this is the case here as well, but surprisingly the basidia themselves are very small. Besides giant spores in *L. echinus*, some smaller spores are also observed, probably produced by 2-spored instead of 2-spored basidia. Four-spored basidia could not be observed, however a single 1-spored basidium was recorded.

### 6. *Lactarius falcatus* Verbeken & Van de Putte, sp. nov.

*MycoBank MB804185; Fig. 8*

**Holotype.** THAILAND, Chiang Mai Prov., Mae Tang District, Ban Mae sae village, 18 June 2008, K. Van de Putte 08-038 (holo GENT; iso MFU).

**Etymology.** Latin for sickle-shaped, curved (like the wings of a falcon), referring to the shape of the spines on the spores.

**Basidiocarp** globose to subglobose, 17–22 mm diam. **Peridium** brown (6E6, but paler) in upper part, part buried in soil paler brown to buff (4A3, with brown tinge), smooth. **Stipe** absent. **Columella** absent. **Gleba** cream-coloured (3A3), discolouring pale greyish brown with light orange pinkish tinge (5A4). **Latex** moderately abundant, immediately bright pale yellow (1A4). **Taste** unknown. **Smell** unremarkable.

**Spores** globose to subglobose, (8.8)9.1–10.1–11.1 × 8.5–9.3–10.1 μm, n = 40, Q = 1.01–1.07–1.13; apiculus 3–4 μm long; ornamentation echinate, composed of long, isolated spines; spines up to 1.5(2) μm long, rather blunt and somewhat irregular, often curved, rounded on top, seldom acute. **Basidia** 2-spored, some subclavate, some with remarkable narrower part in the middle, mostly irregularly shaped, 35–45 × (3)7–10 μm, sterigmata up to 5 μm long. **Cystidia** absent. **Pseudocystidia** present, cylindrical, 2–4 μm diam. **Peridiopellis** a loose layer of intricate hyphae, arranged perincially as well as antically,

### DISCUSSION

A striking diversity of sequestrate *Russulales* was encountered during these expeditions in tropical South East Asian forests. Six new species are described here and are phylogenetically placed in the genus *Lactarius*. We also found one angiocarpous *Russula* species which will be described in a separate paper (Hampe et al. In prep.). Worldwide, all known species of sequestrate milkcaps so far belong to the genus *Lactarius*; none are described in the genus *Lactifluus* (Verbeken & Nuytinck In press). The angiocarpous habit evolved several times in the genus and has been demonstrated in *L. subg. Russularia*, L. subg. *Piperites* and L. subg. *Plinthogalus*. The species described here largely confirm this: *L. echinellus*, *L. echinus*, *L. satuninisorus* and *L. shoreae* are included in *L. subg. Plinthogalus*. *Lactarius falcatus* is a representative of *L. subg. Russularia*. *Lactarius pomiolens* has a rather isolated position and cannot be confined with cer-
tainty to one of the existing subgenera. There seems to be a close relation with the African L. kabansus and L. tenellus, for which recent phylogenetic studies show that a new infrageneric group has to be created (Stubbe 2012). Studying Arcangelelia, Thiers (1984b) stated that there are two major evolutionary lines: A. borziana and A. densa (Thailand) have basidiospores ornamented with spines and rods, while the other line, represented by American and perhaps Australian species, has spores with either a broken or a complete reticulum. Both types of spore ornamentation are also encountered here, but it turns out that they do not represent true evolutionary lines and the spore ornamentation is not a phylogenetic informative feature in these. It is even striking that in L. subg. Phyllophagus, a subgenus characterized so far by reticulate to highly winged spores, two species with isolated spines are occurring.

Wilson et al. (2011) show that the gasteromycete lineages within the Agaricomycetes might now be diversifying at rates comparable to, or exceeding, those of their nongasteroid relatives. Their analyses suggest that the net diversification rate of gasteroid forms exceeds that of nongasteroid forms, and that gasteroid forms will eventually come to predominate over nongasteroid forms in the clades in which they have arisen. The low number of gasteroid forms in the Agaricomycetes as a whole may reflect the relatively recent origin of many gasteroid lineages. The even more recent origin of gasteromycetization in the order Russulales is suggested by several observations. Firstly, the anatomy of the basidiomes is relatively simple and in no way comparable to the complex and specialized tissues found in highly evolved gasteroid groups such as Sclerodermatinae or Phallomyctidae. Secondly, none of the gasteroid lineages in Lactarius has evolved into a clade containing a diversity of species. On the contrary, the gasteroid species appear as independent and isolated incidents within the phylogeny. Hibbett et al. (1994) suggested that the genetic mechanisms resulting in the initial stages of gasteromycetization could be rather simple. It is generally assumed that dry climatic conditions are one of the driving forces that enhances the development of sequestrate fruiting bodies. However, gasteroid species occur also in Europe and North America in temperate climates, and the current findings demonstrate a strong presence in tropical rainforests as well. In Sri Lanka for instance, the number of known sequestrate Lactarius species now exceeds the number of known agaricoid species (Pegler 1986, Stubbe 2012). The exploration rate is rather low and the period of sampling may play an important role in the number of sequestrate fungi we encountered, but we assume that angiocarpous mushrooms are overlooked in these regions and that the phenology and ecology of these tropical rainforest angiocarpous species deserve further investigation. It seems likely to us that more sequestrate species are to be discovered in rainforest biotopes. Another impression we have from several expeditions is that production of angiocarpous basidiomes does not necessarily coincide with the seasonality of the majority of macromycetes. During our expedition in Sri Lanka, agaricoid mushrooms were not abundant, even scarce. The intensified search efforts lead us to find a greater number of small and inconspicuously growing species, among which many false truffles, such as the species presented in this paper. Perhaps the tropical angiocarpous species flourish during periods when unfavourable weather conditions cause too much stress for most agaricoid species. Another hypothesis is that angiocarpous fructifications are less susceptible to – or less dependent on – seasonal changes and are abundant year-round, only to be overlooked during the fructification season of the other mushrooms. The phenology and ecology of these tropical rainforest angiocarpous species deserve further investigation.

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