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Research Article


A new section, *Lactifluus* section *Neotropicus* (Russulaceae), and two new *Lactifluus* species from the Atlantic Forest, Brazil

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Lactifluus is a monophyletic genus of ectomycorrhizal fungi with about 200 described species classified in four subgenera: *L.* subg. *Gymnocarpus*, *L.* subg. *Lactariopsis*, *L.* subg. *Lactifluus*, and *L.* subg. *Pseudogymnocarpus*. Currently *L.* subg. *Lactariopsis* is considered monophyletic with approximately 57 species described and classified in four sections: *Albati*, *Edules*, *Lactariopsis*, and *Russulopsidei*. There are few species of *L.* subg. *Lactariopsis* described from the Neotropical region, however local mycologists commonly collect specimens of this subgenus during their mycological surveys. The goals of the present study are to evaluate the phylogenetic and morphological relationships of the species from *L.* subg. *Lactariopsis* found in Brazil, to contribute to the knowledge of *Lactifluus* in Neotropical ecosystems by describing a new section in this subgenus and two new species from Brazil, and to document *L. neotropicus* as a new record from Brazil. Based on morphological and phylogenetic evidence we propose *Lactifluus* sect. *Neotropicus* as a new section and *Lactifluus catarinensis* and *L. marielleae* as two new species in this section. We provide a key to the species of *L.* sect. *Neotropicus*, which is shown to be a monophyletic group of Neotropical species within *L.* subg. *Lactariopsis*. The most striking characteristic of several species in *L.* subg. *Lactariopsis* is the secondary angiocarpic development, not present in the other subgenera. Species with a secondary veil can be found in two lineages that diversified more recently than the rest of the genus: *L.* sect. *Lactariopsis* and *L.* sect. *Neotropicus*. Species that show morphological affinities with the two new species in *L.* sect. *Neotropicus* described here are commonly found and we expect a greater diversity to be revealed of this section in the Neotropical region.

Key words: ITS, *Lactarius*, milkcaps, morphology, phylogeny, 28S

Introduction

Lactifluus (Pers.) Roussel (Russulaceae, Basidiomycota) is a genus of ectomycorrhizal fungi with about 200 accepted species (He et al., 2019) distributed worldwide, which reaches its greatest diversity in the tropics. Together with members of the genus *Lactarius* Pers., species are popularly known as milkcaps (Verbeke & Nuytinck, 2013). Progress towards generating a

phylogeny-based classification of *Lactifluus* and allied genera has been accelerated recently by several molecular systematic studies of the group (Buyck et al., 2008; De Crop et al., 2017; Verbeke, Stubbe, Van de Putte, Eberhardt & Nuytinck, 2014). *Lactifluus* is monophyletic and classified in four subgenera: *L.* subg. *Gymnocarpi* (R. Heim ex Verbeke) De Crop, *L.* subg. *Lactariopsis* (Henn.) Verbeke, *L.* subg. *Lactifluus* and *L.* subg. *Pseudogymnocarpi* (Verbeke) De Crop (De Crop et al., 2017).

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Lactariopsis (Henn.) Verbeken was erected by Hennings (1901) as a closely related genus to *Lactarius* based on the basidiome development with an involute pileus margin connected with the stipe by a membranaceous velum in *Lactariopsis zenkeri* Henn. The remaining characters described were equal to those found in *Lactarius*. Heim (1937) proposed the term pseudoangiocarpic development to describe the kind of development found in species with a velum. Heim (1938) and Singer (1942) considered pseudoangiocarpic development as a weak character to define the genus *Lactariopsis*, and consequently, Heim (1938) considered *Lactariopsis* as a subgenus in the genus *Lactarius*. Singer (1942) considered them at sectional level: *L. sect. Lactariopsidae*. In a synopsis for *L. subgenus Lactariopsis*, Verbeken (1998a) adopted the term secondary angiocarpic development instead of pseudoangiocarpic development (after Reijnders, 1963) and considered the creation of a subgenus based on the secondary angiocarpic development as artificial. Hence, Verbeken (1998b) emended and proposed a larger definition of the subgenus *Lactariopsis* in which species with secondary angiocarpic development and other species with close morphological affinities were included. After *Lactarius* was split into three genera (Buyck *et al.*, 2008; Buyck, Hofstetter, Verbeken & Walley, 2010), *Lactarius*, *Lactifluus* and *Multifurca* Buyck & V. Hofst., the subgenus containing species with a velum was found to belong within *Lactifluus* and consequently it was recombined to *Lactifluus* subg. *Lactariopsis* by Verbeken, Nuytinck and Buyck (2012).

Currently *L. subgenus Lactariopsis* is considered monophyletic (De Crop *et al.*, 2017) with 57 species described. Four sections have been described, *L. sect. Albati* (Bataille) Verbeken, *L. sect. Edules* (Verbeken) Verbeken, *L. sect. Lactariopsis* and *L. sect. Russulopsidae* (Verbeken) Verbeken. Seven more monophyletic clades were recognized by De Crop *et al.* (2017). Of these seven, two are entirely Neotropical: Clade 2 and Clade 3. Clade 2 contained *L. annulifer* (Singer) Nuytinck, *L. subiculatus* S.L. Mill., Aime & T.W. Henkel, *L. venezuelanus* (Dennis) De Crop and an undescribed species from French Guyana. In Clade 3 grouped *L. multiceps* (S.L. Mill., Aime & T.W. Henkel) De Crop and another undescribed species from French Guyana (De Crop *et al.*, 2017). Sá *et al.* (2019) described *L. caatingae* which also clusters in Clade 2. All species from Clade 2 have thick-walled elements in the pileipellis and this clade includes all known South American taxa with secondary angiocarpic development (De Crop *et al.*, 2017, Sá *et al.*, 2019).

Despite the few species of *L. subgenus Lactariopsis* described from the Neotropical region, it is common to collect specimens of this subgenus in mycological

surveys. It is also usual to find specimens without a specific name in the herbaria, because of the lack of knowledge on the genus in the Neotropics. Based on this, the goals of the present study are: (1) evaluate the phylogenetic and morphological relationships of the species from *L. subgenus Lactariopsis* found in Brazil, (2) contribute to the knowledge of the biodiversity of *Lactifluus* in Neotropical ecosystems by describing a new section in this subgenus and two new species in this section, and by documenting *L. neotropicus* as a new record from Brazil.

Materials and methods

Collect and morphological studies

Specimens were collected in dense ombrophilous forest, Brazilian Atlantic Rain Forest, in Minas Gerais, Rio de Janeiro, Santa Catarina, and Paraíba states (Fig. 1). We used the opportunistic sampling method in seven collecting points: Benjamim Maranhão Botanic Garden (Paraíba State), Mangabeiras City Park (Minas Gerais State), Serra dos Órgãos National Park and Tijuca National Park (Rio de Janeiro State), Lagoa da Conceição, Pântano do Sul to Lagoinha do leste trail and Santo Amaro da Imperatriz (Santa Catarina State). Collection points were georeferenced and mapped through a free and open-source software QGIS 3.6 (QGIS Development Team 2019), with standards base-map of GADM (Global Administrative Areas [GADM], 2019) and WorldClim 2 (Fick & Hijmans, 2017). The specimens were photographed in the field and macroscopically described. The collections were examined following standard mycological methods (Largent, Johnson & Watling, 1980; Mueller, Bills & Foster, 2004), and later dried in a food dehydrator for 8 h at approximately 40 °C. Colour codes follow Kramer (2004; e.g., oac758) or Kornerup and Wanscher (1978; e.g., 6E4) colour guides. Microscopic features were studied from dried material by mounting free-hand sections of the basidiomata in 5% KOH, Melzer's reagent, or Congo red. Cresyl blue was used to verify the presence of ortho- or metachromatic reactions in cross sections. The descriptions of the pellis structures follow Verbeken (1998a). The basidiospore ornamentation is described and illustrated as observed in Melzer's reagent. Basidiospores were measured in side view in Melzer's reagent, excluding the ornamentation. The Q value denotes the length/width ratio of the basidiospores ($n = 30$ per specimen), the value is given as lowest – average – highest Q value of all specimens examined. Basidia, cystidia, marginal cells, and pileipellis were measured as observed in KOH. Measures are given as lowest – average – highest

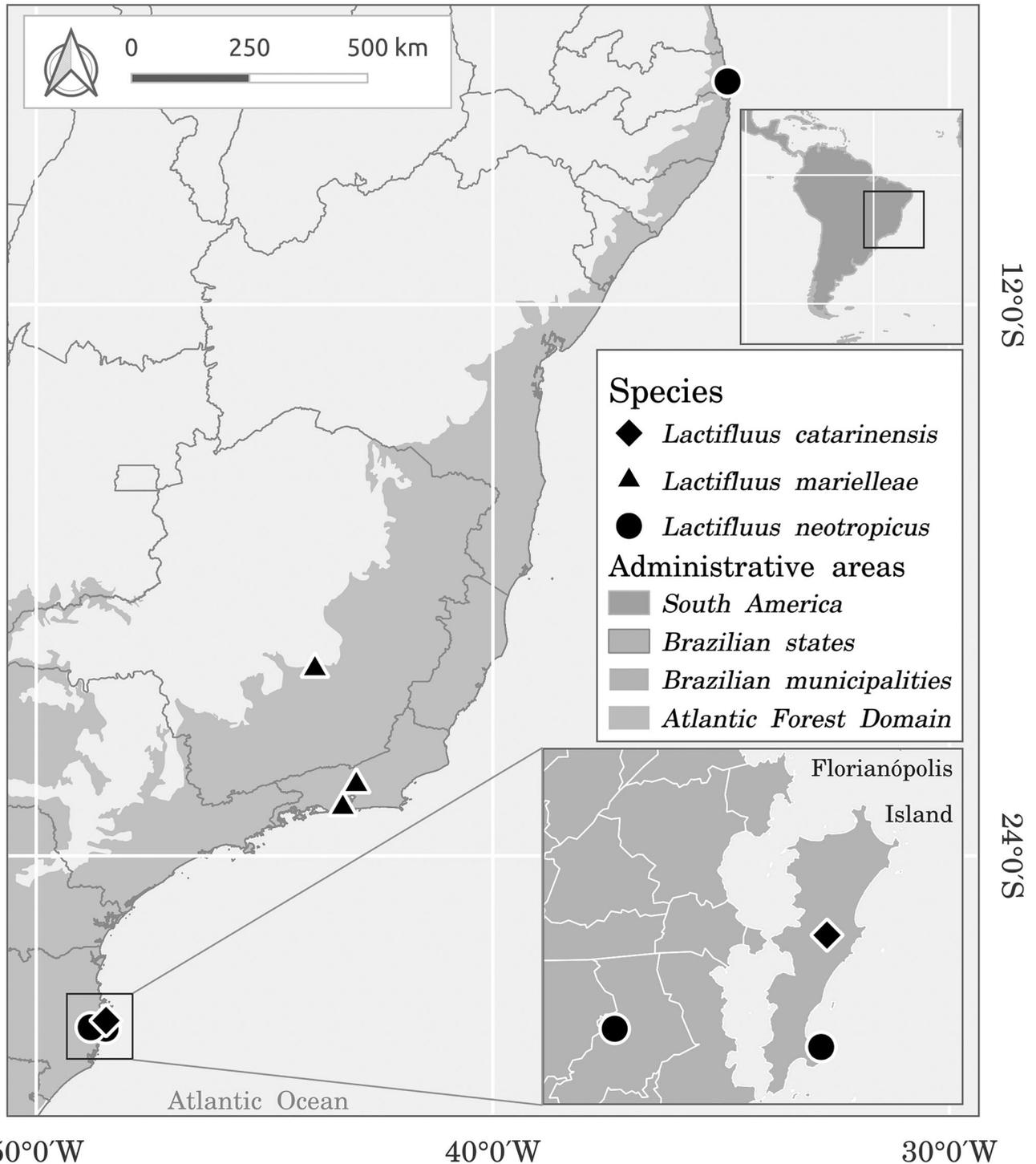


Figure 1. Sampling areas of *Lactifluus catarinensis*, *L. marielleae*, and *L. neotropicus*.

value for length and width ($n=30$ per specimen). To perform the scanning electron microscopy (SEM) studies of spores, fragments of the hymenophore were removed from dried basidiomata, mounted on aluminium stubs with carbon adhesive tabs, and coated with

30 nm of gold with a sputter coater (Balzers SCD030 – Balzers Union FL9496). The basidiospores were examined with a JSM-6360LV scanning electron microscope operating at 10 keV. The specimens are deposited at herbarium FLOR (Universidade Federal de Santa

Catarina, Florianópolis, Brazil) and herbarium RB (Botanical Garden of Rio de Janeiro, Rio de Janeiro, Brazil). Herbaria acronyms follow Thiers (2019).

DNA extraction, PCR, and sequencing

In the field, a small portion of the basidioma was preserved in silica gel, a FTA card (Flinders Technology Associates), or in 2% cetyltrimethylammonium bromide (CTAB) for DNA extraction. DNA extraction was made using one of the following protocols: (i) Dentinger *et al.* (2010), (ii) MasterPure™ Yeast DNA Purification kit (Epicenter, Madison, WI), or (iii) Wizard Genomic DNA Purification kit (Promega Corp., Fitchburg, USA). Two nuclear ribosomal DNA regions were amplified: the internal transcribed spacer (ITS 1-2) regions and the Δ 1/D2 regions of the large subunit rDNA 28S rDNA. The pairs of primers ITS8-F + ITS6-R (Dentinger *et al.*, 2010) and ITS1f + ITS4 (Gardes & Bruns, 1993; White, Bruns, Lee & Taylor, 1990) were used to amplify the ITS region. The 28S region was amplified using the pair of primers LR0R + LR7 (Vilgalys & Hester, 1990) and CTB6 + TW14 (Taylor & Bruns, 1999). The polymerase chain reaction (PCR) followed two protocols: (1) Dentinger *et al.* (2010) protocol; and (2) a protocol that consisted of a mix of 14.3 μ l purified water, 1 μ l bovine serum albumin (10 mg/ml), 0.5 μ l MgCl₂ (25 μ M), 5 μ l green GoTaq buffer (5 \times , Promega Corp., Fitchburg, USA), 0.5 μ l of each primer (50 μ M), dNTP (10 mM) and GoTaq DNA polymerase (1 U/25 μ l, Promega Corp., Fitchburg, USA). The PCR amplifications were performed using an Eppendorf MasterCycler thermal cycler with the following parameters: three minutes of initial denaturation at 94 °C, five denaturation cycles at 94 °C for 45 s, annealing at 55 °C for 45 s, and elongation at 72 °C for 1 min; the reaction continued for 35 (for ITS region) to 40 (for 28S region) cycles. A final elongation was performed at 72 °C for 10 min.

The amplification products were checked on agarose 1% gel with SightDNA (Euromedex, Souffelweiersheim). The PCR products were then sequenced following Dentinger *et al.* (2010) and sent to GATC Biotech (Konstanz, Germany) or Macrogen (Lille, France). Sequence chromatograms were corrected using the software Geneious v. 6.1.8 (Kearse *et al.*, 2012), Sequencher 5.1 (Gene Codes Corp.) or DNASTart from SeqMan (Swindell & Plasterer, 1997).

Phylogenetic analyses

Our analysis included 137 ITS and 112 28S sequences, 23 were newly generated sequences and 113 retrieved

from the GenBank database (Supplementary Table S1). The ITS + 28s final aligned matrices include 137 terminals. Eighty-eight terminals are from taxa in *L.* subg. *Lactariopsis*, corresponding to 56 species from all the sections and clades in De Crop *et al.* (2017), 13 terminals from the subgenus *Gymnocarpi* corresponding to 10 species, five terminals from the subgenus *Pseudogymnocarpi* corresponding to four species and seven terminals from the subgenus *Lactifluus* corresponding to seven species. Twenty-three terminals from species of *Lactarius*, *Multifurca*, and *Russula* were used as outgroup taxa. The alignment was made using MUSCLE (Edgar, 2004), and was then manually adjusted with AliView (Larsson, 2014). The dataset was divided into four data partitions: ITS1, 5.8S, ITS2, 28S. The final alignment and the resulting phylogenetic trees were deposited at TreeBASE (S24546).

Maximum likelihood (ML) analyses were conducted using Standard RAxML version 8.2.11 (Stamatakis, 2014). The ML analyses involved 100 ML searches, each one starting from one randomized step-wise addition parsimony tree, under a GTRGAMMA model, with all other parameters estimated by the software. To verify the reliability of the nodes, multi-parametric bootstrapping replicates under the same model were computed, allowing the program to halt bootstrapping automatically using the autoMRE option.

The Bayesian analyses (BI) were performed with the software MrBayes 3.2.6 (Ronquist & Huelsenbeck, 2003) in the CIPRES Science Gateway 3.1 (Miller, Pfeiffer & Schwartz, 2010). Bayesian analyses were implemented by two independent runs, each one starting from random trees, with four simultaneous independent chains, and performed 20,000,000 generations, keeping one tree every 1,000th generation. Four rate categories were used to approximate the Gamma distribution, and the nucleotide substitution rates were fixed to the estimated values. Of all trees sampled, 25% were discarded as burn-in and checked by the convergence criterion (frequencies of average standard deviation of split <0.01) in Tracer v.1.7.1 (Rambaut & Drummond, 2018). The remaining trees were used to reconstruct a 50% majority-rule consensus tree and to estimate Bayesian posterior probabilities (hereafter BPP) of the branches. A branch was considered strongly supported if it showed a BPP \geq 0.95 and/or bootstrap support (hereafter BS) \geq 90%, while moderate support was considered BPP \geq 0.8 and/or BS \geq 70%. The trees produced were adjusted with FigTree v1.4 (Rambaut, 2012).

Results

Phylogenetic analyses

Our molecular results show that the new section *Neotropicus* in *L.* subg. *Lactariopsis* is strongly supported (BS = 100, BPP = 1; Fig. 2 and Supplementary Figs 1–5). *L.* sect. *Neotropicus* forms a moderately supported group together with *L.* sect. *Lactariopsis*, clades 1, 2, 3 and *L. cocosmus* (BS = 34, BPP = 0.89). *Lactifluus catarinensis* sp. nov., *L. marielleae* sp. nov., *L. venezuelanus* and several unidentified specimens from French Guiana form a well-supported clade (BS = 90, BPP = 1) that is closely related to the clade formed by *L. subiculatus* and two unidentified specimens from Colombia (BS = 100, BPP = 1). *Lactifluus neotropicus* has a closer relationship with *L. annulifer* (BS = 62, BPP = 0.71) than with the rest of the species of *L.* sect. *Neotropicus*. Eight clades and two isolated species were recovered within *L.* subg. *Lactariopsis*. *Lactifluus* sect. *Lactariopsis* and *L.* sect. *Albati* appeared as monophyletic, while *L.* sect. *Edules* and *L.* sect. *Russulopsis* appeared as paraphyletic. The monophyly of *L.* sect. *Lactariopsis* is doubtful, the branch linking *L.* sect. *Albati* with the rest of the subgenus is weakly supported in the Bayesian analysis (BPP = 0.83) and has a low bootstrap support in the ML analysis (BS = 32).

Taxonomy

Genus *Lactifluus* (Pers.) Roussel, Fl. Calvados, Ed. 2: 66. 1806

Basionym. *Agaricus* sect. *Lactifluus* Pers., Syn. Meth. Fung.: 429. 1801.

= *Pleurogala* Redhead & Norvell, Mycotaxon 48: 377. 1993.

≡ *Lactarius* sect. *Panuoidei* Singer, Kew Bull. 7: 301. 1952.

TYPE (automatic). *Agaricus lactifluus* L., Sp. Pl.: 1172. 1753 (= *Lactifluus volemus* (Fr.: Fr.) Kuntze).

Lactifluus subg. *Lactariopsis* (Henn.) Verbeken, Mycotaxon 118: 449. 2011

Basionym. *Lactariopsis* Henn., Bot. Jahrb. Syst. 30: 51. 1901.

≡ *Lactarius* subg. *Lactariopsis* (Henn.) R. Heim, Prodr. Fl. Mycologique Madagascar 1: 36. 1938.

= *Lactarius* sect. *Edules* Verbeken, Belg. J. Bot. 132: 176. 2000 (1999).

≡ *Lactifluus* subg. *Edules* (Verbeken) Verbeken, Mycotaxon 118: 448. 2011.

= *Lactarius* subg. *Russulopsis* Verbeken, Mycotaxon 77: 439. 2001.

≡ *Lactifluus* subg. *Russulopsis* (Verbeken) Verbeken, Mycotaxon 118: 452. 2011.

TYPE. *Lactariopsis zenkeri* Henn., Bot. Jahrb. Syst. 30: 51. 1902 (1901) (≡ *Lactifluus zenkeri* (Henn.) Verbeken).

Lactifluus sect. *Neotropicus* J. Duque, L. Delgat, A. Verbeken, M. A. Neves & Anibal A. Carvalho Jr. sect. nov. – MycoBank: MB 831448.

TYPE: *Lactarius neotropicus* Singer, Kew Bull. [7]: 299 (1952). (≡ *Lactifluus neotropicus* (Singer) Nuytinck).

ETYMOLOGY: *neotropicus*, because the type species is *Lactifluus neotropicus* and it contains only neotropical species.

DIAGNOSIS: Secondary veil may be present or absent. The amyloid ornamentation of the basidiospores is composed of hemispherical to irregularly shaped low warts and ridges, forming an incomplete reticulum. Pileipellis a palisade to lampropalisade, with thin- to thick-walled hair-shaped terminal elements and the subpellis composed of thick-walled isodiametric cells.

Description

Agaricoid basidiomata; pileus small to medium sized, firm; surface dry, azonate, with pale yellow, orange, brownish orange, ochraceous, light brown, rusty brown, brownish red, greyish red to dull red colours. Context white, pale orange to cream, unchanging; latex scant to absent, watery, unchanging. Lamellae adnate, adnexed to decurrent, close to subdistant, occasionally forking. Secondary velum present or absent. Stipe central, cylindrical, firm, dry, more or less concolorous with pileus. Basidiospores globose, subglobose, broadly ellipsoid to ellipsoid; ornamentation amyloid, composed of hemispherical to irregularly shaped low warts and ridges, forming an incomplete reticulum; sometimes with an amyloid suprahilar spot. Basidia clavate, one to four spored. Macrocystidia sometimes present. Pseudocystidia present, scarce to abundant. Pileipellis a palisade to lampropalisade, with thin to thick-walled hair-shaped terminal elements; subpellis with thin- to thick-walled isodiametric cells.

Key to the Neotropical species of *Lactifluus* section *Neotropicus*:

The characters of *L. caatingae* are taken from Sá et al. (2019). The characters of *L. annulifer* are taken from Singer (1952). The characters for *L. subiculatus* are taken from Miller, Aime and Henkel (2012) and for *L. venezuelanus* Dennis (1970) and Pegler and Fiard (1979):

1. Secondary velum present. Pileus ochraceous, brownish red, greyish red, dull red to flesh colour. Pileipellis a lamprotrichopalissade2

1'. Secondary velum absent. Pileus lemon colour, pale yellow, ferruginous, brownish orange, ochraceous-buff, light orange, reddish orange, brownish orange, light



Figure 2. ITS + 28s ML tree for the genus *Lactifluus* showing the relationship of the section *Neotropicus*. Branches with strongly supported values, bootstrap values (BS) $\geq 90\%$ and posterior probabilities (BPP) = 0.9, are in bold. The supports are shown on the branches as BS/BPP. Species in bold letters represent the new produced sequences. * is in the representative specimen for type specimens for a species. a. Isolated species 1, b. Isolated species 2, c. species classified in *L.* sect. *Edules*, d. species classified in *L.* sect. *Russulopsidei*.

brown to yellowish brown. Pileipellis a palisade to lampropalisade3

2. Pileus ochraceous to flesh colour. Basidiospores $8.8\text{--}13 \times 8\text{--}10 \mu\text{m}$. Macrocystidia present. Elements of the suprapellis $57\text{--}187 \times 5.3\text{--}9.3 \mu\text{m}$, sometimes forked..

L. annulifer

2'. Pileus brownish red, greyish red to dull red. $5.9\text{--}9.6 \times 5.4\text{--}8.8 \mu\text{m}$. Macrocystidia absent. Elements of the suprapellis $67.77\text{--}120.25 \times 8.6\text{--}8.6 \mu\text{m}$, not forked..... *L. neotropicus*

3. Macrocystidia present (scarce). Needle-shaped elements in the pileipellis absent..... *L. catarinensis*

3'. Macrocystidia absent. Needle-shaped elements in the pileipellis present 4

4. Pileus reddish orange, light orange to tan colour with brownish tinges. Pileipellis a palisade with thick-walled isodiametrical cells; needle-shaped elements in the pileipellis with thin wall..... *L. marielleae*

4'. Pileus ferruginous, brownish orange, light brown, yellowish brown, lemon to ochraceous-buff colour. Pileipellis a palisade to lampropalisade with thin-walled isodiametrical cells; needle-shaped elements in the pileipellis with thickened wall55'. Pileus light brown to yellowish brown. A distinct subiculum present. Basidia of $45\text{--}60 \times 15\text{--}18 \mu\text{m}$ *L. subiculatus*

Pileus rusty brown, brownish orange, lemon to ochraceous-buff colour. A distinct subiculum absent. Basidia of $28\text{--}40 \times 6.5\text{--}11 \mu\text{m}$ 6

Basidiospores $6.1\text{--}7.1 \times 5.6\text{--}6.6 \mu\text{m}$. Basidia $28\text{--}40 \times 8\text{--}11 \mu\text{m}$ *L. caatingae*

6'. Basidiospores $7\text{--}9.7 \times 6\text{--}7.8 \mu\text{m}$. Basidia $30\text{--}40 \times 6.5\text{--}9 \mu\text{m}$ *L. venezuelanus*

Lactifluus catarinensis J. Duque, M.A. Neves & M. Jaegger sp. nov. Figs 3, 5.1, 5.2, 6.3 and Supplementary Figs 6–7.

HOLOTYPE: BRAZIL, SANTA CATARINA: Florianópolis, Lagoa da Conceição, Morro da Lagoa Trail, 20 March 2014, Altielys Magnago ACM993 (FLOR61778!) GenBank accession nos: ITS = MK937538, 28S = MK937556, MycoBank: MB 831447.

PARATYPES: BRAZIL, SANTA CATARINA: Florianópolis, Lagoa da Conceição, Morro da Lagoa Trail, 3 March 2011, Melissa Jaegger MJ22 (FLOR49445!), 10 March 2011, Melissa Jaegger MJ23 (FLOR49447!), MJ24 (FLOR4448!); 21 April 2011, Maria Alice Neves MAN754 (FLOR49461!); 11 May 2012, Altielys Magnago ACM333 (FLOR49457!); 19 March 2014, Jaime Duque J.Duque 44 (FLOR61780!), Jaime Duque J.Duque 48 (FLOR6177!); 10 November 2015, Jaime Duque J.Duque 119 (FLOR61428!), Jaime Duque J.Duque 120 (FLOR61429!); 29 December 2015 Mary L. Vanegas-León MVL31 (FLOR61423!).

ETYMOLOGY: *catarinensis*, referring to the type locality, Santa Catarina State.

DIAGNOSIS: The species is characterized by the brownish orange, pale orange to pale yellow colour of the basidiomata, the presence of two and four-spored basidia, the presence of macrocystidia, the palisadic pileipellis with a slightly gelatinous layer on the surface.

Description

Pileus 13–58 mm diam., convex, depressed, moderately depressed to infundibuliform; surface azonate, dry, smooth, brownish orange (6C6 to 7C6) in the centre, pale yellow to pale orange toward the margin; margin incurved to uplifted, rimose, smooth to finely striate.

Context firm, 1–5 mm thick, cream colour; odour none; taste not remarkable. **Lamellae** up to 3 mm wide, decurrent, close, cream to white; margin even. **Lamellulae** of different lengths. **Stipe** central, 2–43 × 3–13 mm, equal to tapering toward the base; surface longitudinally ribbed, concolorous with the pileal surface, solid to fistulose. **Latex** not observed. Basidiomata firm. **Spore print** not obtained. Solitary or gregarious. On soil.

Basidiospores (5.19–)5.58–7.47–9.74(–10.39) ±0.71 × (4.75–)5.02–6.44–8.26(–8.78) ±0.58 μm (Q = 1.01–1.16–1.32 ±0.058), globose to ellipsoid; ornamentation amyloid, composed of isolated low warts (0.14–0.39–0.86 ±0.16 μm) or united by ridges, forming an incomplete reticulum; suprahilar spot smooth, amyloid or inamyloid. **Basidia** 33.44–45.90–58.42 ±5.43 × 7.07–9.98–12.59 ±0.93 μm, clavate to subclavate, at times somewhat constricted at the apex, two to four spored. **Macrocystidia** 73–91.61–100.11 × 8.68–10.43–11.88 μm, rare, fusiform, occasionally branched, needle-like to granular content.

Pleuropseudocystidia scarce, 8.32–12.05–12.98 ±1.31 μm diameter, emerging 6.5–36.85–51.89 ±3.75 μm, arising from the laticiferous system, fusiform; content amorphous, refringent. **Lamella-edge** sterile; marginal cells 11.25–24.12–37.2 × 4.21–5.52–7.54 μm, subfusiform to fusiform, somewhat clavate, thin-walled, hyaline. **Hymenophoral trama** heteromerous, composed of sphaerocytes and scarce to abundant laticiferous hyphae.

Pileipellis a palisade; suprapellis composed of erect to oblique, slender, subcylindrical or subfusiform terminal elements, 11.3–68.29 (±13.60) × 3.35–8.55 (±1.29) μm, often septate, thin-walled, with a slightly gelatinized layer; subpellis, composed of more or less anticline series of somewhat irregular isodiametric cells, somewhat elongated, thin-walled. **Stipitipellis** a palisade; suprapellis of stout, broadly fusiform or subclavate terminal elements; subpellis thin, composed of one to three layers of globose or subglobose, cellular elements and inflated hyphae.

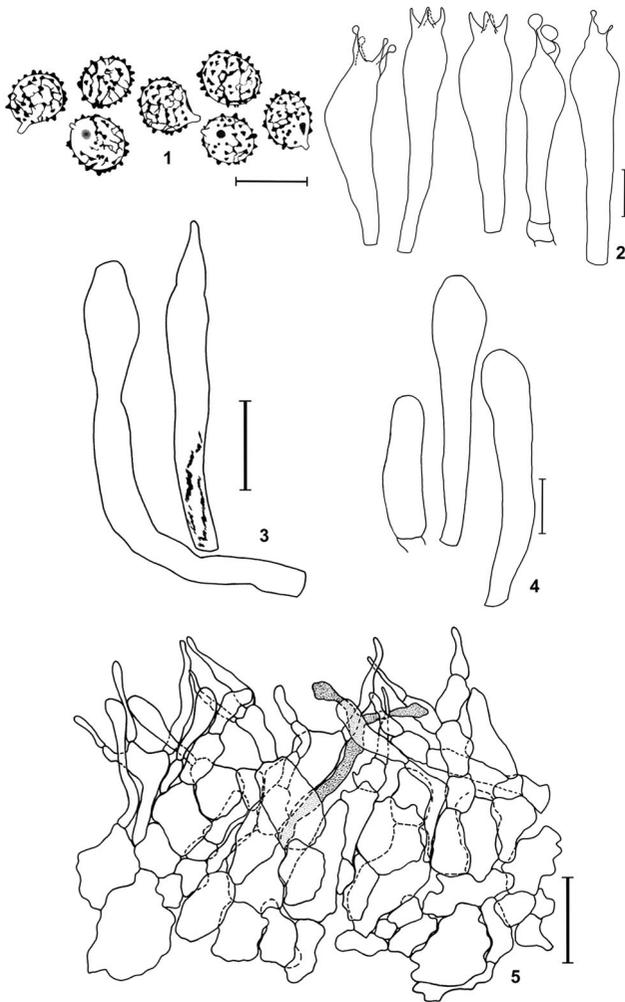


Figure 3. Microscopic structures of *Lactifluus catarinensis*. 1. Basidiospores. 2. Basidia. 3. Macrocystidia. 4. Basidiola. 5. Pileipellis. Scale bars in 1, 2 and 4 = 10 µm; 3 and 5 = 20 µm.

REMARKS: The morphologically most closely related species to *L. catarinensis* are *L. caatingae* Sá and Warchow, *L. marielleae* J. Duque sp. nov., *L. venezuelanus* and *Lactarius mamorensis* Singer. Before making any comparisons we want to take into account the following: first, *Lactarius mamorensis* probably belongs to *Lactifluus*, because of the characters used by Verbeke and Nuytink (2013): azonate, dry pileal surface and a heteromerous hymenophoral trama composed of sphaerocytes, uninflated and lacticiferous hyphae. Secondly, we base our concept of *L. venezuelanus* on the poor protologue of Dennis (1970) and the more elaborate description of Pegler and Fiard (1979). To facilitate the discussion the respective citation is included in the cases where both characters compared are found in both studies, in the remaining cases the comparison is made according to Pegler and Fiard (1979).

The main morphological characteristic to differentiate *L. catarinensis* from the other four species is the presence of a gelatinous layer in the pileipellis surface in *L. catarinensis* that is absent in the other four species. The needle-shaped elements in the pileipellis are absent in *L. catarinensis* and present in the other four species. There are macrocystidia present in *L. catarinensis* and *Lactarius mamorensis*, and absent in *L. caatingae*, *L. marielleae*, and *L. venezuelanus*. The pileipellis in *L. catarinensis*, *L. marielleae*, and *L. venezuelanus* is a palisade, and a lampropalisade in *L. caatingae*. The pileipellis description of *Lactarius mamorensis* in the protologue (Singer, Araujo & Ivory, 1983) is not unambiguous as to the type of pileipellis, however it could be interpreted as a palisade.

The morphological similarities between the five species are the azonate, dry, glabrous, smooth pileal surface, the incurved pileal margin when young, the absence of latex, except in *L. mamorensis*, the ornamentation composed of low warts and ridges that form an incomplete reticulum and the basidia with two to four sterigmata, except in *L. caatingae* which has four-spored basidia only. Additional shared characteristics between *L. catarinensis* and *Lactarius mamorensis* are the longitudinally ribbed stipe surface and the presence of macrocystidia. An additional shared characteristic between *L. catarinensis*, *L. marielleae*, and *L. venezuelanus* is the low abundance of pleuropseudocystidia and the pileipellis palisadic structure. A shared characteristic between *L. catarinensis* and *L. marielleae* is the orange colours of the basidiomata. The basidia are of similar size, $32.84\text{--}43.33\text{--}58.47 \times 8.38\text{--}9.96\text{--}12.96 \mu\text{m}$ in *L. marielleae* and $33.44\text{--}45.90\text{--}58.42 \pm 5.43 \times 7.07\text{--}9.98\text{--}12.59 \mu\text{m}$ in *L. catarinensis*. Both species have pleuropseudocystidia with amorphous, refringent content.

Lactifluus marielleae J. Duque & M.A. Neves sp. nov. Figs 4, 5.3, 5.4, 6.1, 6.2 and Supplementary Fig. 8.

HOLOTYPE: BRAZIL, MINAS GERAIS: Belo Horizonte, Mangabeiras City Park, Tatu Trail, 16 April 2011, *Leal-Dutra Caio CALD27* (FLOR49462!) GenBank accession nos: ITS = MK937527, 28S = MK937547, MycoBank: MB 831449.

PARATYPES: BRAZIL. RIO DE JANEIRO: Rio de Janeiro, Tijuca National Park, on soil, 7 February 2017, *Heiseke Celeste CHC376* (RB786092!); Teresópolis, Serra dos Orgãos National Park, Primavera trail, November 2016, Jaime Duque *J.Duque 258* (RB786090!); Jaime Duque *J.Duque 259* (RB786091!).

ETYMOLOGY: *marielleae*, in honour of the Brazilian politician, feminist, and human rights activist Marielle Franco killed on 14 March 2018.

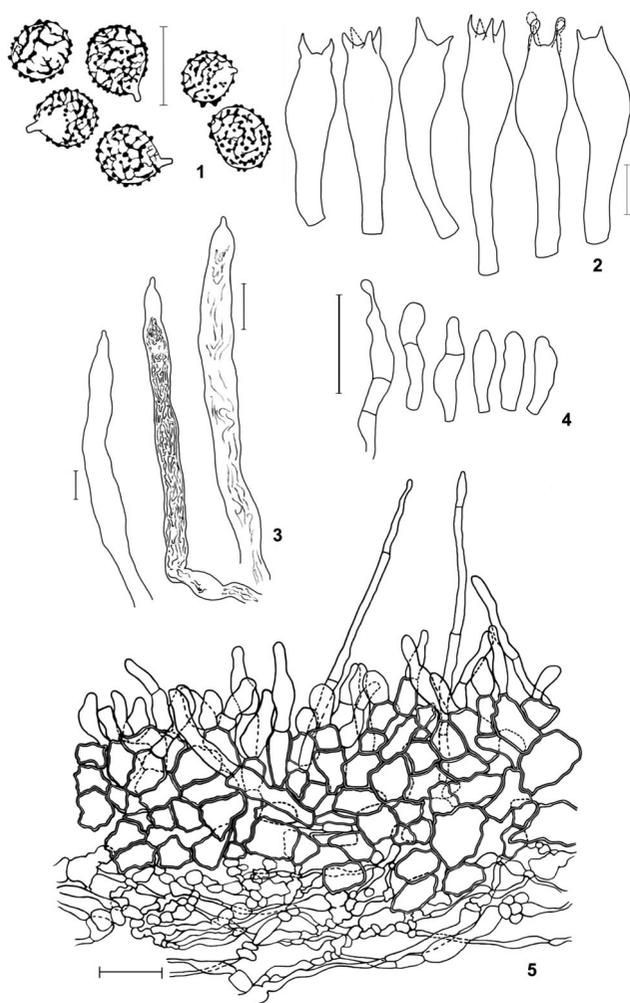


Figure 4. Microscopic structures of *Lactifluus marielleae*. 1. Basidiospores. 2. Basidia. 3. Pseudocystidia. 4. lamellae marginal cells. 5. Pileipellis. Scale bars in 1, 2 and 3 = 10 μ m; 4 and 5 = 20 μ m.

DIAGNOSIS: The species is characterized by the orange colour of the basidiomata, the presence of two and four-spored basidia, the thick-walled pileipellis isodiametric cells and thin-walled needle-shaped elements present in the pileipellis.

Description

Pileus 20–90 mm diameter, convex when young, depressed in the centre to infundibuliform when mature; surface dry, smooth to slightly rugose in the centre, azonate, pale orange (oac764-5A5) to tan colour (oac766) with brownish (oac667) tinges in younger basidiomata, light orange (5A4) to reddish orange (7C7) in older basidiomata; margin incurved at first and straight to uplifted when mature, sulcate up to mid-radius, greyish orange (5B5). **Context** 1–4 mm wide, solid, cream colour, unchanging when exposed; odourless, no taste. **Lamellae** 1–4 mm wide, adnexed in younger

basidiomata to decurrent in older basidiomata, sub-distant, sometimes forked toward the margin, cream (oac815) to pale orange (oac969 – 4B4) with reddish orange (7C7) to brownish (oac667) tinges; margin entire. **Lamellulae** one to three between lamellae, of two different lengths. **Stipe** central, 15–49 \times 5–17 mm, equal to tapering downwards, surface longitudinally fibrous to rugulose, concolorous with lamellae. **Latex** absent. Basidiomata firm. **Spore print** not obtained. Solitary or gregarious. In soil.

Basidiospores 6.45–7.56–9.78 \pm 0.68 \times 5.48–6.54–7.62 \pm 0.53 μ m (Q = 1.01–1.15–1.30 \pm 0.061), globose, subglobose to broadly ellipsoid; ornamentation amyloid, composed of low warts (0.17–0.39–0.74 \pm 0.14 μ m) and ridges, forming an incomplete reticulum; suprahilar spot smooth, mainly inamyloid, amyloid in few spores and rarely weakly amyloid. **Basidia** 32.84–43.33–58.47 \pm 6.72 \times 8.38–9.96–12.96 \pm 0.84 μ m, clavate to subclavate, mostly 4-spored, occasionally 2-spored. **Macrocystidia** absent. **Pleuropseudocystidia** 6.24–10.63–16.09 \pm 2.54 μ m diameter, emerging 8.4–28.95–72.43 \pm 16.29 μ m, abundant, arising from the lactiferous system, fusiform to cylindrical, occasionally branched, apex obtuse, subacute to mucronate; content amorphous, refringent. **Lamella-edge** sterile; marginal cells 7.72–12.85–21.01 \pm 3.54 \times 3.59–4.70–5.76 \pm 0.60 μ m, subcylindric, subfusiform, thin-walled, hyaline. **Hymenophoral trama** heteromerous, composed of sphaerocytes and abundant lactifers hyphae. **Pileipellis** a palisade; suprapellis composed of erect to oblique terminal elements, 10.53–24.31–52.37 \pm 10.35 \times 3.53–5.67–9.26 \pm 1.45 μ m, subfusiform to subcylindrical, thin-walled; needle-shaped, septate, thin-walled, swollen base elements often present, 52.4–103.01–168.87 \pm 30.69 \times 3.38–4.93–7.01 μ m, scattered; subpellis, composed of several layers of isodiametric or irregularly shaped, slightly thick-walled cells, 7.15–15.57–34.28 \pm 5.76 μ m diameter, wall 0.71–1.32–2.57 \pm 0.41 μ m. **Stipitipellis** a palisade, composed of elements similar to those of the suprapellis of the pileipellis.

REMARKS: The morphologically most similar species to *L. marielleae* are *L. caatingae*, *L. catarinensis*, *L. venezuelanus*, and *Lactarius mamorensis*. For the morphological comparison between *L. marielleae* and *L. catarinensis* see the comments for *L. catarinensis*. The main differences between *L. marielleae*, *L. caatingae*, *L. venezuelanus*, and *Lactarius mamorensis* are the pileus colour, pale orange to reddish orange with some brownish tinges in *L. marielleae*, rusty brown, brownish orange, reddish brown to fuscous red in *L. caatingae*, brownish to ferruginous becoming ochraceous to lemon in *L. venezuelanus*, pinkish cinnamon, flesh ochre, cream to orange yellow in *Lactarius mamorensis*. The

basidia in *L. marielleae* are bigger than in *L. caatingae*, *L. venezuelanus*, and *Lactarius mamorensis*; $32.84\text{--}43.33\text{--}58.47 \times 8.38\text{--}9.96\text{--}12.96 \mu\text{m}$ in *L. marielleae*, $28\text{--}40 \times 8\text{--}11 \mu\text{m}$ in *L. caatingae*, $30\text{--}40 \times 6.5\text{--}9 \mu\text{m}$ (Pegler & Fiard, 1979) and $25\text{--}40 \times 8 \mu\text{m}$ (Dennis, 1970) in *L. venezuelanus*, $23\text{--}47 \times 8.5\text{--}13.3 \mu\text{m}$ in *Lactarius mamorensis*. The pileipellis isodiametric cells in *L. marielleae* have a slightly thickened wall, they are thin-walled in the other species. The needle-shaped, septate elements in the pileipellis are thin-walled in *L. marielleae* and *L. mamorensis* but thick-walled in the other species. The pleuropseudocystidia are abundant, with amorphous and refringent content in *L. marielleae* and *L. caatingae*, but are scattered, sometimes rare, with granular content in *L. venezuelanus*. There is no reference to pleuropseudocystidia in the description for *Lactarius mamorensis*. The marginal cells are smaller in *L. marielleae* ($7.72\text{--}12.85\text{--}21.01 \times 3.59\text{--}4.70\text{--}5.76 \mu\text{m}$) and *L. caatingae* ($15\text{--}22 \times 6\text{--}9 \mu\text{m}$) than in *L. venezuelanus* ($32\text{--}47 \times 2\text{--}6 \mu\text{m}$).

Lactifluus marielleae, *L. caatingae*, *L. venezuelanus*, and *Lactarius mamorensis* share the similar basidiospores size, $6.45\text{--}7.56\text{--}9.78 \times 5.48\text{--}6.54\text{--}7.62 \mu\text{m}$ in *L. marielleae*, $(5.6\text{--})6.1\text{--}7.1(-8.2) \times 5.6\text{--}6.6(-8.2) \mu\text{m}$ in *L. caatingae*, and $6\text{--}9 \mu\text{m}$ diameter (Dennis, 1970) and $7\text{--}9.7 \times 6\text{--}7.8 \mu\text{m}$ (Pegler & Fiard, 1979) in *L. venezuelanus*. However they are slightly bigger in *Lactarius mamorensis* ($6.7\text{--}10.7(-11.2) \times 5.8\text{--}9.3(-10)$). The pileipellis is a palisade in the four species. The needle-shaped, septate elements in the pileipellis are present in all species but are more abundant in *L. venezuelanus*. According to the description in Pegler and Fiard (1979) the *L. venezuelanus* lamellae fork toward the margin, which has also been found in some of the specimens examined for *L. marielleae*, however the forking lamellae are not described in the protologue of *L. venezuelanus*. Other shared characteristics for *L. caatingae*, *L. marielleae*, *L. venezuelanus*, and *Lactarius mamorensis* were presented in the comments for *L. catarinensis*.

Lactifluus neotropicus (Singer) Nuytinck, in Verbeken, Nuytinck & Buyck, Mycotaxon 118: 450 (2011) Figs 5.5, 5.6, 6.4 and Supplementary Figs 9–10.

≡ *Lactarius neotropicus* Singer, Kew Bull. [7]: 299 (1952).

Pileus 11–89 mm diam., convex, slightly depressed to infundibuliform; surface dry, minutely velutinous, slightly scrobiculate and entire at the centre; pellis dehiscent near the margin, disrupting into squamules toward the margin; overall surface of young basidiomata brownish red (oac586 to oac659/9C7), in mature basidiomata brownish red (oac586 to oac659/9C7) at centre, greyish red (9B5) to dull red (8B3) at the mid radius,

dull red at margin (oac653/9B3), greyish white (1B1) at margin between the squamulose surface; margin inrolled to incurved. **Annulus** present, apical, fibrillose, scaly, closed in the young basidiomata. **Context** 1–4 mm thick, solid, cream, unchanging when exposed; odour nil, taste spicy. **Lamellae** 1–4 mm wide, adnexed, adnate to adnate with a tooth, closed; cream colour; margin forked, even. **Lamellulae** of different lengths. **Stipe** central, $25\text{--}50 \times 10\text{--}11 \text{mm}$, equal to tapering at base; surface glabrous to minutely velutinous, concolorous with the pileus margin. **Context** solid; cream colour. **Latex** absent. **Spore print** not obtained.

Basidiospores $5.91\text{--}7.49\text{--}9.63 \pm 0.80 \times 5.37\text{--}6.55\text{--}8.78 \pm 0.57 \mu\text{m}$ ($Q = 1.0065\text{--}1.14\text{--}1.29 \pm 0.077$), globose to broadly ellipsoid; ornamentation amyloid, composed of isolated low warts ($0.14\text{--}0.36\text{--}0.66 \pm 0.13 \mu\text{m}$) or united by ridges, forming an incomplete reticulum; suprahilar spot smooth, inamyloid. **Basidia** $40.27\text{--}48.43\text{--}63.26 \pm 6.28 \times 8.59\text{--}10.17\text{--}11.56 \pm 0.95 \mu\text{m}$, clavate, one, two to four spored. **Macrocytidia** absent. **Pleuropseudocystidia** $5.6\text{--}11.57\text{--}16.87 \pm 2.25 \mu\text{m}$ diameter, emerging $12.47\text{--}50.34\text{--}64.77 (-112.91) \pm 14.34 \mu\text{m}$, abundant, arising from the lactiferous system, fusiform to cylindrical, occasionally branched, apex obtuse, subacute to mucronate, more rarely forking; content, amorphous, granular to needle-like, refringent. **Lamella-edge** infertile, sometimes forking, marginal cells $9.83\text{--}20.61\text{--}38.24 \pm 9.61 \times 5.56\text{--}8.37\text{--}13.55 \pm 2.63 \mu\text{m}$, subfusiform to fusiform, somewhat clavate, thin-walled, hyaline. **Cheilopseudocystidia** like pleuropseudocystidia. **Hymenophoral trama** heteromerous, composed of sphaerocytes and abundant lactifers hyphae. **Pileipellis** a lamprotrichopalysade, two-layered, elements of suprapellis $67.77\text{--}94.63\text{--}120.25 \pm 17.32 \times 8.6\text{--}7.14\text{--}8.6 \pm 1.11 \mu\text{m}$, long, slender, hair-shaped and tapering upwards, at times tortuous, very thick-walled (up to $3 \mu\text{m}$), at times arising from the subpellis; subpellis composed of narrow cells, slightly thick-walled to thin-walled; pseudopileocystidia present, scarce. **Stipitipellis** a lamprotrichopalysade, similar to the pileipellis but at the base of the stipe consisting of narrower and longer thick-walled, branched hyphae. **Annulus** composed of thin-walled, hyaline hyphae, that are densely interwoven, often septate, sometimes branched and $2\text{--}5 \mu\text{m}$ diam.

SPECIMENS EXAMINED: BRAZIL, PARAIBA: João Pessoa, Benjamin Maranhão Botanic Garden, 10 September 2011, Altielys Magnago ACM323 (FLOR49440!); SANTA CATARINA: Florianópolis, Pântano do Sul to Lagoinha do Leste Trail, 4 January 2012, Melissa Jaegger MJI20 (FLOR49433!); 21 March 2014, Jaime Duque J.Duque 52 (FLOR61781!); 4 January 2016, Mary Vanegas-León MVL43 (FLOR61424!); 13 April 2017, Maria Alice Neves

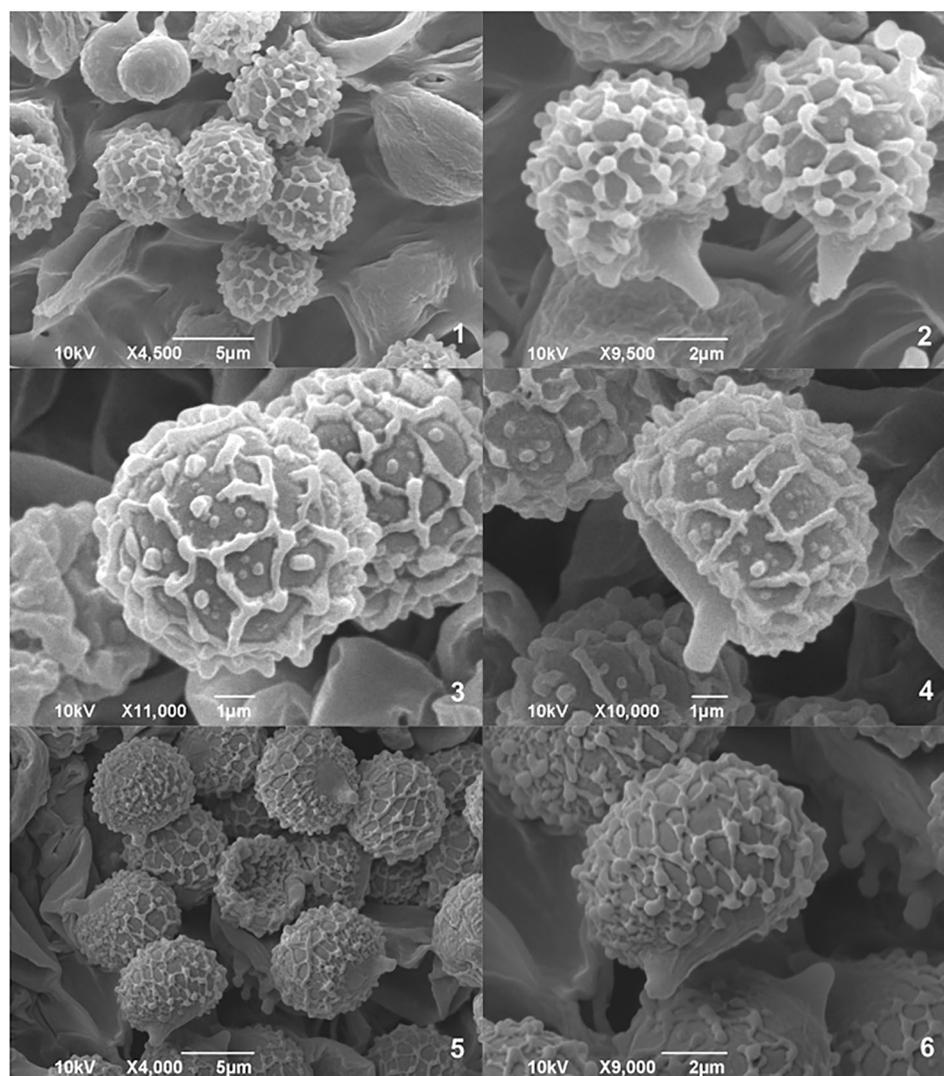


Figure 5. SEM of the basidiospores. 1–2. *Lactifluus catarinensis* (MJ24). 3–4. *L. marielleae* (CALD27). 5–6. *L. neotropicus* (MJ120).

MAN1222 (FLOR 67061!); Santo Amaro da Imperatriz, Plaza Caldas da Imperatriz Resort & Spa. Trail, 13 March 2012, MJ139 (FLOR49439!).

Notes: *Lactifluus neotropicus* has been reported from Trinidad and Dominica (Singer, 1952; Pegler & Fiard, 1979) and this is the first record from Brazil. The most striking characteristic of this species is the presence of a secondary velum. There are six other described species with secondary velum in the whole genus: *L. annulatoangustifolius* (Beeli) Buyck, *L. annulifer*, *L. heimii* (Verbeken) Verbeken, *L. pelliculatus* (Beeli) Buyck, *L. velutissimus* (Verbeken) Verbeken, and *L. zenkeri* (Henn.) Verbeken. Only *L. annulifer* and *L. neotropicus* have a Neotropical distribution, the other five species are endemic to tropical Africa. There are other morphological similarities between the six species (we did not

consider *L. pelliculatus* because it was not possible to make any morphological comparison), with the pellis rupturing near the margin, except for *L. annulifer* which has an even surface; spores ornamentation composed of isolated low warts (up to 0.2 µm in *L. heimii* and *L. velutissimus*, up to 0.1 µm in the rest of the species) and short ridges forming an incomplete reticulum, and the pileipellis structure is a lamprotrichopalisade to lampropalisade.

Lactifluus neotropicus can be differentiated from the other five species by the reddish pileus colour, while in *L. annulatoangustifolius* it is yellow to brownish orange, flesh to ochre colour in *L. annulifer*, brownish orange to greyish orange in *L. heimii*, yellowish colours in *L. velutissimus* and yellowish brown to pale ochraceous in *L. zenkeri*. Latex is absent in *L.*



Figure 6. Basidiomata. 1–2. *Lactifluus marielleae* (JDuque259 and JDuque258 respectively). 3. *L. catarinensis* (ACM993). 4. *L. neotropicus* (MAN1222).

neotropicus while it is present in the remaining species. *Lactifluus neotropicus* has one to four-spored basidia, *L. annulatoangustifolius* and *L. annulifer* have two to four, and *L. heimii* and *L. velutissimus* have only four-spored basidia. The apical elements of the pileipellis do not or very rarely fork in *L. neotropicus* while forked elements in the pileipellis are more common in the other species.

Discussion

We described and evaluated the phylogenetic relationships of the new section *Neotropicus* based on new sampling in the Brazilian Atlantic forest. We also describe two new species, document *L. neotropicus* for the first time from Brazil and propose a key for the species in the section found in Brazil. Our results showed *Lactifluus* sect. *Neotropicus* as a monophyletic group of Neotropical species in the subgenus *Lactariopsis*. These results corroborate that *L. annulifer*, *L. subiculatus*, and *L. venezuelanus* are closely related species, De Crop *et al.* (2017) considered this group of species in ‘Clade 2’ in the subgenus *Lactariopsis*. This clade is also recovered in an ITS analysis in Sá *et al.* (2019). We do not test the relationship of *L. caatingae* in the present study, however, we included *L. caatingae* in sect. *Neotropicus* because Sá *et al.* (2019) show that *L. caatingae* is

closely related to *L. annulifer*, *L. subiculatus*, *L. venezuelanus*, and other *Lactifluus* spp. that we included here.

We found a moderately supported clade in the Bayesian analysis formed by *L. sect. Neotropicus*, *L. sect. Lactariopsis*, clade 1, clade 2, and clade 3. Our results are similar to those in De Crop *et al.* (2017), who found the same grouping; the main difference is that the group formed by *L. annulifer*, *L. subiculatus*, and *L. venezuelanus* formed a strongly supported clade with *L. multiceps* and *L. sp. G3264* in their study. Differences are probably explained by the fact that they included more genes in the analysis (ITS, LSU, RPB2, and RPB1).

Three of the species included in *L. sect. Neotropicus* were placed in other sections before. *Lactifluus annulifer* and *L. neotropicus* were treated within *L. sect. Lactariopsidae* (synonym of *L. sect. Lactariopsis*) by Pegler and Fiard (1979) and Singer *et al.* (1983). *Lactifluus venezuelanus* was placed in sect. *Polysphaerophori* by Pegler and Fiard (1979) and Singer *et al.* (1983), however this section is now classified in *L. subgenus Pseudogymnocarpi*, typified by the Mexican species *L. veraecrusis*. *Lactifluus subiculatus* is not included in any section, however, Miller *et al.* (2012) suggested a close relationship between *L. subiculatus* and *L. neotropicus* based on molecular evidence and the combination of long thick-walled hairs in the suprapellis arising from an epithelioid subpellis.

The most striking characteristic of several species in *L.* subg. *Lactariopsis* is the secondary angiocarpic development, unique within the genus. Verbeken (1998b) and De Crop et al. (2017) already discussed that this feature has several origins. Our results are similar, since species with secondary angiocarpic development can be found in *L.* sect. *Lactariopsis* and *L.* sect. *Neotropicus*. However, it can be hypothesized that this characteristic has an evolutionary origin in the crown group of *Lactifluus*. The question is, did the secondary angiocarpic development arise several times or was it present in a common ancestor to *L.* sects. *Lactariopsis* and *Neotropicus* and was it subsequently lost in several lineages? Most of the time the species in *L.* subg. *Lactariopsis* can be found under the litter or even slightly buried under the soil, eaten by animals, mostly insect larvae. The secondary angiocarpic development may serve as a defence mechanism to protect the hymenophore.

The morphology of *Lactarius mamorensis* indicates that it may be a *Lactifluus* species, however, it was not possible to test the phylogenetic relationship of *Lactarius mamorensis* with the species treated in the present analyses. In our analyses we included sequences of two probably new species from Brazil and Colombia, in addition to several sequences retrieved from the GenBank data base of unidentified specimens collected in French Guiana. Based on the common collecting of specimens with morphological affinities to *L.* sect. *Neotropicus* in mycological surveys in different South American countries, a greater diversity of the section in the Neotropical region is here predicted. The short branches of several lineages indicate that the diversification process in *L.* sect. *Neotropicus* may have happened at a fast rate in a short period of time, however, a broader sampling and analyses including calibration methods are needed to depict the evolutionary process behind the high diversity observed in *L.* sect. *Neotropicus*.

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Disclosure statement

No potential conflict of interest was reported by the authors.

Supplemental material

Supplemental material for this article can be accessed here: <https://dx.doi.org/10.1080/14772000.2020.1769221>.

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