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Two new species of *Calocybe* (Lyophyllaceae) from northeast China

JIZE XU[a*], XIAODONG YU[b], CHUNLAN ZHANG[c*] & YU LI[b]
[a] *Jilin Agriculture Science and Technology University, Jilin 132101, China.*
[b] *Jilin Agricultural University, Changchun 130118, China.*
[c] *Changchun University, Changchun 130022, China.*
**Corresponding author: Jize Xu: xujz802@nenu.edu.cn*

JIZE XU and XIAODONG YU contributed equally to this work.

Abstract

Two new species of *Calocybe* (*Calocybe erminea sp. nov.* and *C. badiofloccosa sp. nov.)* are described from Liaoning province, China. *Calocybe erminea* is characterized mainly by its light khaki to dirty white pileus, slightly hygrophanous stipe, small basidiospores and cellular epicutis. The key characters of *C. badiofloccosa* are its light ocher yellow and rough pileus, villose stipe and large basidiospores. The results of phylogenetic analyses based on the internal transcribed spacer (ITS) and the nuclear large subunit rDNA (nrLSU) region indicated that they belong to *Calocybe*, but they are distinct from other species in the genus. They are compared morphologically with other species which are phenotypically similar and the phylogenetic relationships between them and allied taxa are discussed. These results confirm the species is new to science.

Keyword: Agaricales, Basidiomycota, morphology

Introduction

The genus *Calocybe* Kühner ex Donk (1962: 42) is typified with *C. gambosa* (Fr.) Donk and is distributed worldwide (Singer 1986). The genus *Calocybe* was first erected (but invalidly published) by Kühner (1938). Kühner (1938) proposed that *Calocybe* is a section of the genus *Lyophyllum*, but Singer (1962a) treated *Calocybe* as a genus of Lyophyllaceae because of the differences to species of the genus *Lyophyllum* in the color of the pileus (the colours are brighter in *Calocybe*). The phylogenetic analyses of Lyophyllaceae were made by Hofstetter *et al*. (2002) based on combined nrLSU, ITS and the mitochondrial ribosomal small subunit gene (mtSSU) sequence data. The results of their study suggested that *Calocybe* was a monophyletic group and could be considered as a genus. Moncalvo *et al*. (2002) also supported the result of Hofstetter *et al*. (2002) based on the phylogenetic analyses of the nrLSU sequences. Bellanger *et al*. (2015) confirmed the taxonomic status of *Calocybe* based on a five-gene phylogenetic analysis of the Lyophyllaceae. For infrageneric classification, Singer (1962a) divided *Calocybe* into five sections, based on the color of pileus, the surface of the spores and the type of epicutis, namely Sect. *Calocybe*, Sect. *Echinosporae* (Lange) Sing, Sect. *Heterosporae* (Lange) Singer, Sect. *Pseudoflammulae* Sing, and Sect. *Carneoviolaceae* Sing. Singer (1986) included Sect. *Heterosporae* (Lange) Singer in *Lyophyllum* and therefore only four sections remain in *Calocybe*. However, Singer's classification system did not correspond to the five clades recognized by Li *et al*. (2017), resulting from the phylogenetic analyses of molecular data.

The main morphological features of *Calocybe* are the tricholomatoid basidiomata, brightly coloured or white pileus and (usually) small spores (Singer 1986). Currently, there are 102 records of *Calocybe* listed in Index Fungorum (http://www.indexfungorum.org). Kirk *et al*. (2008) accepted 40 species in the genus. In China, however, only 8 species have been reported so far: *Calocybe aurantiaca* X.D. Yu & Jia J. Li (2017: 58), *Calocybe carnea* (Bull. 1792: 533) Donk (1962: 43), *Calocybe convexa* X.D. Yu & Jia J. Li (2017: 60), *Calocybe chrysenteron* (Bull. 1821: 126) Singer (1962: 47), *Calocybe decolorata* X.D. Yu & Jia J. Li (2017: 61), *Calocybe gambosa* (Fr. 1821: 50) Donk (1962: 43), *Calocybe ionides* (Bull. 1792: 533) Donk (1962: 43), and *Calocybe naucoria* (Murrill 1914: 15) Singer (1962: 47) (Huang 1998; Mao 2000; Liu 2004; Dai *et al*. 2010; Wu 2010; Li *et al*. 2017). The species diversity of *Calocybe*

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in China has not been properly assessed. In the present study, based on the phylogenetic analyses and morphological characters, two new species of *Calocybe* are described from Liaoning province, China.

Material and methods

Sampling

The samples used in this study were collected in 2016 from Haitang Mountain and Bailang Mountain, Liaoning province, China. Dried specimens have been deposited in the Herbarium Mycology of Jilin Agricultural Science and Technology University (HMJU).

Morphology

Macroscopic descriptions of the basidiomata are based on field notes and color photographs which were taken with a Canon 80D camera. The names of colors used in the descriptions are from Kornerup and Wanscher (1978). Microscopic examination was from dried specimens using light and scanning electron microscopy (SEM). 5% KOH was used as the mounting medium, and Melzer's reagent was used to examine any amyloid or dextrinoid reactions. The Q value (length/width ratio) was calculated from 30 mature basidiospores. Spore measurements were recorded as (a) b–c × d–e (f), where (a) represents the minimum, (f) represents the maximum, b–c and d–e covers 90% of the data. Scanning electron microscope (SEM) images of basidiospores were taken using a FEI Quanta 200 FE-SEM.

DNA extraction, PCR and sequencing

DNA was extracted from dried specimens following the procedure described by Zhao *et al*. (2011). Polymerase chain reaction (PCR) was performed to amplify sequences of the ITS and nrLSU regions. Primers ITS1 and ITS4 (White *et al*. 1990) were used for the ITS region while primers LROR (Rehner & Samuels 1994) and LR7 (Vilgalys & Hester 1990) were used for the nrLSU region. Thermal cycling conditions were as follows, for ITS: initial denaturation at 94 ℃ for 4 min, followed by 40 cycles of denaturation at 94 ℃ for 1 min, annealing at 56 ℃ for 1 min and extension at 72 ℃ for 1 min and final extension step at 72 °C for 5 min; nrLSU: initial denaturation at 94 ℃ for 4 min, followed by 40 cycles of denaturation at 94 ℃ for 90 s, annealing at 56 ℃ for 90 s and extension at 72 ℃ for 90 s and final extension step at 72 °C for 5 min. The products of PCR amplification were examined on a 1% agarose gel and stored at –4 ℃. Sequencing was completed by BGI Co., Ltd, Beijing, China.

Nucleotide sequence analysis

The ITS, nrLSU and the combined ITS/nrLSU datasets were analysed separately. For ITS, 38 sequences representing 23 species were selected, of which 36 sequences were retrieved from GenBank and six species of *Lyophyllum* were used as outgroups. For nrLSU, 27 sequences representing 19 species were selected, of which 25 sequences were retrieved from GenBank and five species of *Lyophyllum* were used as the outgroups. For the combined ITS/nrLSU, 72 sequences representing 26 species were incorporated, of which 68 sequences were retrieved from GenBank and *Agaricus campsetris* L. (1753: 1173) was used as the outgroup. Sequences downloaded from GenBank and the newly generated sequences obtained in this study were aligned manually using BioEdit 7.2.5 (Hall 1999). The conservative region was selected in Gblock (http://www.phylogeny.fr/one_task.cgi?task_type=gblocks) and the gaps were treated as missing data (Talavera & Castresana 2007). Saturation was tested by DAMBE 5.2 (Posada 1998). The selection of best-fitting model was completed by MrModelTest 2.3 based on AIC (Akaike Information Criterion) and BIC (Bayesian Information Criterion) (Nylander 2004). The GTR + I + G model was chosen for the ITS alignment and nrLSU alignment, and the GTR+ I model for the combined ITS/nrLSU alignment. Maximum likelihood (ML) analysis was performed with Mega 7.0. The ML tree was evaluated by bootstrap analysis with 1000 replicates (Stamatakis 2006). Bootstrap values greater than or equal to 60% are indicated along nodes. All the sequences used in this study are listed in Table 1.

TABLE 1. Taxa, vouchers and GenBank accession numbers used in the molecular analyses.

Species	Collection	Public database accession number	
		ITS	LSU
Calocybe aurantiaca	SYAU-FUNGI 006	NR_156304	NG_058937
Calocybe aurantiaca	SYAU-FUNGI-005	—	KU528833
Calocybe badiofloccosa	HMJU00098	SF593738	SF593740
Calocybe carnea	CBS552.50	AF357028	AF223178
Calocybe chrysenteron	AMB 17092	KP885639	KP885628
Calocybe chrysenteron var. cerina	L 05-87	KP885640	KP885629
Calocybe chrysenteron	G0271	—	MK277667
Calocybe chrysenteron	FR2014053	KP192603	—
Calocybe chrysenteron	4398	JF907772	—
Calocybe convexa	SYAU-FUNGI 008	NR_156303	NG_058936
Calocybe convexa	SYAU-FUNGI-007	—	KU528830
Calocybe decolorata	SYAU-FUNGI-003	—	KU528834
Calocybe decolorate	SYAU-FUNGI 004	NR_156302	NG_058938
Calocybe erminea	HMJU00100	SF593739	SF593741
Calocybe fallax	5972	JF907774	—
Calocybe gambosa	HC78/64	AF357027	AF223177
Calocybe gambosa	8064	JF907775	—
Calocybe gambosa	WA0000071035	MK028896	—
Calocybe graveolens	FR2014044	KP192590	—
Calocybe gambosa	TUB 011576	—	DQ071716
Calocybe gambosa	TAA179639	—	AM946414
Calocybe cf. gaveolens	FR2014043	KP192609	—
Calocybe hypoxantha var.occidentalis	SCM 1580	KP885636	—
Calocybe hypoxantha	G0278	—	MK278319
Calocybe hypoxantha	EC 20140228	KP885635	—
Calocybe ionides	13284	JF907780	—
Calocybe indica	Bengaluru	JN874408	—
Calocybe indica	cbetnau1523	MH327767	—
Calocybe indica	cbetnau1521	MH327733	—
Calocybe ionides	HC77/133	AF357029	AF223179
Calocybe ionides	G0764	—	MK277668
Calocybe naucoria	AMB 17094	KP885642	KP885630
Calocybe naucoria	AMB 17093	KP885641	—
Calocybe naucoria	CL 941120-13	KP885643	—
Calocybe naucoria	HC80/103	AF357030	AF223180
Calocybe onychina	CL 121115-07	KP885644	KP885632
Calocybe obscurissima	HC79/181	AF357031	AF223181

.....continued on the next page

TABLE 1 (Continued**)**

Species	Collection	Public database accession number	
		ITS	LSU
Calocybella pudica	AMB 15994	KP858000	KP858005
Calocybella pudica	AMB 15995	KP858001	KP858006
Calocybella pudica	AMB 15996	KP858002	KP858007
Calocybella pudica	AMB 15997	KP858003	KP858008
Calocybe persicolor	FR2013243	KP192564	—
Calocybe persicolor	FR2013197	KP192536	—
Calocybe persicolor	HC80/99	AF357026	AF223176
Calocybe pilosella	TR gmb 00931	KJ576811	—
Lyophyllum ambustum	FR2014080	KP192636	—
Lyophyllum caerulescens	15759	JF908339	—
Lyophyllum amariusculum	FR2013215	KP192544	—
Lyophyllum cf. aemiliae	AB07-10-136	KP192591	—
Lyophyllum aemiliae	FR2014017	KP192596	—
Lyophyllum cf. helvella	GC07101301	KP192625	—
Lyophyllum caerulescens	HC 80.140	AF357052	AF223209
Lyophyllum deliberatum	G0631	—	MK278318
Lyophyllum atratum	PDD:87010	KJ461895	KJ461896
Lyophyllum aff. decastes	PBM3069	—	HQ832459
Lyophyllum decastes	BAYER G 738	—	AY207228
Agaricus campestris	HMAS 272458	KP229418	KP331530
Asterophora parasitica	CBS683.82	AF357038	AF223191
Gerhardtia borealis	AMB 15993	KP858004	KP858009
Gerhardtia citrinolobata	JBSD 126508	KY363576	NG_060686
Gerhardtia highlandensis	PBM2806 (CUW)	GU734744	EF535275
Gerhardtia sinensis	GDGM 46394	KX882033	KX882034
Gerhardtia sp.	HC01/025	EF421103	EF421091
Ossicaulis lignatilis	D604	DQ825426	AF261397
Ossicaulis sp.	JMB-2019a	MK650044	MK650043
Tephrocybella griseonigrescens	TO:HG 21112014	KR105775	NG_059989
Tricholomella constricta	HC 84.75	DQ825429	AF223188
Ugola praticola	CBS 705.82	MH861543	MH873284

Results

Phylogeny

The phylogenetic tree that resulted from the analysis of the combined ITS-LSU dataset indicated that *Calocybe* is a monophyletic genus (Fig. 1). The genus *Calocybe* contained two clades (A and B) in all phylogenetic trees (Figs

1–3). In the ITS tree (Fig. 2), *C. indica* Purkay. & A. Chandra (1974: 415) was found to be distant from all other species of *Calocybe*, which is consistent with the result of Li *et al*. (2017). The two new species represented distinct monophyletic lineages with high statistical support. *Calocybe badiofloccosa* was nested in Clade A while *C. erminea* was placed in Clade B.

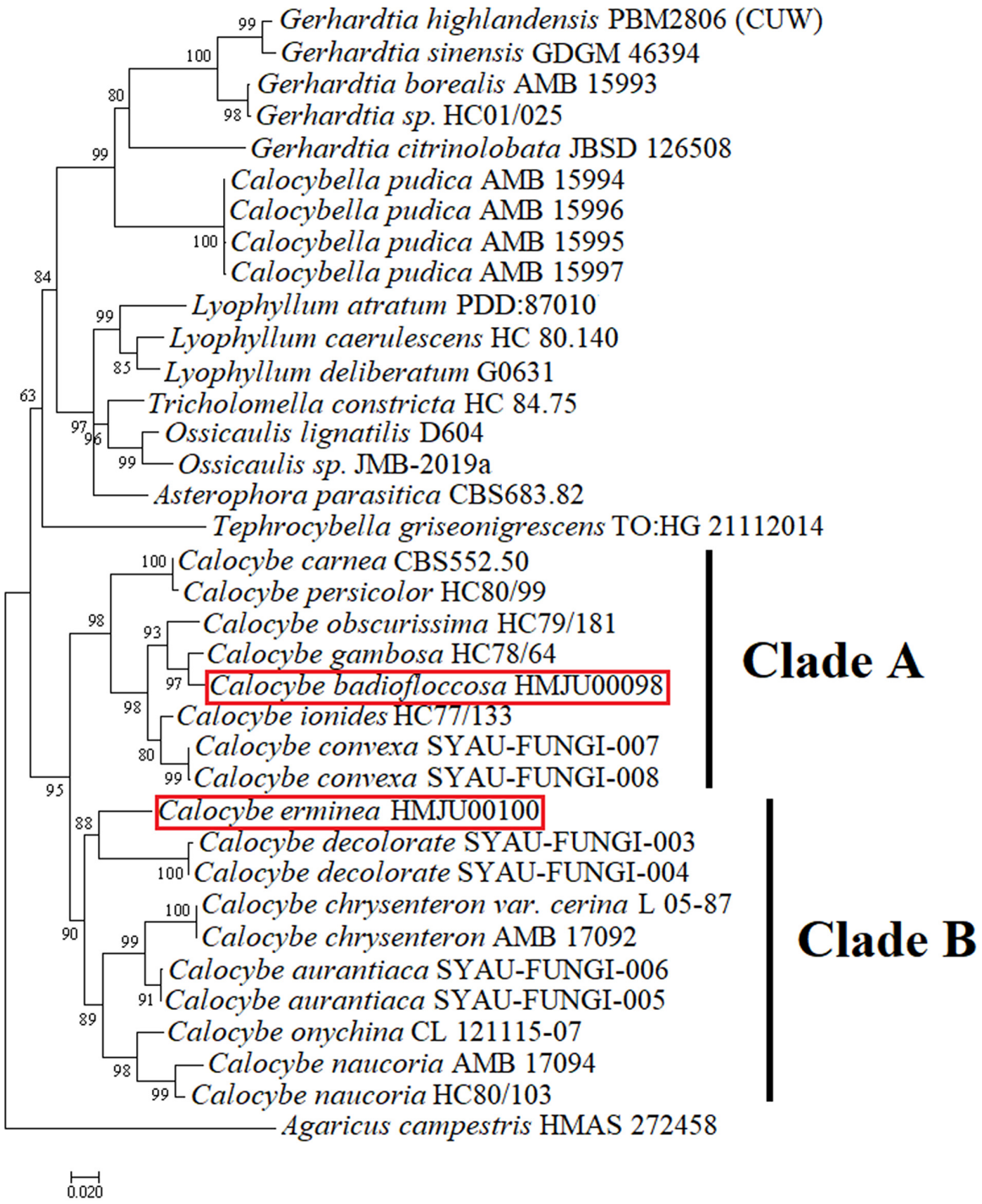

FIGURE 1. Maximum likelihood tree resulting from analysis of ITS+LSU dataset. *Agaricus campestris* was used as the outgroup. The new species are in red.

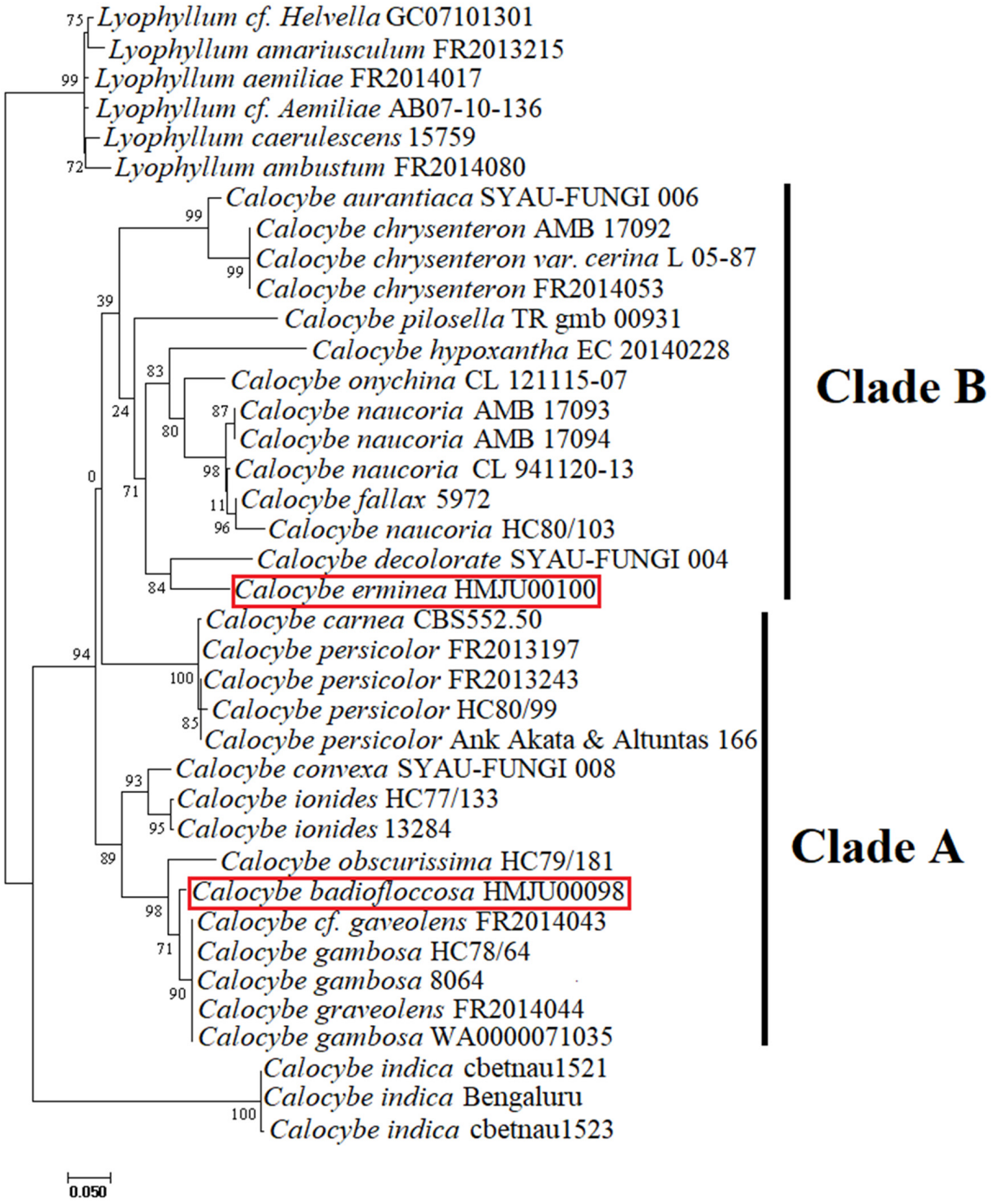

FIGURE 2. Maximum likelihood tree resulting from analysis of ITS sequence data with the outgroup *Lyophyllum*. The new species are in red.

Taxonomy

Calocybe erminea J. Z. Xu & Yu Li, *sp. nov*. **(**Figs. 4–6)

Mycobank No: MB 831885

Diagnosis:—Pileus light khaki (4A4) to dirty white (4B1), yellowish ocher (3A3) at the center. Lamellae 0.3–0.5 cm wide, dirty white (1A2), decurrent. Stipe 3–5 cm long and 0.5–0.8 cm wide, dark brown (4C2). Basidiospores (2.9) 3.2–3.8 (4.7) × 2.5–3.0 (3.55) µm, subglobose, inamyloid.

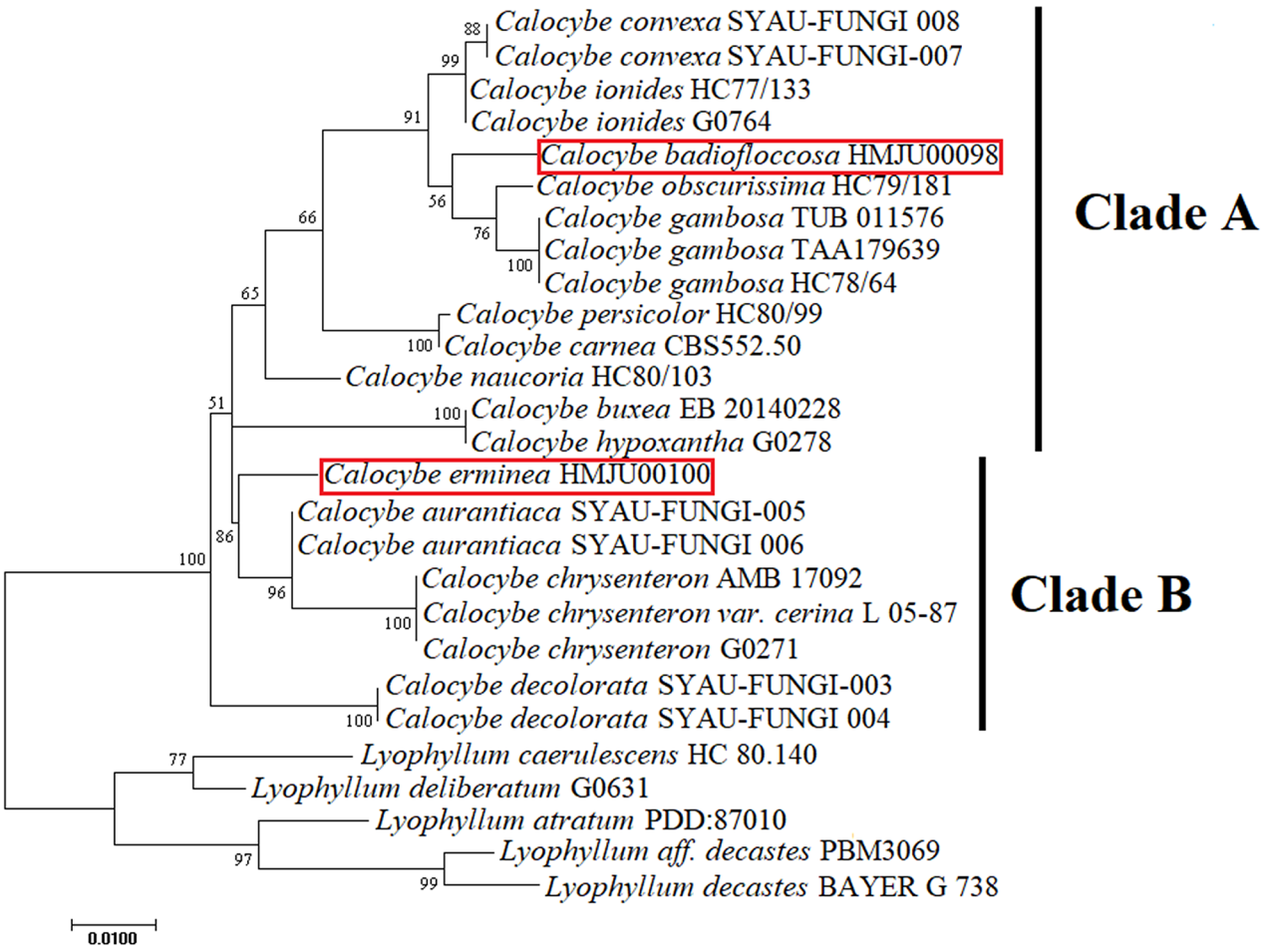

FIGURE 3. Maximum likelihood tree resulting from analysis of LSU sequence data with the outgroup *Lyophyllum*. The new species are in red.

Holotype:—CHINA. Liaoning province, Fumeng Town, Haitang Mountain, 6 August 2016, *Jize Xu HMJU00100*.

Etymology:—From the Latin 'ermineus'. The species is named for its dull white pileus.

Description:—*Pileus* 3–4 cm diam., flattened to slightly depressed at the center, light khaki (4A4) to dirty white (1A2) at first, dirty white (4B1) when mature, yellowish ocher (3A3) at the center; margin regular, slightly incurved; surface smooth glabrous, not sticky, nearly leathery when mature. *Lamellae* decurrent, broad, 0.3–0.5 cm wide, close to distant, dirty white (1A2); edges entire. *Stipe* 3–5 cm long and 0.5–0.8 cm wide, equal or attenuated towards the base; surface dark brown (4C2), slightly hygrophanous, longitudinally fibrillose-striate, whitish (1B2) pruinose at apex, hollow. Context nearly fibrous.

Basidiospores small, (2.9) 3.2–3.8 (4.7) × 2.5–3.0 (3.5) µm, Q= (1.08) 1.24–1.32 (1.50) (n=30), subglobose, smooth, thin-walled, inamyloid. *Basidia* (10.9) 12.6–15.0 (16.0) × 3.4–4.3 (4.5) µm, clavate, four-sterigmate. *Cystidia* absent. Hymenophoral trama regular, hyaline, hyphae cylindrical, 5.6–9.8 µm wide, not pigmented. *Pileipellis* cellular, composed of hyphae 6.3–10.5 µm diam, thin-walled, not pigmented. *Clamp connections* absent.

Habitat and distribution:—Solitary, on the ground in mixed forest. Known from Liaoning Province in China.

Additional specimens examined:—CHINA. Liaoning Province, Fumeng town, Haitang Mountain, 6 August 2016, *Jize Xu HMJU00099*.

Remarks:—*Calocybe* has been divided into Sect. *Pseudoflammulae* and three other sections. Species of Sect. *Pseudoflammulae* have a cellular epicutis (Singer 1986). *Calocybe erminea* belongs to Sect. *Pseudoflammulae* because of the cellular epicutis. Five species of the section are similar to *C. erminea* in size and shape of the basidiocarp: *C. caucasica* Singer (1962: 47), *C. chrysenteron*, *C. cyanea* Singer ex Redhead & Singer (1978: 501), *C. naucoria* and *C.*

onychina (Fr. 1938: 41) Donk (1962: 43). The pileus of *C. erminea* is light khaki to dirty white at first becoming dirty white when mature; however, in *C. caucasica*, the pileus is egg yellow-brown (Singer 1962b), in *C. chrysenteron*, it is brownish yellow, brownish orange or orange-brown (Arnolds 2006), in *C. naucoria* it is dull saffron (Singer 1962b), in *C. onychina* it is purple-brown (Singer 1978), and in *C. cyanea*, it is violet (Singer 1978). *Calocybe erminea* has decurrent gills; in Sect. *Pseudoflammulae* apart from *C. erminea*, only *C. caucasica* has decurrent gills, the gills of the other species are adnate or adnexed. The gills of *C. caucasica* are narrow and crowded (Singer 1962b), whereas, the gills of *C. erminea* are wide and not crowded. *Calocybe erminea* has a dark brown stipe which is equal or attenuated towards the base, and the surface is longitudinally fibrillose-striate or has dirty white fibrous appendages. While, *C. caucasica* has a subcylindrical and glabrous stipe (Singer 1962b), *C. chrysenteron* has a cylindrical and brownish yellow or brownish orange stipe (Arnolds 2006), the stipe of *C. cyanea* is cylindrical, violet at apex and tomentose at base (Singer 1978). *Calocybe erminea* is distinct from *C. onychina* in having a non-pigmented cellular epicutis, the epicutis hyphae in *C. onychina* often have with reddish contents (Singer 1978).

FIGURE 4. Basidiomata of *Calocybe erminea* J. Z. Xu & Yu Li (HMJU00100, holotype). Scale bars: 2 cm (**a–d**). Photos by: J. Z. Xu

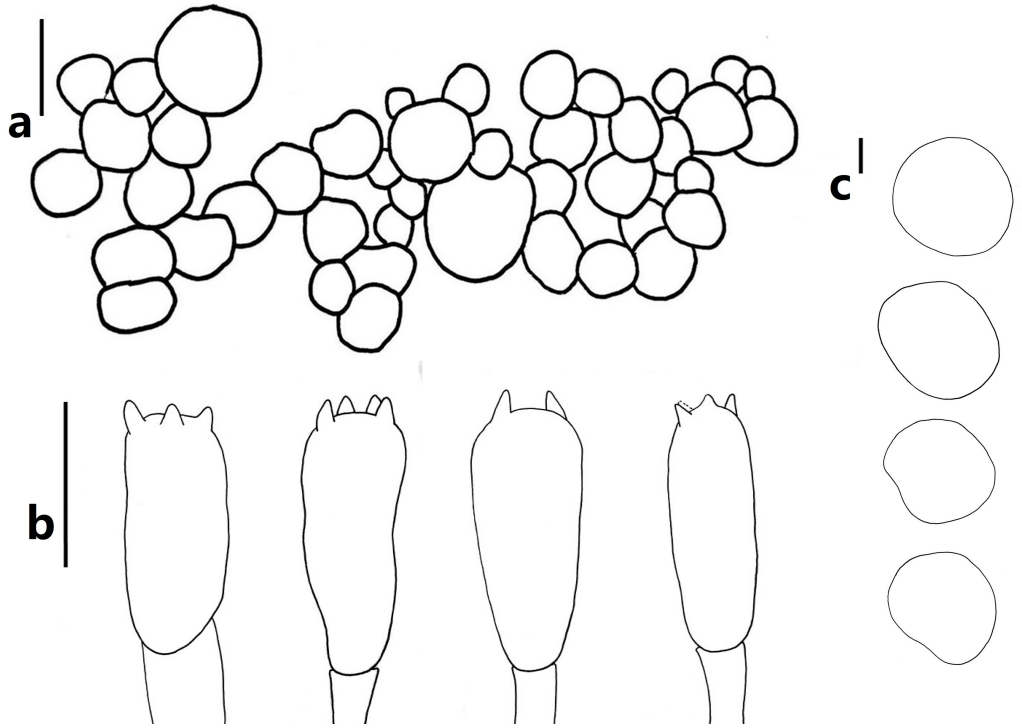

FIGURE 5. Microscopic features of *Calocybe erminea* J. Z. Xu & Yu Li (HMJU00100, holotype). Scale bars: 10 μm (**a–b**); 1 μm (**c**). Drawings by: X. D. Yu

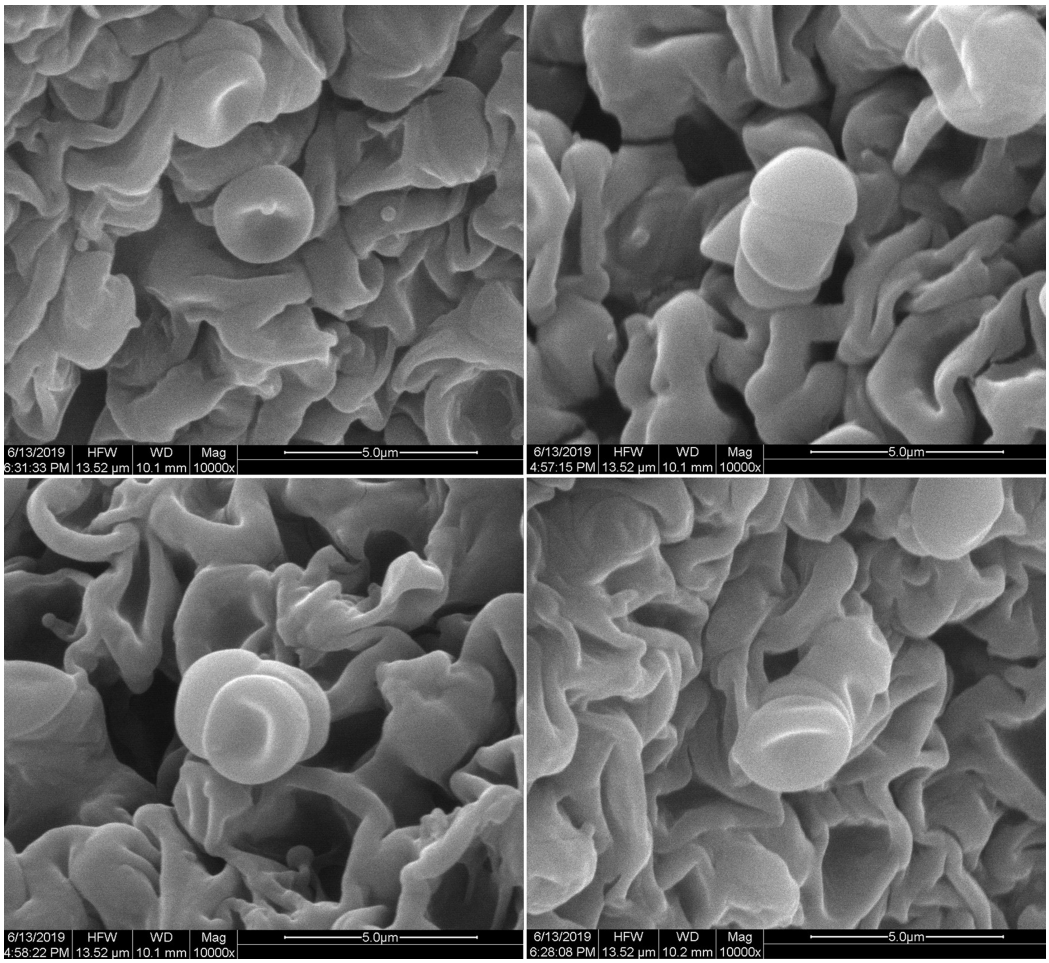

FIGURE 6. Scanning electron microscope images of basidiospores of *Calocybe erminea* J. Z. Xu & Yu Li (HMJU00100, holotype). Photos by: J. Z. Xu

Calocybe badiofloccosa J. Z. Xu & Yu Li, *sp. nov.* (Figs. 7–9)
Mycobank No: MB 831886

Diagnosis:—Pileus 2–4.5 cm diam., light ocher yellow (4A4). Lamellae dirty white (4B3), adnate to decurrent. Stipe 4–8 cm long, darker than pileus, with obvious white pubescence at base. Basidiospores (4.8) 5.0–6.1 (7.0) × 2.7–3.6 (4.2) µm, Q= (1.35) 1.51–1.75 (2.24) (n=30), ellipsoid. Cystidia and clamp connections absent.

Holotype:—CHINA. Liaoning province, Huludao city, Bailang Mountain, 17 August 2016, *Jize Xu HMJU00098*.

Etymology:—Refers to the pubescence at the base of stipe.

Description:—*Pileus* 2–4.5 cm diam., light ocher yellow (4A4), convex to flattened at first, when mature, umbonate at the center, margin becomes inflexed, surface with radial stripes, not smooth. *Lamellae* dirty white (4B3), adnate to decurrent, somewhat broad, edges irregular. *Stipe* 4–8 cm long, the color darker than the pileus and become lighter towards the base, longitudinally fibrillose-striate, with obvious white pubescence at the base, solid. *Context* nearly fibrous.

FIGURE 7. Basidiomata of *Calocybe badiofloccosa* J. Z. Xu & Yu Li (HMJU00098, holotype). Scale bars: 2 cm (**a–c**). Photos by: J. Z. Xu

Basidiospores (4.8) 5.0–6.1 (7.0) × 2.7–3.6 (4.2) µm, Q= (1.35) 1.51–1.75 (2.24) (n=30), ellipsoid, smooth, thin-walled, inamyloid. *Basidia* (11.4) 15.6–18.0 (18.3) × 3.0–4.2 (4.9) µm, clavate, four-sterigmate. *Cystidia* absent. Hymenophoral trama regular, hyphae cylindrical, hyaline, 5.2–9.7 µm wide. *Pileipellis* consisting of dense radially parallel, repent hyphae, 5.1–8.3 µm wide, hyaline, thin-walled, not pigmented. *Clamp connections* absent.

Habitat and distribution:—Single, on humus or ground of broad-leaved forest. Known from Liaoning Province in China.

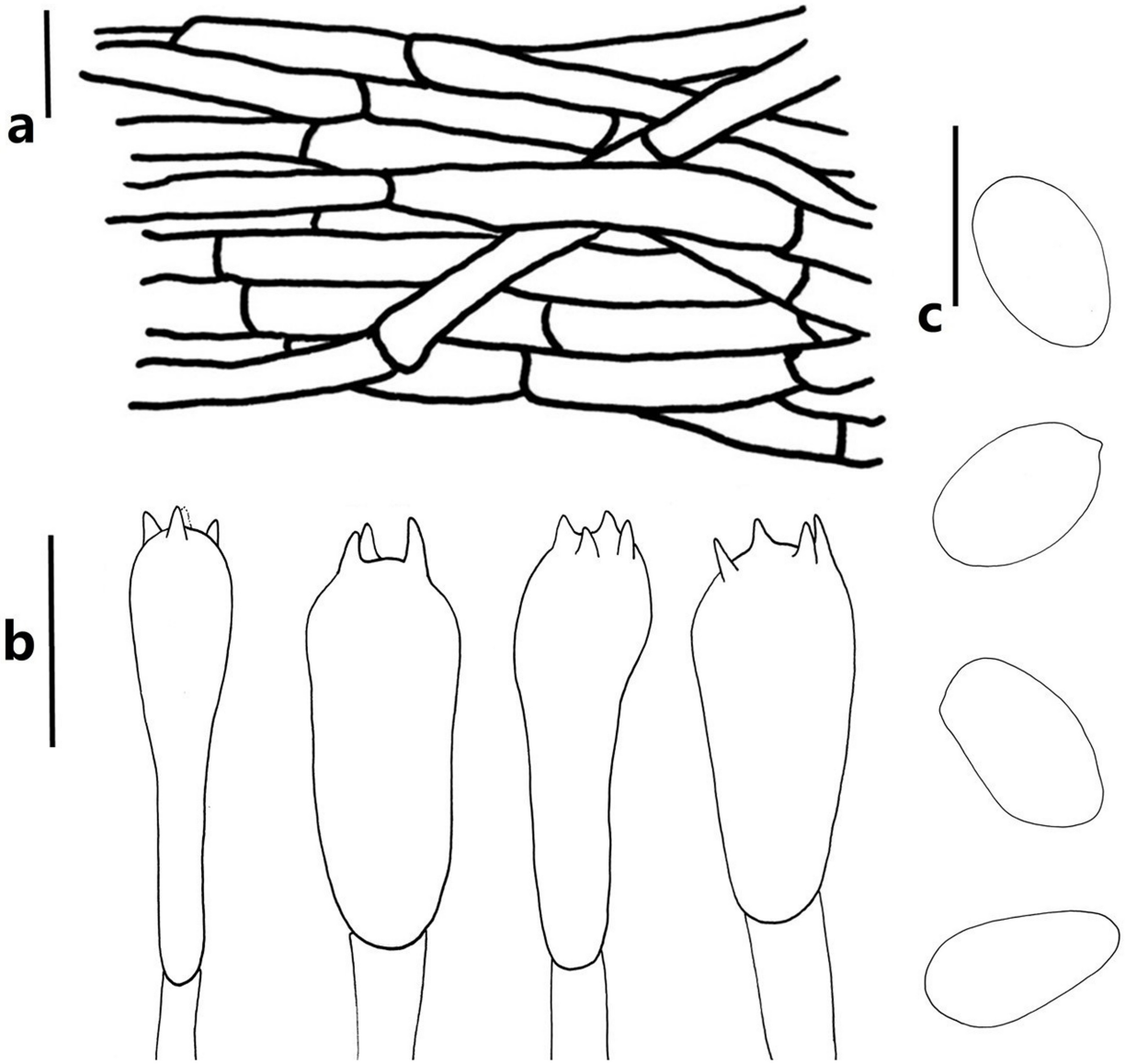

FIGURE 8. Microscopic features of *Calocybe badiofloccosa* J. Z. Xu & Yu Li (HMJU00098, holotype). Scale bars: 10 μm (**a–b**); 5 μm (**c**). Drawings by: X. D. Yu

Remarks:—*Calocybe badiofloccosa* has big basidiospores. Within the genus, *C. africana* Singer (1978: 268), *C. carnea*, *C. coniceps* Singer (1978: 268), *C. gambosa*, *C. heterospora* Singer (1962: 46) and *C. indica* also have large basidiospores (Singer 1978; Singer 1962b; Purkayastha & Chandra 1974). Among the species mentioned above, only *C. coniceps* has cystidia; cystidia are absent in *C. badiofloccosa* and other species. The pileus and stipe of *C. africana* are pale violet (Singer 1978). The pileus of *C. indica* is white or whitish but dirty white with age (Purkayastha & Chandra 1974), whereas, the pileus of *C. badiofloccosa* is light ocher yellow and in this feature it is similar to *C. carnea*, *C. coniceps*, *C. gambosa*, and *C. heterospora*, but it never becomes pale violet or whitish to dirty white. *Calocybe badiofloccosa* has an equal stipe, but the stipes of *C. carnea* and *C. heterospora* are attenuated towards the base (Singer 1962b) and the stipe of *C. indica* is subbulbous at the base (Purkayastha & Chandra 1974). Additionally, *C. heterospora* differs from *C. badiofloccosa* in having white extended rhizomorphs (Singer 1962b). The lamellae of *C. badiofloccosa* are not crowded, are medium broad and with irregular margin. However, the lamellae of both *C. carnea* and *C. gambosa* are crowded, narrow and with entire margin (Singer 1962b; Mao 2000). Furthermore, *C. badiofloccosa* is distinct from *C. carnea* and *C. gambosa* in having radial stripes at the surface of the non-smooth pileus (Singer 1962b; Mao 2000).

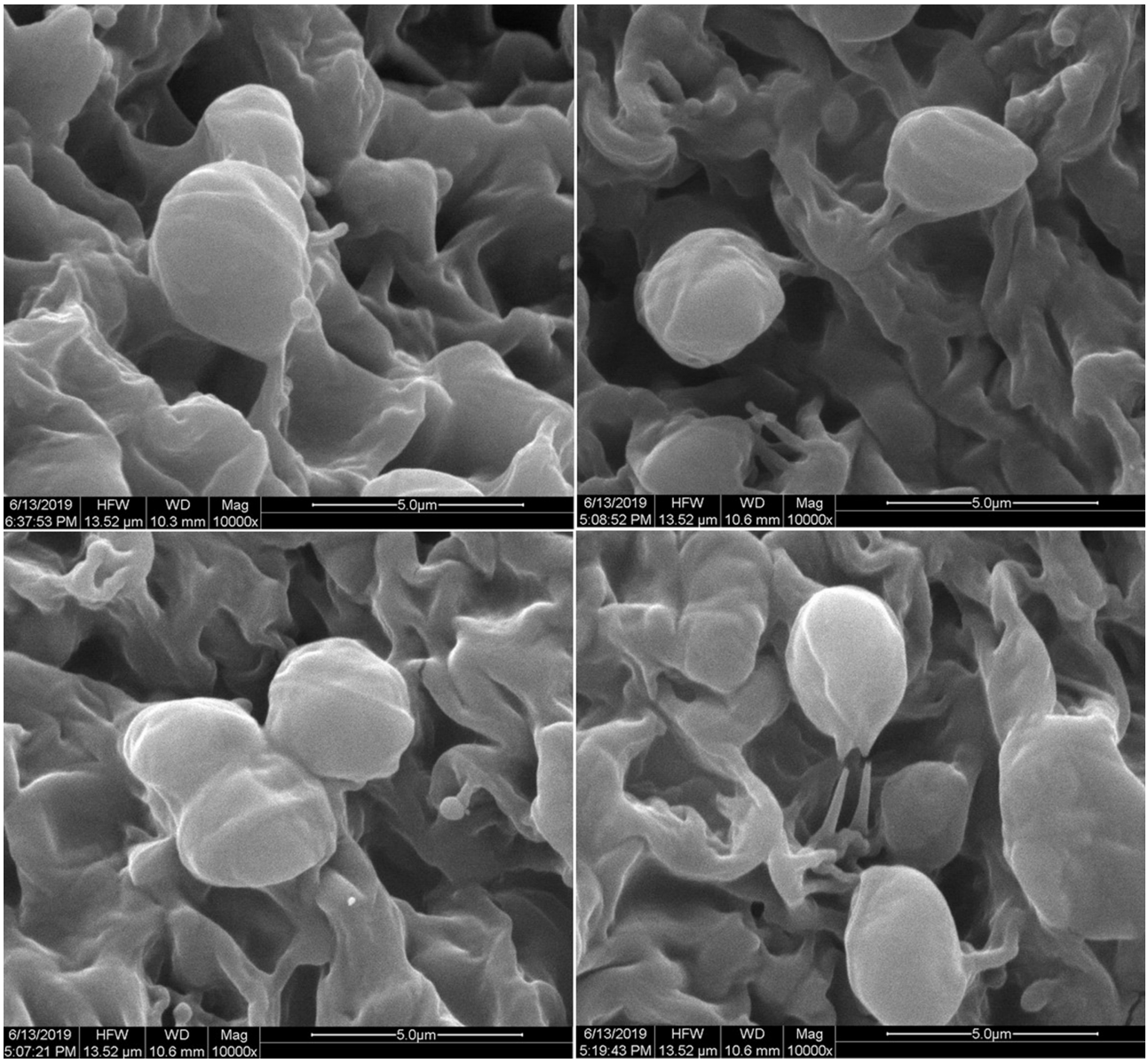

FIGURE 9. Scanning electron microscope images of basidiospores of *Calocybe badiofloccosa* J. Z. Xu & Yu Li (HMJU00098, holotype). Photos by: J. Z. Xu

Discussion

Several recent phylogenetic studies indicated that *Calocybe* formed a distinct clade within the Lyophyllaceae and could be considered as a distinct genus (Hofstetter *et al*. 2002; Moncalvo *et al*. 2002; Bellanger *et al*. 2015; Li *et al*. 2017). However, the infrageneric classification of *Calocybe* is still dubious. The phylogenetic trees of Li *et al*. (2017) recognized five clades within *Calocybe*. However, our combined dataset (ITS+nrLSU) analyses strongly support *Calocybe* as a monophyletic group that contains 2 major clades (A and B). *Calocybe badiofloccosa* is in Clade A. The species of Clade A have white, yellowish to orange-buff pileus and a non-cellular epcutis. *Calocybe erminea* is in Clade B. The epicutises of Clade B species are cellular with the exception of *C. aurantiaca* and *C. decolorata* (Li *et al*. 2017). The morphologically-based infrageneric classification of *Calocybe* by Singer (1986) and the results of our phylogenetic analyses are not congruent. Nevertheless, it shows that the structure of the epicutis is indeed an important character for infrageneric classification. More molecular data are needed to clarify the infrageneric classification of the genus.

Acknowledgements

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