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Six unrecorded macrofungi from the Royal Tombs (Donggureung and Seooreung) of the Joseon Dynasty and Jongmyo Shrine, Korea

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Macrofungi are visible to the naked eye and play important ecological roles in nutrient cycles as decomposers and symbionts in forest ecosystems. Collectively, macrofungi have great potential as valuable resources for food, cosmetics, and medicinal uses. We surveyed the Royal Tombs (Donggureung and Seooreung) of the Joseon Dynasty and Jongmyo Shrine, where the surrounding vegetation is well-preserved, to investigate indigenous macrofungi. During surveys in 2015-2016, we discovered six macrofungi that were previously not recorded to Korea. They were identified to the species level using morphological features and phylogenetic analysis based on the internal transcribed spacer region: *Cruentomycena kedrovaya*, *Dacrymyces aureosporus*, *Laetiporus versisporus*, *Parasola setulosa*, *Piptoporellus soloniensis*, and *Pluteus longistriatus*. The detailed morphological descriptions and molecular analysis are provided in this study.

Keywords: Donggureung, indigenous fungal species, ITS, Jongmyo Shrine, Seooreung, unrecorded species

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Introduction

Macrofungi are visible to the naked eye ($\geq$1 cm in size) and fruit on woody substrate, leaf litter, decaying organic matter, soil or fungi (Kirk *et al*., 2008). Most macrofungi belong to the Basidiomycota, but some to the Ascomycota. They play important ecological roles in nutrient cycles as decomposers and symbionts in forest ecosystems globally (Dix and Webster, 1995). Moreover, macrofungi have great potential in food production, cosmetics, and medicinal uses (Wasser, 2002; Parvez *et al*., 2007). As a result, a great deal of effort has been dedicated to the excavation of indigenous species at local and regional scales.

Macrofungi have been identified based on macro and microscopic characteristics. However, morphological identification is not be reliable when basidiocarps are collected in an immature state and many have different morphology depending on the environment (Grand *et al*., 2011; Park *et al*., 2014; Grilli *et al*., 2016). In addition, some species have similar morphological features, which may result in misidentification (Reeves and Welden, 1967; Grand *et al*., 2011). Accumulated DNA sequence information could help to facilitate species identification (Moncalvo *et al*., 2000; Taylor *et al*., 2000; Nilsson *et al*., 2008; Schoch *et al*., 2012; Kõljalg *et al*., 2013) and the internal transcribed spacer (ITS) region has been proposed as a fungal barcode region (Schoch *et al*., 2012).

The National Institute of Biological Resources organized several projects to discover Korean indigenous macrofungi. The results of these projects have been a tremendous increase in the inventory of macrofungi in Korea. Both morphological characters and molecular analysis have been used to discover new species or unrecorded species in Korea (Park *et al*., 2014; Lee *et al*., 2015; Cho *et al*., 2016). In this study, we surveyed macrofungi in the Royal Tombs of the Joseon Dynasty and Jongmyo Shrine, designated as World Heritage sites by UNESCO (http://whc.unesco.org/en/statesparties/kr), and discovered six previously unrecorded species in Korea. We provide morphological characteristics in detail and phylogenetic relationship with closely related species.

Materials and Methods

Sampling and morphological observation

Macrofungi were collected during the period from 2015

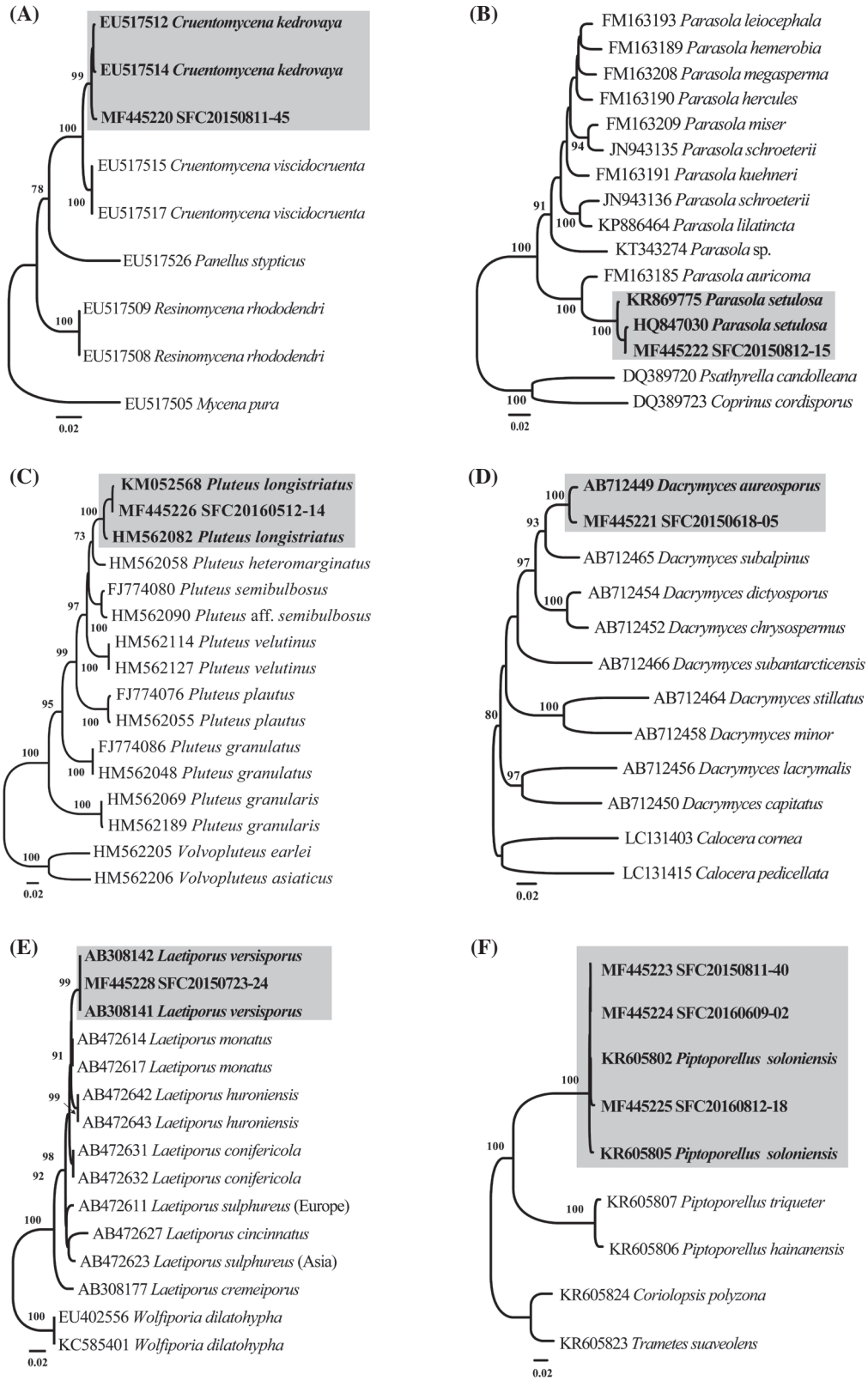


Fig. 1. Neighbor-joining trees based on the sequences of the internal transcribed spacer (ITS) region. Bootstrap scores of >70 are presented at the nodes. The scale bar indicates the number of nucleotide substitutions per site. Gray box indicates taxon which is including specimens of newly recorded species. A, *Cruentomycena kedrovaya*; B, *Parasola setulosa*; C, *Pluteus longistriatus*; D, *Dacrymyces aureosporus*; E, *Laetiporus versisporus*; F, *Piptoporellus soloniensis*.



Fig. 2. Six newly recorded species in Korea: A, *Cruentomycena kedrovaya* (SFC20150811-45); B, *Parasola setulosa* (SFC20150812-15); C, *Pluteus longistriatus* (SFC20160512-14); D, *Dacrymyces aureosporus* (SFC20150618-05); E, *Laetiporus versisporus* (SFC20150723-24); F, *Piptoporellus soloniensis* (SFC20150811-40). Scale bars = 1 cm.

to 2016 in a region of two Royal Tombs (Donggureung and Seooreung) and Jongmyo Shrine. Dried specimens were deposited in the Seoul National University Fungus Collection (SFC). The specimens were initially identified on the basis of their macro- and microscopic features according to the published descriptions (Largent and Thiers, 1977; Breitenbach and Kränzlin, 1984-2000; Gilbertson and Ryvarden, 1986; Hongo and Izawa, 1994; Lee *et al*., 2015). Microscopic observation was performed using Eclipse 80i light microscope (Nikon, Tokyo, Japan). We measured basidia (n = 20/specimen), cystidia (n = 20/specimen), and basidiospores (n = 20/specimen). Quotient (Q) was the ratio of the difference in the mean basidiospore length to the mean spore width of the specimens studied. The 'Methuen and book of Colour' (Kornerup and Wanscher, 1983) was used as a reference for the color standard.

DNA sequence-based identification

Genomic DNA was extracted using a modified CTAB extraction protocol (Rogers and Bendich, 1994). PCR amplification of the ITS region were performed as previously described by Park *et al*. (2013) using primer sets, ITS1F/ITS4 or ITS1F/ITS4B (White *et al*., 1990; Gardes and Bruns, 1993). DNA sequencing was performed at the DNA Synthesis and Sequencing Facility, Macrogen (Seoul, Korea), using an automated DNA sequencer (ABI PRISM 3730XL Analyzer; Applied Biosystems, Foster City, CA, USA). For molecular identification, each sequence was compared with reference sequences in GenBank by using BLAST. Sequences were edited and aligned using MAFFT v7 (Katoh and Standley, 2013). Neighbor-joining phylogenetic analysis was also performed in MEGA5 using the Kimura 2-parameter model of evolution for tree inference with 1,000 bootstrap replicates (Kimura, 1980). All the sequences have been deposited in GenBank (accession Nos. MF445220-MF440226, MF445228).

Results and Discussion

During the survey of indigenous macrofungi from the Royal Tombs and Jongmyo Shrine in Korea from 2015 to 2016, we discovered six species previously unrecorded species in Korea using a combination of ITS se-

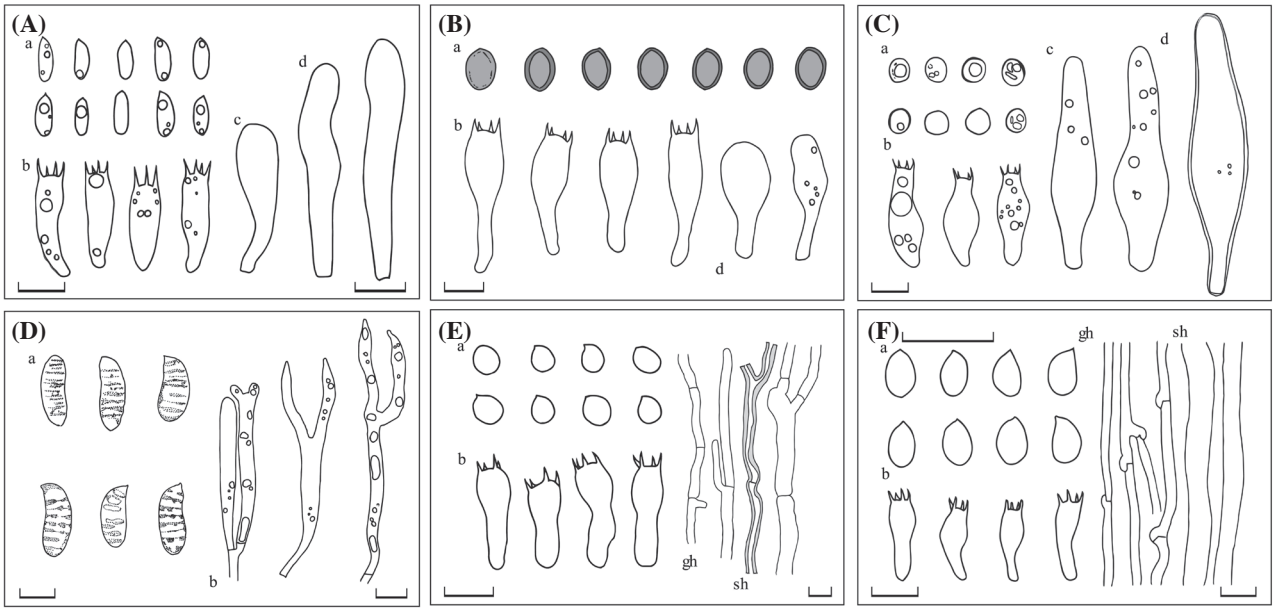


Fig. 3. Microscopic features of six newly recorded species in Korea: A, *Cruentomycena kedrovaya* (SFC20150811-45); B, *Parasola setulosa* (SFC20150812-15); C, *Pluteus longistriatus* (SFC20160512-14); D, *Dacrymyces aureosporus* (SFC20150618-05); E, *Laetiporus versisporus* (SFC20150723-24); F, *Piptoporellus soloniensis* (SFC20150811-40). a, basidiospores; b, basidia; c, cheilocystidia; d, pleurocysidia; gh, generative hyphae; sh, skeletal binding hyphae. Scale bars = 10 μm.

quence analysis and morphological characteristics. Here, we provide morphological characteristics and phylogenetic trees of these six unrecorded macrofungi. They were divided into three morphological groups: agaricoid fungi, jelly fungi, and wood decay fungi.

Specimens of agaricoid fungi were initially identified as *Cruentomycena*, *Parasola*, and *Pluteus* based on their basidiocarp shapes. *Cruentomycena* specimen (SFC20150811-45) formed a monophyletic group with *Cruentomycena kedrovaya* with 99% bootstrap support. This specimen showed sequence similarity of 99.3-99.7 % to *C. kedrovaya* (EU517512, EU517514) and 98.7% to *C. vicidocruenta* (EU517515, EU517517) as a sister group. *Cruentomycena* (Basidiomycota, Agaricales, Mycenaceae) is a genus of agaricoid fungi with only three species (Index Fungorum; http://www.indexfungorum.org). The genus *Cruentomycena* is characterized by mycenoid to marasmielloid basidiomata, bloody red colored, viscid pileus, colorless cheilocystidia, absence of pleurocystidia and elongated amyloid basidiospores (Petersen *et al*., 2008).

The *Parasola* specimen (SFC20150812-15) formed a monophyletic group with *Parasola setulosa* with 100% bootstrap support. This specimen showed sequence similarity of 99.1-99.9% to *P. setulosa* (HQ847030, KR869775) and 96.0% to *P. auricoma* (FM163185) as sister group. *Parasola* (Basidiomycota, Agaricales, Psathyrellaceae) is a genus of agaricoid fungi with 24 species (Index Fungorum; http://www.indexfungorum.org). Two species of this genus, *P. conopilus* and *P. plicatilis*, have been reported in Korea (Lee *et al*., 2015). The genus *Parasola* is characterized by small coprinoid basidiomata, nondeliquescent during sporulation, completely absence of veil, irregularly trimorphic basidia, and black spore print (Redhead *et al*., 2001).

The *Pluteus* specimen (SFC20160512-14) clustered as a monophyletic group with reference sequences of *Pluteus longistriatus* with 100% bootstrap support. This specimen showed sequence similarity of 98.0-99.8% to *P. longistriatus* (HM562082, KM052568). *Pluteus* (Basidiomycota, Agaricales, Plutaceae) is a genus of agaricoid fungi with over 500 species (Index Fungorum; http://www.indexfungorum.org), 32 of which have been reported in Korea. The genus *Pluteus* is characterized by medium to small sized floccose-fibrliiose pileus, free and pinkish lamellae, and absence of annulus and volva (Peck, 1885).

Jelly fungi (SFC20150618-05) were identified morphologically as a species of *Dacrymyces*. This specimen clustered as a monophyletic group with *Dacrymyces aureosporus* with 100% bootstrap support. These specimens showed sequence similarity of 98.5% to *D. aureosporus* (AB712449) and 93.1% to *D. subalpinus* (AB712465) as sister group. *Dacrymyces* (Basidiomycota, Dacrymycetales, Dacrymycetaceae) is a genus of jelly fungi with about 70 species (Index Fungorum; http://www.indexfungorum.org). Four species of this genus, *D. capitatus*, *D. palmatus*, *D. stillatus*, and *D. variisporus*

have been reported in Korea (Lee *et al*., 2015). The genus *Dacrymyces* is characterized by gelatinous and yellow colored basidiocarps, dolipore-type septa in hyphae surrounded by parenthesomes, and causing brown rot (Shirouzu *et al*., 2009).

Wood decay fungi were initially identified as *Laetiporus sulphureus*. However, they were confirmed as *Laetiporus versisporus* and *Piptoporellus soloniensis*. *Laetiporus* specimen (SFC20150723-24) formed a monophyletic group with *Laetiporus versisporus* with 100% bootstrap support. These specimens showed sequence similarity of 100% to *L. versisporus* (AB308141, AB308142). *Laetiporus* (Basidiomycota, Polyporales, Fomitopsidaceae) is a genus of polyporoid fungi with 16 species (Index Fungorum; http://www.indexfungorum.org). Two species of this genus, *L. miniatus* and *L. sulphureus*, have been reported in Korea. The genus is characterized by annual and soft to corky yellowish orange basidiocarp, dimitic hyphal system, absence of clamp connection, irregular and fragile pores, and causing brown rot (Han *et al*., 2016).

Three specimens (SFC20150811-40, SFC20160609-02, and SFC20160812-18) formed a monophyletic group with *Piptoporellus soloniensis* with 100% bootstrap support. These specimens showed sequence similarity of 98.9-99.8% to *P. soloniensis* (KR605802, KR605805) and 77.2-79.3% to *P. hainanensis* (KR605806) and *P. triqueter* (KR605807) as sister group. *Piptoporellus* (Basidiomycota, Polyporales, Fomitopsidaceae) is a genus of polyporoid fungi with only three species (Index Fungorum; http://www.indexfungorum.org). This genus is new to Korea. The genus *Piptoporellus* is characterized by annual and corky pale orange basidiocarp, dimitic hyphal system, generative hyphae with clamp connection, small and fragile pores, absence of cystidia, and causing brown rot (Han *et al*., 2016).

In conclusion, six unrecorded macrofungi were discovered from World Heritage sites in Korea: the Royal Tombs of the Joseon Dynasty (Donggureung and Seooreung) and Jongmyo Shrine. These areas are also known as biodiversity hotspots of Korea. Over 200 macrofungi have been reported from the Donggureung and Seooreung (Yang, 2003). More comprehensive studies of the diveristy of macrofungi in Royal Tombs of the Joseon Dynasty and Jongmyo Shrine are currently underway and will be presented in near future.

Taxonomy

Cruentomycena kedrovaya R.H. Petersen, Kovalenko & O.V. Morozova, Mycotaxon 105: 127 (2008)
Hierarchy: Basidiomycota, Agaricales, Mycenaceae
Specimens examined: KOREA, Gyeonggi-do, Guri-si, Donggureung, 37°37′06″ N, 127°07′58″ E, 11 Aug 2015, Hae Jin Cho, Young Woon Lim, SFC20150811-45 (GenBank accession No. MF445220), on the leaf litter of *Quercus* spp. and *Alnus japonica*.
Pileus 7-11 mm, first convex, then applanate with central depression in age, surface glabrous, dull, margin slightly crenate, striate, pastel red (8A5) to brownish red (9C8). *Lamellae* decurrent, distant, concolorous with pileus, occasionally forked, lamellulae present. *Stipe* 12-20 × 0.4-0.8 mm, terete, tough, hollow, slightly darker than pileus.
Basidiospores 8.4-9.2-10.3 × 3.2-3.6-4.0 μm, Q = 2.24-2.54-2.91, oval, sometimes obclavate, thin-walled, hyaline. *Basidia* 4-spored, 20.2-22.8-25.8 × 5.2-6.4-7.6 μm, narrowly clavate, contents with small guttules. *Cheilocystidia* 24.0-29.9-39.9 × 8.2-8.9-9.4 μm, pedicellate, clavate to broadly clavate. *Pleurocystidia* 32.2-36.0-40.3 × 6.9-7.2-7.4 μm, rare, pedicellate, clavate. *Caulocystidia* 32.8-41.4-61.2 × 6.9-7.9-9.0 μm, very thin-walled, broadly clavate, clamped.
Remarks: This study is the first report of genus *Cruentomycena* in Korea. The key features of this genus are bloody red basidiocarps and absence of pleurocystidia. Petersen *et al*. (2008) reported that almost all basidiomata of *C. kedrovaya* were collected on catkins of *Alnus* in Russia. Korean sample of this species were also collected on the litter layer of *Quercus* spp. and *Alnus japonica*. However, pleurocystidia are observed in Korean samples. Further studies for *Cruentomycena* on the presence or absence of pleurocystidia are needed.

Parasola setulosa (Berk. & Broome) Redhead, Vilgalys & Hopple, Taxon 50(1): 236 (2001)
Hierarchy: Basidiomycota, Agaricales, Psathyrellaceae
Specimens examined: KOREA, Seoul, Jongmyo shrine, 37°34′24″ N, 126°59′47″ E, 12 Aug 2015, Hae Jin Cho, Hyun Lee, Young Woon Lim, SFC20150812-15 (GenBank accession No. MF445222), on the ground of deciduous forest.
Pileus 10-25 mm, first conical to convex, becoming applanate with central depression, hygrophnous, pruinose, plicate-striate, veil absent, margin crenulate, orange white (5A2) to brownish orange (6C5). *Lamellae* free to adnexed, close, concolorous with pileus, black in age, lamellulae present. *Stipe* 35-75 × 1-2 mm, smooth, filiform, hollow, white.
Basidiospores 9.7-10.4-11.2 × 6.9-7.5-8.1 μm, Q = 1.28-1.38-1.54, ellipsoidal, germ-pore central, dark brown. *Basidia* 22.9-27.9-36.7 × 9.0-9.8-10.8 μm, clavate. *Cheilocystidia* not seen. *Pleurocystidia* 22.8-29.3-32.3 × 8.7-9.5-10.5 μm, cylindrical to slightly cymbiform with acute apex. *Pileipellis* hymeniform, dermatocystidia 117.4-119.3-140.9 × 7.3-8.2-8.9 μm, lanceolate with thick walls.
Remarks: *Parasola setulosa* has thicker cell walls than

those of its sister species, *P. auricoma*. *Parasola setulosa* is similar to *P. mirabilis* in morphology but *P. mirabilis* has smaller basidiospores (8.5-10.6 × 5.9-6.6 μm) (Nagy *et al*., 2010).

Pluteus longistriatus (Peck) Peck, Annual Report on the New York State Museum of Natural History 38: 137 (1885)
Hierarchy: Basidiomycota, Agaricales, Plutaceae
Specimens examined: KOREA, Gyeonggi-do, Guri-si, Donggureung, 37°37′17″ N, 127°07′19″ E, 12 May 2016, Hae Jin Cho, Hyun Lee, Young Woon Lim, SFC20160512-14 (GenBank accession No. MF445226), in the rotten knot of live *Acer tataricum* subsp. *ginnala*.
Pileus 20-35 mm, convex when young, becoming broadly convex to flat in age, umbonate to papillate, radially fibrillose, margin rugulose, orange grey (6B2) to brownish grey (7D2). *Lamellae* free, close, lamellulae abundant, white at first, becoming pinkish in age. *Stipe* 40-70 × 5-8 mm, cylindrical, equal, vertically fibrillose, white to whitish brown.
Basidiospores 6.4-7.0-7.7 × 5.4-6.0-6.9 μm, Q = 1.10-1.16-1.27, subglobose to broadly ellipsoidal, anamyloid. *Basidia* 20.4-24.5-28.0 × 8.4-9.5-10.6 μm, clavate to ventricose. *Cheilocystidia* 43.1-56.0-67.2 × 10.2-13.0-22.5 μm, subclavate to clavate. *Pleurocystidia* 49.1-60.6-77.3 × 11.3-13.5-16.8 μm lageniform to subcylindric.
Remarks: *Pluteus longistriatus* is characterized by brownish grey color and the long, radial striations that extend from margin to center of the pileus, exposed white flesh between the striation cracks. This species is similar to *P. plautus* in morphology. However, the cheilocystidia of *P. longistriatus* is subclavate to clavate while the cheilocystidia of *P. plautus* is lageniform (Breitenbach and Kränzlin, 1995).

Dacrymyces aureosporus Shirouzu & Tokum., Persoonia 23: 22 (2009)
Hierarchy: Basidiomycota, Dacrymycetales, Dacrymycetaceae
Specimens examined: KOREA, Gyeonggi-do, Goyang-si, Seooreung, 37°38′20″ N, 126°54′02″ E, 18 Jun 2015, Hyun Lee, Young Woon Lim, SFC20150618-05 (GenBank accession No. MF445221), on the rotten log of deciduous tree.
Basidiocarps gregarious, sometimes joined together, turbinate to convoluted, sessile or stipitate bearing a cerebriform pileus, orange white (6A2) to pale red (7A3), gelatinous, 1-3 mm high, 1-6 mm diameter.
Sterile parts of basidiocarps covered with simple cylindrical to clavate, septate, hyaline, thin walled marginal hyphae. Internal hyphae thin-walled, branched, septate, hyaline, 2.5-3 μm diam. All hyphae without clamp connections. *Basidia* 41.1-53.5-72.2 × 4.5-5.3-6.1 μm, cylindrical, bifurcate. *Probasidia* similar to basidia, becoming bifurcate. *Basidiospores* 17.7-19.9-22.4 × 7.1-8.0-9.7 μm, Q = 2.16-2.48-2.86, allantoid, 7-septate, thin-walled
Remarks: *Dacrymyces aureosporus* is characterized by turbinate to convoluted basidiocarps, and 7-septate basidiospores. This species is similar to *D. chrysospermus*. However, *D. chrysospermus* has terminal cells with thick wall on the sterile part of the basidiocarps (Shirouzu *et al*., 2009) while *D. aureosporus* has thin-walled terminal cells.

Laetiporus versisporus (Lloyd) Imazeki, Bulletin of the Tokyo Science Museum 6: 88 (1943)
Hierarchy: Basidiomycota, Polyporales, Fomitopsidaceae
Specimens examined: KOREA, Seoul, Jongmyo shrine, 37°34′30″ N, 126°59′32″ E, 23 Jul 2015, Hyun Lee, Young Woon Lim, SFC20150723-24 (GenBank accession No. MF445228), on the live stem of *Quercus* spp.
Basidiocarps annual, laterally substipitate, sometimes imbricate. *Pileus* dimidiate to flabelliform, 70-210 mm, surface glabrous, azonate, radially furrowed, vivid yellow (3A8) to deep orange (5A8) when fresh, becoming paler in age. Pore surface pale yellow to yellow, pores angular, 4-5 per mm, with entire dissepiments that easily torn.
Hyphal system dimitic, clamp connection absent. Generative hyphae in context hyaline, thin-walled, sometimes branched, 7-11 μm in diameter. Skeletal hyphae in context thick-walled, much branched, 8-16 μm in diameter. Generative hyphae in tube hyaline, thin-walled, 2.5-4 μm in diam. Skeletal hyphae in tube thick-walled, solid, 2-5 μm in diameter. *Cystidia* absent. *Basidia* 14.8-18.0-21.3 × 6.1-6.7-7.8 μm, clavate, 2 or 4-spored, simple septate at the base. *Basidiospores* 5.3-6.0-7.2 × 4.4-5.1-5.2 μm, Q = 1.0-1.2-1.3, globose to broadly ellipsoidal.
Remarks: *Laetiporus versisporus* used to be misidentified to *L. sulphureus* in Korea for a long time because of similar morphology. In the Indexfungorum (http://www.indexfungorum.org/), *L. versisporus* is also recorded as a synonym of *L. sulphureus*. However, recent phylogenetic studies showed that *L. sulphureus* and *L. versisporus* are different species (Ota *et al*., 2009; Song *et al*., 2014). Moreover, European *L. surphureus* differs from *L. versisporus* by more ellipsoidal basidiospores. Sometimes *L. versisporus* makes anamorphic form of basidiocarp which has brownish and powdery pileus (Ota *et al*., 2009).

Piptoporellus soloniensis (Dubois) B.K. Cui, M.L. Han & Y.C. Dai, Fungal Diversity 80(1): 343 (2016)
Hierarchy: Basidiomycota, Polyporales, Fomitopsidaceae
Specimens examined: KOREA, Gyeonggi-do, Guri-si,

Donggureung, 37°36′50″ N, 127°07′51″ E, 11 Aug 2015, Hae Jin Cho, Young Woon Lim, SFC20150811-40 (GenBank accession No. MF445223), on the dead branch of broad-leaved tree; KOREA, Seoul, Jongmyo shrine, 37°34′26″ N, 126°59′44″ E, 9 Jun 2016, Nam Kyu Kim, Young Woon Lim, SFC20160609-02 (GenBank accession No. MF445224), on the dead log of *Quercus* spp.; KOREA, Gyeonggi-do, Guri-si, Donggureung, 37°37′15″ N, 127°07′33″ E, 12 Aug 2016, Young Woon Lim, SFC 20160812-18 (GenBank accession No. MF445225), on the fallen branch of broad-leaved tree.

Basidiocarps annual, pileate, solitary, corky, hard corky and light in weight when dry. *Pileus* 70-200 mm, dimidiate, semicircular, applanate to slightly convex, surface sponge-like when young, warty in age, sometimes slightly zonate near margin, orange white (6A2) to light orange (5A5), partly orange red (8A7) when young. Pore surface yellowish white to cream, pores round to angular or irregular, 3-4 per mm, dissepiments entire.

Hyphal system dimitic, generative hyphae with clamp connections. Generative hyphae in context rare, hyaline, thin-walled, sometimes branched, 2.5-3.5 μm in diam. Skeletal hyphae in context abundant, thick-walled, sometimes branched, 2-6 μm in diam. Generative hyphae in tube hyaline, thin-walled, sometimes branched, 2.5-3.5 μm in diam. Skeletal hyphae in tube dominant, thick-walled, flexuous, 2-3 μm in diam. Cystidia absent. Cystidioles present. *Basidia* 14.0-17.2-22.4 × 5.6-6.6-7.6 μm, clavate, 4-spored, clamp connection at the base. *Basidiospores* 5.2-5.4-5.7 × 3.0-3.1-3.3 μm, Q = 1.43-1.56-1.67, ellipsoidal to oval, apiculus acute.

Remarks: *Piptoporellus soloniensis* was misidentified to *Laetiporus sulphureus* in Korea. However, they can be distinguished by type of attachment to the substratum and basidiospore shape. *Piptoporellus soloniensis* forms almost solitary basidiocarps and its shape is applanate to slightly convex and has ellipsoidal to oval basidiospore. However, *L. sulphureus* forms almost imbricate and has a guttate basidiospore shape (Ota *et al*., 2009).

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