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Lactarius cucurbitoides (Russulales, Basidiomycota), a new species from South Korea supported by molecular and morphological data

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Abstract

A new species belonging to *Lactarius* subg. *Plinthogalus* was discovered during a long-term project on the diversity of Korean *Lactarius*. This species is proposed here as *Lactarius cucurbitoides*. The status of *L. cucurbitoides* as a new species is supported by molecular data and morphological features. Phylogenetic analysis based on internal transcribed spacer (ITS) sequences shows that *L. cucurbitoides* is closely related to *L. subplinthogalus*, *L. friabilis,* and *L. oomsisiensis*, with pairwise distances of 2.8–4.3%. Morphological characters of L. cucurbito*ides* that distinguish it from these closely related species are a pale yellow to pale orange colored pileus and non-discoloration of white latex. The new species is described and illustrated in the present paper.

Key words: ectomycorrhizal fungi, Korea barcode of life, taxonomy, white latex, sp. nov.

Introduction

The genus *Lactarius* Pers., also known as "milkcaps" for its production of latex, is a group of ectomycorrhizal fungi with an estimated 500 species worldwide (Le *et al*. 2007). Recent molecular analyses show that the milkcaps do not form a monophyletic group. A new genus, *Multifurca*, was raised by Buyck & V. Hofstetter (also including some former *Russula* species), and *Lactarius* was divided into two genera—*Lactarius* sensu novo and *Lactifluus* (Pers.) Roussel (Buyck *et al*. 2010, Verbeken *et al*. 2011, Stubbe *et al*. 2012, Verbeken *et al*. 2012). The total number of described species belonging to *Lactarius* sensu stricto accounts for 75–80% of the currently described species (Buyck *et al.* 2010, Verbeken & Nuytinck 2013).

Lactarius subg. *Plinthogalus* (Burl.) Hesler & A.H. Sm. was first considered a separate genus *Lactariella* (Schröter 1889), then Burlingham made it a group "*Plinthogalae*" in the genus *Lactarius* (Burlingham 1908). *Lactarius* subgenus *Plinthogalus* (Berk.) Hesler & A. H. Sm. sensu lato has been defined by its distinct appearance including greyish or brownish pigments, velvety aspect of pileus and stipe, and heavily ornamented basidiospores. The group was elevated to the level of section by Singer (1942) and to subgenus by Hesler & Smith (1979). Recently, Verbeken added several sections to this group (Verbeken 2000).

Species in this subgenus are characterized by a grey or brown-colored pileus, stipe with a typical velvety and dry texture, striking color change of the white latex when exposed to air, and heavily ornamented and reticulate spores (Le *et al*. 2007, Stubbe *et al*. 2007). In addition to these striking morphological characters, *L.* subg. *Plinthogalus* is well supported by molecular data (Eberhardt & Verbeken 2004, Le *et al*. 2007, Buyck *et al*. 2008, Stubbe *et al*. 2012).

The subgenus is rather well-studied in Europe and North America, but studies in Asia have been limited. Many new species of this particular group have been reported in Asia in recent years. Das & Sharma (2004) initially extended our knowledge by including a new Indian species. Subsequently, six new species were reported from Thailand (Le *et al*. 2007), eleven new species were identified in Malaysia (Stubbe *et al*. 2007, Stubbe *et al*. 2008), and two new species were reported from India (Das & Verbeken 2012).

In Korea, 52 *Lactarius* species have been reported to date (Kim *et al*. 2013). This inventory has been taken based on comparisons of macromorphology to European and North American *Lactarius* species. However, morphological

classification may be misleading because recent studies found that Asian *Lactarius* species are not conspecific with North American and European species with similar morphology (Le *et al*. 2007, Stubbe *et al*. 2008). Nonetheless, taxonomy of *Lactarius* in western countries was often applied to other continents. Recently, a Korean barcode of life project has been organized by the National Institute of Biological Resources (www.nibr.go.kr) to promote the exploration of biodiversity and biological resources. Part of the fungal barcode project is a long-term study to evaluate Korean *Lactarius* species. We discovered a new species that is genetically and morphologically distinct from known *Plinthogalus* species during this project. We document and describe this new *Lactarius* species in the present paper.

Materials and Methods

Sampling

The three specimens used in this study were originally identified by macromorphology as follows: two *L. subplinthogalus* (SFC20110818-21, SFC20110825-14) from Chiak National Park, Wonju-si, Gangwon-do, South Korea, and one *L. oomsisiensis* (SFC20130719-110) from Seongjubong Natural Recreation Forest, Sangju-si, Gyeongsangbuk-do, South Korea. The specimens have been deposited in the Seoul National University Fungus Collection (SFC).

Molecular procedures and phylogenetic analysis

Genomic DNA of the three specimens was extracted from fresh or dried pieces of fruit bodies following a modified CTAB extraction protocol (Rogers & Bendich 1994). The internal transcribed spacer (ITS) was sequenced for the three specimens. ITS was amplified using primers ITS1F or ITS5 and ITS4 or ITS4B (White *et al*. 1990, Gardes & Bruns 1993). PCR reactions were performed in a C1000 thermal cycler (Bio-Rad, Hercules, CA, USA) using AccuPower PCR premix (Bioneer Co., Daejeon, Korea) in a final volume of 20 µl containing 10 pmol of each primer and 1 µl of genomic DNA. PCR amplification was performed as described by Park *et al*. (2013). The PCR products were electrophoresed through a 1% agarose gel stained with EcoDye DNA staining solution (SolGent Co., Daejeon, Korea) and purified with the Expin PCR Purification Kit (GeneAll Biotechnology, Seoul, Korea) according to manufacturer's instructions. DNA sequencing was performed in both directions using the PCR primers at Macrogen (Seoul, Korea) in an ABI3700 automated DNA Sequencer.

Sequences were assembled, proofread, and edited using MEGA 5 software (Tamura *et al*. 2011) and deposited in GenBank (Fig. 1). Based on previous studies on *L.* subg. *Plinthogalus* (Le *et al*. 2007, Stubbe & Verbeken 2012), we obtained relevant ITS sequences of closely related species from GenBank. Multiple sequence alignments were conducted using MAFFT v7 (Katoh & Standley 2013) with the default settings, checked by eye, and adjusted manually. Maximum likelihood (ML) phylogenetic analyses were performed using RAxML (Stamatakis 2006) with the GTRGAMMA model of nucleotide substitution and 1000 bootstrap replicates.

Morphological study

Field characters were described and photographed using fresh specimens. The Methuen Handbook of Color (Kornerup & Wanscher 1978) was used as the color standard for descriptions of the specimens. Measurements of microscopic structures were made from slide preparations mounted in 3% (w/v) KOH and 1% (w/v) phloxine or Melzer's reagent (IKI) (Largent *et al*. 1977) with a light microscope (Nikon Eclipse 80i). Quotient (Q), which is the ratio of basidiospore variation between mean length and mean width, was calculated for each of the three specimens studied. For scanning electron microscope (SEM), dried pieces of lamellae with spores were attached to aluminum stubs using double sided adhesive tape, coated with gold in a sputter coater (Bal-Tec SCD 050, Lichtenstein), and then examined with a SEM (Zeiss Sigma, Carl Zeiss, Germany).

Results

Molecular data

ITS sequences of the three specimens were nearly identical, and their length was 646 bp. ML analysis of ITS data recovered the three specimens forming a monophyletic clade with strong support (Bootstrap support = 100%). This

clade was nested in *L.* subg. *Plinthogalus* and distinct from other species with available ITS data (Fig. 1). The intra-specific variation of ITS for these specimens was 0.2%. These results provide molecular support that the three *Lactarius* specimens represent a new species. The phylogeny suggests that this species is closely related to *L. subplinthogalus* Coker, *L. friabilis* H.T. Le & Stubbe, and *L. oomsisiensis* Verbeken & Halling (bootstrap support = 91%) (Fig. 1). The phylogenetic relationships within this strongly supported group could not be resolved with ITS. Comparisons of the ITS sequences of this new *Lactarius* species with *L. subplinthogalus*, *L. friabilis*, and *L. oomsisiensis* revealed sequence dissimilarities of 2.8–3.0%, 3.3–3.6%, and 2.8–4.3%, respectively.

Macro- and micromorphological features of this potentially new species were compared with *L. subplinthogalus*, *L. friabilis*, and *L. oomsisiensis* and found to be distinct (Table 1).

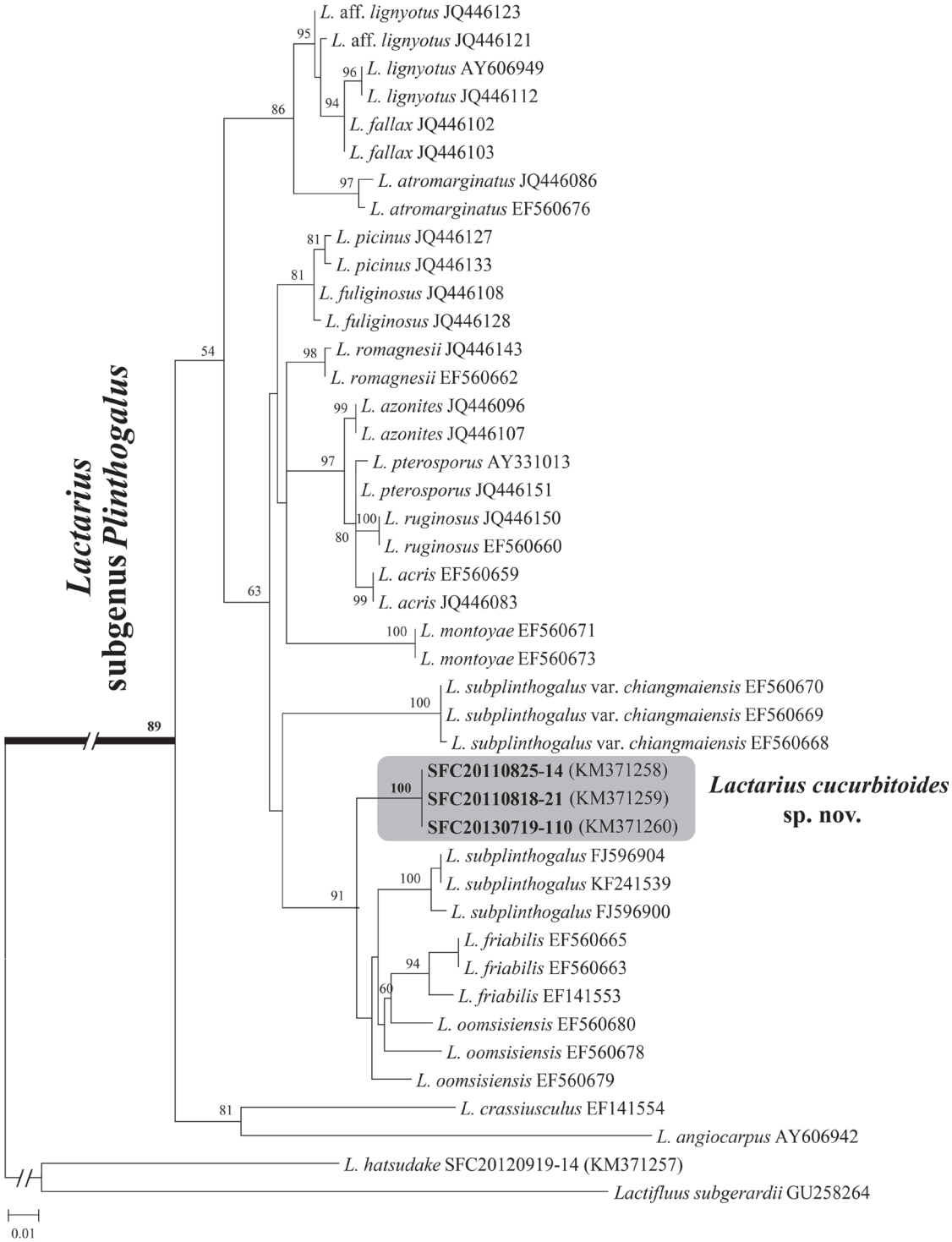

FIGURE 1. Maximum likelihood tree inferred from ITS dataset for *Lactarius cucurbitoides* and allied species**.** Bootstrap scores are presented at the nodes only if >50. The scale bar indicates the number of nucleotide substitutions per site. Accession numbers of the representative specimens are marked following the species name.

TABLE 1. Macroscopic and microscopic features of *Lactarius cucurbitoides* and closely related species *L. oomsisiensis, L. subplinthogalus, L. friabilis*, *L. montoyae,* and *L. subplinthogalus* var. *chiangmaiensis*. Some characters were the same for all species in the table: gills distant and pleurocystidia absent.

Character	***L. cucurbitoides***	***L. oomsisiensis*** (Verbeken *et al.* 2002)	***L. subplinthogalus*** (Hesler & Smith 1979)	***L. friabilis*** (Le *et al.* 2007)	***L. montoyae*** (Das & Sharma 2004)	***L. subplinthogalus* var. *chiangmaiensis*** (Le *et al.* 2007)
Pileus size	30–60 mm	20–60 mm	30–50 (–105) mm	25–55 mm	30–60 mm	15–40 mm
Pileus color*	**Pale yellow (4A3) to pale orange (6B3)**	**Yellowish orange (4A4) to pale brown (6D7)**	**Whitish to yellowish (light buff)**	**Dark cream-colored (4B3/4, 5A2) to pale yellowish brown (5DE4-5)**	**Gray yellowish to dark gray yellowish brown**	**Pale greysh brown to greysh orange (5BCD3/4)**
Stipe length	30–50 mm	25–50 mm	30–80 mm	20–45 mm	25–58 mm	13–30 mm
Stipe thickness	7–10 mm	7–10 mm	7–15 mm	4–10 mm	7–12 mm	3–8 mm
Spore size	7.0–8.4 (8.8) × (6.4) 6.8–8.0 (8.2) µm	6.6–7.0–7.6 × 5.9–6.6–7.4 µm	7.5–9 (9.5) × 7.8 µm	7.8–7.9–8.4–9.1 × 7.1–7.5–7.8–8.7 µm	7.5–10.0 (10.3) × (7.0) 7.3–9.3 µm	7.9–8.3 (8.8) × (6.2) 7.1–7.3 µm
Spore ornamentation height	Up to 2.2 µm	Up to 2.0 µm	1.5–2.5 µm	1.5–2 (2.5) µm	Up to 2.7 µm	2 (2.2) µm
Basidia size	48.1–59.7 × 11.0–15.6 µm	55–80 × 10–15 µm	52–60 × 12–14 µm	35–75 × 10–15 µm	44–60 × 9–12.5 µm	35–55 × 11–15 µm
Latex color	White	White	White	White or watery white	White	Watery white
Latex discoloration*	**None**	**Slowly pinkish**	**Deep rosy salmon**	**Drying Pinkish**	**None**	**Pale pinkish**
Context discoloration when bruised	Pale pink	Brownish grey, brownish pink	Rosy salmon	Dirty orange pink to orange	None	Yellowish grey or pale yellowish brown to pinkish brown

*Characters with bold font are distinguishing features between the species.

Taxonomy

Lactarius cucurbitoides H. Lee & Y.W. Lim, *sp. nov.* (Figs. 2 & 3)
MycoBank: MB 809952

Diagnosis: *Lactarius cucurbitoides* differs morphologically from the closely related species of *L.* subg. *Plinthogalus* by non-discoloration of the white latex when exposed to air.

Type:—SOUTH KOREA. Gyeongsangbuk-do, Sangju-si, Seongjubong Natural Recreation Forest, 233 m elev., N36°32'02" E128°02'29", 19 July 2013, SFC20130719-110 (Holotype, SFC!)

Pileus 30–60 mm in diam., first convex, then plane, finally with slightly uplifted margin and concave center; surface dry, very delicately appressed subtomentose when young, with age becoming rugose to rugulose, evenly pale yellow (4A3) to pale orange (6B3) when old. *Stipe* 30–50 mm long, 7–10 mm broad, uniform, regular; surface dry, pubescent, white, slightly staining pinkish or unchanging when bruised; context first solid, then hollow. *Lamellae* decurrent to adnate, 3–7 mm broad, distant, with 2–3 series of lamellulae, greyish orange (6B4) when young, darker (6B6) with age, slowly staining pinkish when bruised; edges even. *Context* in pileus white, up to 7.5 mm thick at the center, slowly changing pale pinkish on exposure in pileus. *Latex* white, abundant; taste acrid. *Spore print* white or creamy.

Basidiospores globose to subglobose, 7.0–8.4 (8.8) × (6.4) 6.8–8.0 (8.2) µm (Q = 1.02–1.12(1.13), n = 20); ornamentation amyloid, made up of broad, quite irregular and little sharp ridges, locally up to 2.2 µm in height; ridges often with a paternoster-like (string of beads) aspect, as if made up of several verrucae, forming a fragmentary reticulum, with some helically arranged aspect; isolated verrucae and fragmented ridges abundant. *Basidia* narrowly clavate to subcylindric, 48.1–59.7 × 11.0–15.6 µm, 4-spored; sterigmata 6.5–9.3 × 2.0–3.1 µm. *Pleuromacrocystidia* absent. *Pleuropseudocystidia* abundant, sometimes diverging toward top, usually spherical on top, 3.8–7.2 µm in diam. broad, with refractive and guttate content. *Lamellar edge* sterile; marginal cells generally cylindric, 13.5–26 × 3–6 µm. *Hymenophoral trama* filamentous, septate, transparent hyphae, 2.5–3.2 µm wide and numerous lactiferous hyphae, 7–13 µm wide. *Pileipellis* a 60–70 µm thick trichopalisade, consisting of interwoven hyphae; tip of elements cylindric, 11.5–19 × 4.2–6.8 µm. *Stipitipellis* a trichoderm, 40–80 µm thick; thin walled hyphae, 11.4–25.6 × 4.0–6.3 µm. *Clamp connection* absent.

Habitat:—Solitary or sparse on ground in deciduous forest dominated by *Quercus* spp.

Etymology:—Referring to the pileus shape being similar to a pumpkin.

Additional studied material:—SOUTH KOREA. Gangwon-do, Wonju-si, Chiak National Park, 496 m elev., N37°16'36" E128°4'47", 18 August 2011, SFC20110818-21 (Paratype SFC!); Ibid., 505 m elev., N37°16'37" E128°4'52", 25 August 2011, SFC20110825-14 (Paratype SFC!).

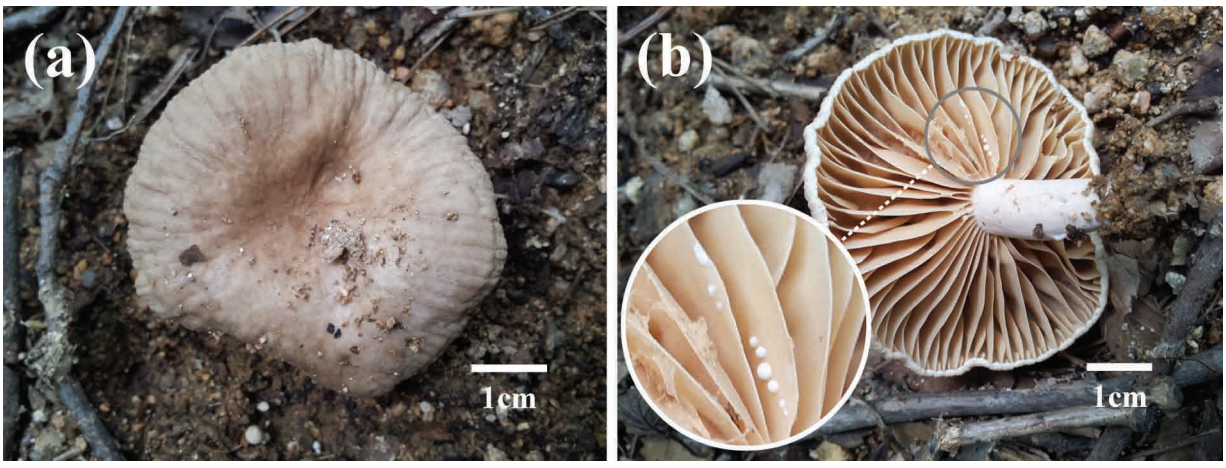

FIGURE 2. Fruit body of *Lactarius cucurbitoides* (SFC20130730-110, holotype): (a) surface of pileus and (b) lamellae with white latex.

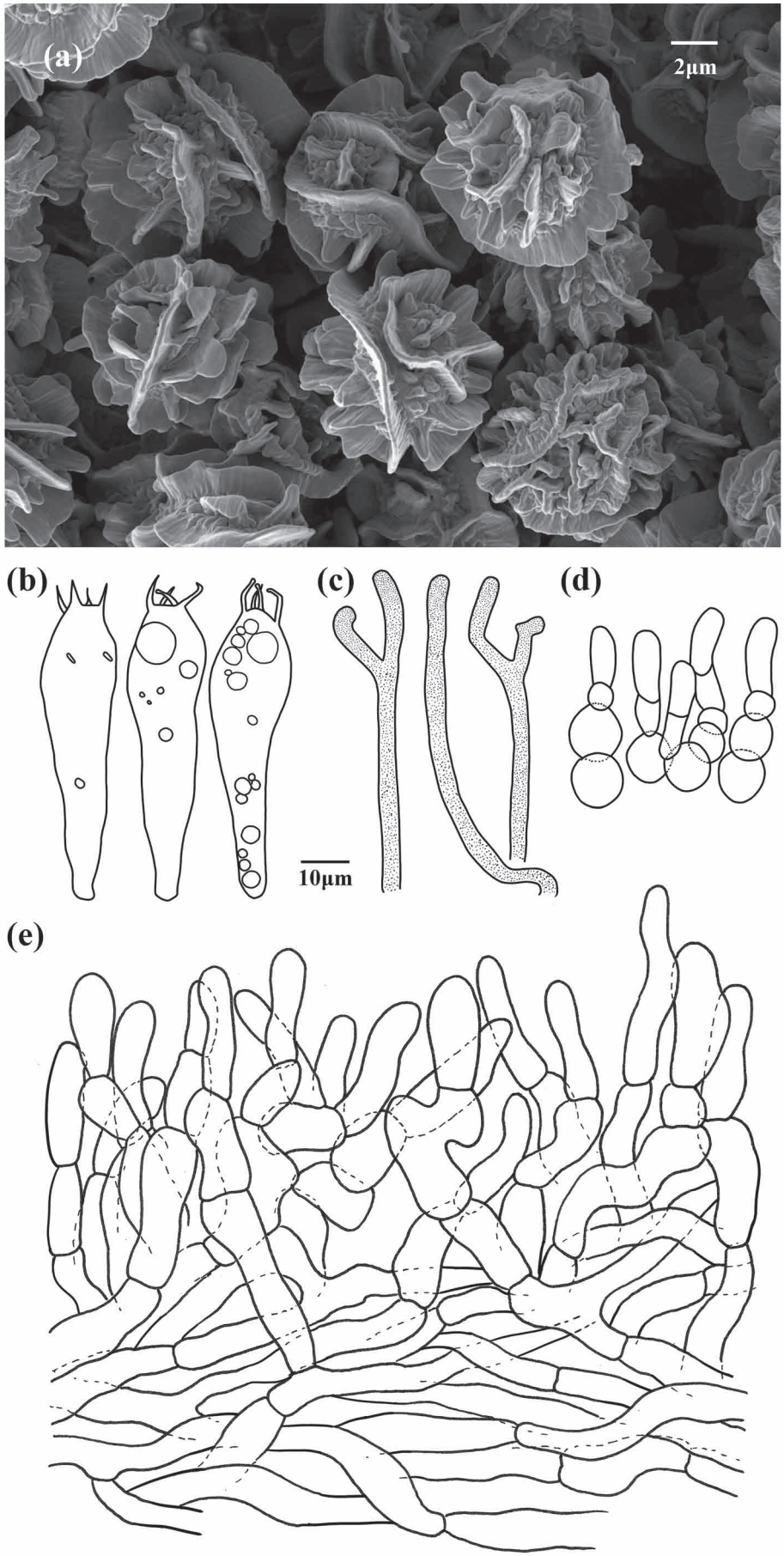

FIGURE 3. Microscopic features of *Lactarius cucurbitoides* (SFC20130719-110, holotype): (a) basidiospores (SEM), (b) basidia, (c) pleuropseudocystidia, (d) pileipellis, and (e) marginal cells.

Comment:—The pale yellow (4A3) to pale orange (6B3) color of the pileus and non-discoloration of white latex are characteristics of *L. cucurbitoides* that distinguish it from other taxa in the subgenus *Plinthogalus*. Although phylogenetically closely related to *L. subplinthogalus*, *L. friabilis*, and *L. oomsisiensis*, *L. cucurbitoides* can be distinguished from these species by color change of context: *L. cucurbitoides* has relatively pale discoloration of context and unchanged color of latex when bruised, whereas the other three species has pinkish like discoloration of the exposed context and latex. Microscopically, *L. cucurbitoides* has slightly larger spores and much shorter basidia compared to *L. oomsisiensis*. For the comparison of *L. cucurbitoides* and two Asian species with similar morphology, *L. montoyae* and *L. subplinthogalus* var. *chiangmaiensis*, *L. cucurbitoides* had smaller, less ornamented spores compared to *L. montoyae* and a relatively larger fruit body compared to *L. subplinthogalus* var. *chiangmaiensis*. Despite a slightly pale color compared to *L. oomsisiensis*, the dark lamellae of *L. cucurbitoides* are also striking in character.

Discussion

Phylogenetic analysis of ITS showed that *L. cucurbitoides* is monophyletic with strong support (bootstrap support = 100%) and distinctive from other species. The distinct morphology and ITS sequences provide evidence that this is a new species. *Lactarius cucurbitoides* is characterized by a pale yellow to pale orange colored dry pileus, stipe with velvety texture, white latex, and heavily ornamented and reticulate spores that are typical characteristics of the subgenus *Plinthogalus* (Smith & Hesler 1962, Heilmann-Clausen *et al*. 1998, Verbeken *et al*. 2002, Le *et al*. 2007). Moreover, *L. cucurbitoides* is distinguished from closely related species in the subgenus *Plinthogalus* in several aspects: pileus with pale yellowish orange color and a white latex that is unchanging when exposed to air and at most stains the context slightly pinkish.

Lactarius cucurbitoides was previously identified as *L. subplinthogalus* Coker in Korea due to similar morphology (Lim & Kim 1972, Jang 2014). However, upon closer examination, *L. cucurbitoides* can be distinguished morphologically from *L. subplinthogalus* by latex color—latex of *L. subplinthogalus* turns from white to deep rosy salmon when exposed to air (Hesler & Smith 1979), but the latex of *L. cucurbitoides* does not change color (Table 1).

Lactarius cucurbitoides can be distinguished morphologically from *L. oomsisiensis* based on pileus color and latex color—the pileus is yellowish orange to pale brown and the latex changes from white to pinkish for *L. oomsisiensis* (Verbeken *et al*. 2002, Le *et al*. 2007), but the pileus is pale yellow to pale orange and the latex does not change color for *L. cucurbitoides* (Table 1). Additionally, *L. cucurbitoides* and *L. oomsisiensis* are distinct in the phylogeny (Fig. 1). Interestingly, the type specimen of *L. oomsisiensis* (GenBank Accession number: EF560680) from Papua New Guinea (Verbeken *et al*. 2002) and two additional specimens (EF560678, EF560679) from Thailand (Le *et al*. 2007) did not form a monophyletic clade (Fig. 1). Therefore, we could not determine the species boundary of *L. oomsisiensis* with these reference sequences. Further studies are needed to clarify this problem.

A third closely related species, *L. friabilis*, can be distinguished morphologically from *L. cucurbitoides* based on the color of pileus and latex. Darker color of the pileus and pinkish discoloration of watery abundant, white latex are distinctive characters of *L. friabilis* (Le *et al*. 2007) and distinguish it from *L. cucurbitoides*.

Molecular and morphological data confirm *L. cucurbitoides* as a new species of *L.* subg. *Plinthogalus*. This result highlights the need for additional studies of *Lactarius* and other fungal taxa in Asia because there is high possibility of non-conspecificity with North American and European species despite similar morphologies (Imazeki *et al*. 1988, Wu & Mueller 1997, Lee *et al*. 2002, Das & Sharma 2005, Le *et al*. 2007). Currently there are 52 recognized *Lactarius* species in Korea, but the results of this study suggest that there are more species awaiting discovery. Studies of *Lactarius* diversity of Korea in comparison to North America and Europe are currently underway and will be presented in future studies.

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