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Research Article

Molecular Taxonomical Re-classification of the Genus *Suillus* Micheli ex S. F. Gray in South Korea

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Abstract The fungal genus *Suillus* Micheli ex S. F. Gray plays important roles in the survival and growth of plant seedlings. Humans have utilized these ectomycorrhizal fungi to enhance the nutrient uptake and defense systems of plants, particularly in the reforestation of coniferous forests. The genus *Suillus* is easily distinguishable by its distinctive morphological features, although the morphology of the fruiting body does not facilitate reliable interspecies discrimination. On the basis of micro-morphological features and internal transcribed spacer sequence analysis, we found that 51 of 117 Korean *Suillus* specimens had initially been misidentified. The list of the 12 *Suillus* species previously recorded in Korea was re-evaluated and revised to only eight distinct species: *S. americanus*, *S. bovinus*, *S. granulatus*, *S. grevillei*, *S. luteus*, *S. pictus*, *S. placidus*, and *S. viscidus*. We provide taxonomical descriptions for six of these species from the sample specimens.

Keywords Ectomycorrhizal fungi, Internal transcribed spacer, Seoul National University Fungal Collection, *Suillus*, *S. luteus*

Suillus species are common, edible, and ecologically important ectomycorrhizal fungi that are associated primarily with coniferous trees in northern temperate and boreal regions [1, 2]. Symbiotic plant-*Suillus* associations positively impact the survival and growth of the plant by improving water and nutrient uptake [3, 4], and enhancing resistance to stresses such as heavy metal toxicity [5, 6], drought [7], plant pathogens [8], and salinity [9]. For these reasons, *Suillus* has often been used in plant nurseries and the reforestation of pine forests.

The genus *Suillus* Micheli ex S. F. Gray is characterized by its fleshy pileus, glutinous or tomentose cap (in the presence or absence of a veil), and small, glandular dots on the stipe [10]. Micheli [11] first applied the generic name *Suillus* to *Boleti*, but Gray [12] later confined the nomenclature of *Suillus* to *Suillus luteus*. Moreover, *S. luteus* (L.:Fr.) S. F. Gray was classified as the type species of *Suillus*.

Currently, approximately 98 *Suillus* species have been reported worldwide [13], with 12 species reported in South Korea alone: *S. americanus*, *S. bovinus*, *S. cavipes*, *S. granulatus*, *S. grevillei*, *S. luteus*, *S. placidus*, *S. pictus*, *S. subluteus*, *S. tomentosus*, *S. viscidus*, and *S. viscidipes* [14-17]. The generic name of *Suillus* was first applied to these species in South Korea by Lee *et al.* in 1959 [18]. Before 1959, several species had been classified into other genera. In 1940, *S. bovinus*, *S. grevillei*, and *S. luteus* were first reported as members of the genus *Boletus* [19], but were amended in 1959 to be members of the genus *Suillus* [18]. Concomitantly, two other species had been classified as *Boletinus*: *B. pictus* in 1958 [20], and *B. cavipes* in 1959 [18]. The classification of the former was amended to *Suillus* in 1981 [21]. The classification of the latter was amended to *Suillus cavipes* by Smith and Thiers in 1964 [1], although it continued to be classified as genus *Boletinus* in South Korea until the early 1990s [22]. Since 1964, an additional seven species have been classified: *S. granulatus* in 1977 [23], *S. aeruginascens* as a synonym for *S. viscidus* in 1978 [24], *S. placidus* in 1984 [25], *S. americanus*, *S. subluteus*, and *S. tomentosus* in 1991 [26], and *S. viscidipes* in 1995

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[15]. Finally, *S. sibiricus* was classified by Lee and Kim [27] in 1985 during a study of ectomycorrhizal fungi associated with Korean pure-stand forests, although there have been no subsequent reports from South Korea using the new species names or taxonomical descriptions.

The classification of *Suillus* has been a controversial issue [2, 28-30] that was clarified with the addition of DNA data [30-32]. Comparing newly sequenced specimens to DNA sequence databases has been beneficial for accurately re-classifying members of this genus [33]. The internal transcribed spacer (ITS) region has been used as a barcode marker for species identification in fungi because both loci ITS1 and ITS2 are variable and provide interspecific resolution [34-36].

While this genus is easily distinguishable by its distinctive morphological features, identifying *Suillus* to the species level based solely on fruiting body morphology remains a challenge. The present study re-evaluates Korean *Suillus* classification using a combination of micro-morphological features and ITS sequence analysis.

MATERIALS AND METHODS

Collection of *Suillus* specimens and microscopic observation. A total of 117 *Suillus* specimens were examined in this study (Table 1). Specimens were obtained from the Korea National Arboretum (KA), the Herbarium Conservation Center of the National Academy of Agricultural Sciences (HCCN), and the Seoul National University Fungal Collection (SFC). Specimens were collected from across South Korea from 1988 to 2013. Based on morphology, 15 of the 117 *Suillus* specimens could not be identified beyond the genus. The remaining 102 specimens were each initially classified as one of 12 species: *S. americanus* (n = 10), *S. bovinus* (n = 19), *S. granulatus* (n = 13), *S. grevillei* (n = 14), *S. luteus* (n = 17), *S. pictus* (n = 11), *S. placidus* (n = 5), *S. subluteus* (n = 1), *S. tomentosus* (n = 3), *S. viscidipes* (n = 3), *S. viscidus* (n = 1), and *S. sibiricus* (n = 5).

For observations of micro-morphological features, dried tissue from voucher specimens was rehydrated in 3% KOH, stained in Congo red solution, and observed under an 80i light microscope (Nikon, Tokyo, Japan). Up to 20 basidiospores, 10 basidia, and 10 cystidia were measured per specimen. Each collection was assigned a species designation upon identification according to the macro- and micro-morphological characteristics described in previous studies [2, 10, 37].

Molecular approach. Genomic DNA was extracted using a modified cetyltrimethylammonium bromide extraction protocol [38]. To avoid amplification of contaminating species, two *Suillus*-specific primers were newly designed for this study: SI1R (5′-TACACGGTCCAGCGCGGAAG-3′) and SI2R (5′-CATCACAYAGCMCTGGBMGTC-3′). The annealing sites of these primers correspond to the terminus of the ITS2 region. The ITS region was amplified using the forward primer ITS1F or ITS5 [39] with the reverse primer SI1R or SI2R. PCR reactions were performed on a

Table 1. *Suillus* specimens used in this study

Scientific name	Collection No.
S. americanus	**SFC20120919-30**, HCCN02685, HCCN00116, HCCN01032, HCCN01223, HCCN02079, HCCN02400, HCCN07942, HCCN10003, HCCN11268, HCCN11796, HCCN13203, HCCN15955, HCCN16006, HCCN18804, HCCN18949, HCCN19197, HCCN20678, HCCN20820, HCCN20839, HCCN23777, HCCN23971, HCCN24079, HCCN24092, SFC20120919-28, SFC20120929-04, SFC20120929-07, SFC20121001-02
S. bovinus	**SFC20130926-03**, HCCN13678, HCCN13747, HCCN16489, HCCN18059, HCCN18065, HCCN18985, HCCN20536, HCCN20632, HCCN24060, KA12-1590, SFC20121001-04
S. granulatus	**SFC20120922-10**, HCCN03171, HCCN03694, HCCN05963, HCCN10888, HCCN10903, HCCN11671, HCCN13033, HCCN14034, HCCN14350, HCCN14451, HCCN14511,HCCN14827, HCCN18079, HCCN18130, HCCN18461, HCCN20187, HCCN23305, HCCN23311, HCCN23389, HCCN23433, HCCN23453, HCCN23596, HCCN24004, KA12-1193, SFC20110818-20, SFC20110818-69, SFC20120719-07, SFC20120727-02, SFC20120814-15, SFC20120827-06, SFC20120904-03, SFC20120904-04, SFC20120905-18, SFC20120915-22, SFC20120918-07, SFC20120930-01
S. grevillei	**SFC20130917-34**, HCCN19126, KA12-1537, HCCN24140, KA12-1709
S. luteus	**SFC20130926-19**, HCCN13739, HCCN15711, HCCN18238, HCCN18952, HCCN18972, HCCN20830, HCCN21008, HCCN23289, HCCN23928, HCCN24248, KA12-1569, SFC20121031-01
S. placidus	**HCCN23560**, HCCN02278, HCCN24084
S. pictus	**SFC20120922-06**, HCCN03600, HCCN03758, HCCN06311, HCCN07034, HCCN13635, HCCN23401, HCCN23554, HCCN23759, HCCN23750, HCCN23933, HCCN24076, HCCN24109, SFC20120919-31, SFC20120922-06, SFC20120925-11
S. viscidus	**HCCN24143**, HCCN13812, HCCN16543, HCCN10904

Specimens denoted in bold text were those chosen as representatives for morphological and molecular analysis.
KA, Korean National Arboretum; HCCN, Herbarium Conservation Center of National Academy of Agricultural Sciences; SFC, Seoul National University Fungus Collection.

T-Professional thermocycler (Biometra, Gottingen, Germany) using Accupower PCR PreMix (Bioneer, Seoul, Korea) with a final reaction volume of 20 μL containing 10 pmol of each primer and 1-μL DNA suspension. The PCR conditions used were 95°C for 5 min; followed by 35 cycles of 95°C for 40 sec, 55°C for 40 sec, and 72°C for 1 min; and a final extension step at 72°C for 10 min. The PCR products were electrophoresed in a 1% agarose gel stained with EcoDye DNA staining solution (Solgent, Seoul, Korea) and purified using the Expin PCR Purification Kit (GeneAll Biotechnology, Seoul, Korea) according to the manufacturer's instructions. Sequencing was performed in the reverse direction for each sample by using either SI1R or SI2R. The DNA sequencing was performed by a private DNA sequencing facility (Macrogen, Seoul, Korea) using an ABI3700 automated DNA Sequencer (Applied Biosystems, Foster City, CA, USA).

Sequence data were edited and aligned using MEGA5.2 [40]. Reference sequences were selected based on previous *Suillus* phylogenetic studies and available GenBank data [30, 33]. Multiple sequence alignments were performed using the default settings of MAFFT v7.122 [41]. Alignments were checked by eye and modified manually. A neighbor joining phylogenetic analysis was performed using MEGA5.2 [42] with 1,000 bootstrap replicates.

RESULTS AND DISCUSSION

Based on phylogenetic analysis of the ITS data, the 117 *Suillus* specimens, which were initially classified as one of 12 species (n = 102) or as an unidentified species (n = 15), were re-confirmed as eight distinct species: *S. americanus*, *S. bovinus*, *S. granulatus*, *S. grevillei*, *S. luteus*, *S. pictus*, *S. placidus*, and *S. viscidus* (Fig. 1). The phylogenetic relationships between these eight species were similar to previous results [30, 33]. *Suillus* specimens that were re-classified by sequence analysis were verified by their morphological characteristics. Classification based on molecular data was consistent with micro- and macroscopic features of the eight species. Although the microscopic characteristics of eight Korean *Suillus* species were consistent with published data, no apparent differences were observed in the sizes and shapes of the basidia, spores, and cystidia among these species. This result indicates that macroscopic features, along with sequence data, are important for classification purposes, while microscopic features are not diagnostic for these *Suillus* species.

All of the available specimens previously classified as *S. tomentosus*, *S. sibiricus*, and *S. subluteus* were re-classified as *S. americanus* (Fig. 2). Previous reports of these three species [26, 27] did not include voucher specimens,

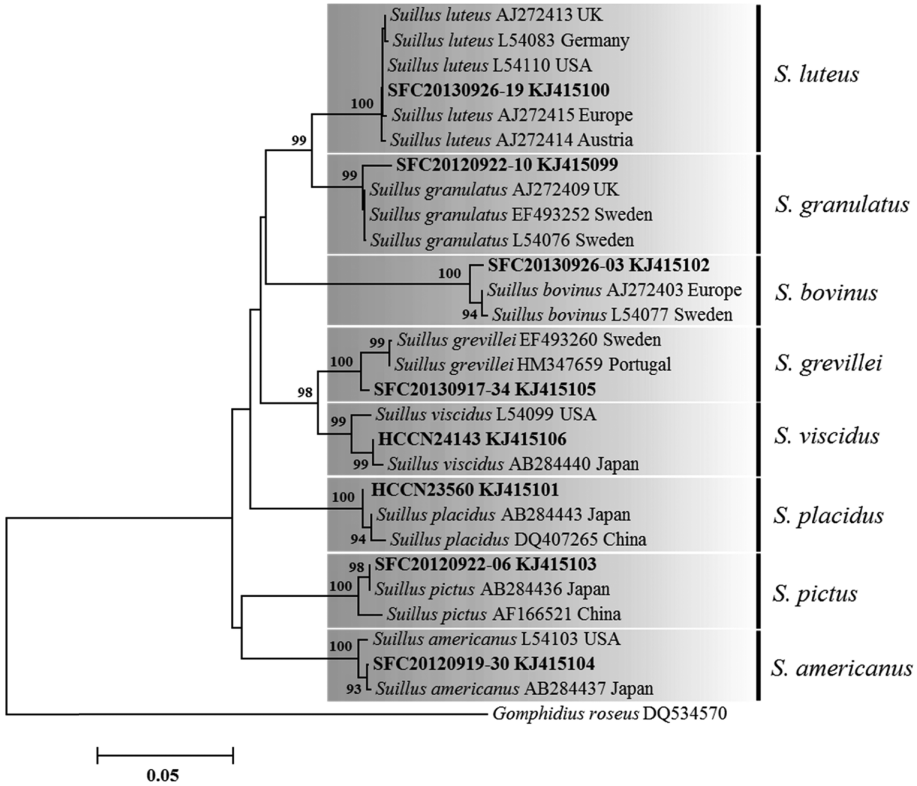

Fig. 1. Molecular classification of 117 Korean *Suillus* specimens based on neighbor joining analysis of the internal transcribed spacer (ITS) region. Numbers above the branches are the frequencies (%) of statistical occurrence based on 1,000 bootstrap replicates. All newly sequenced specimens in this study are labeled in bold text. *Gomphidius roseus* (DQ534570) was used as the outgroup.

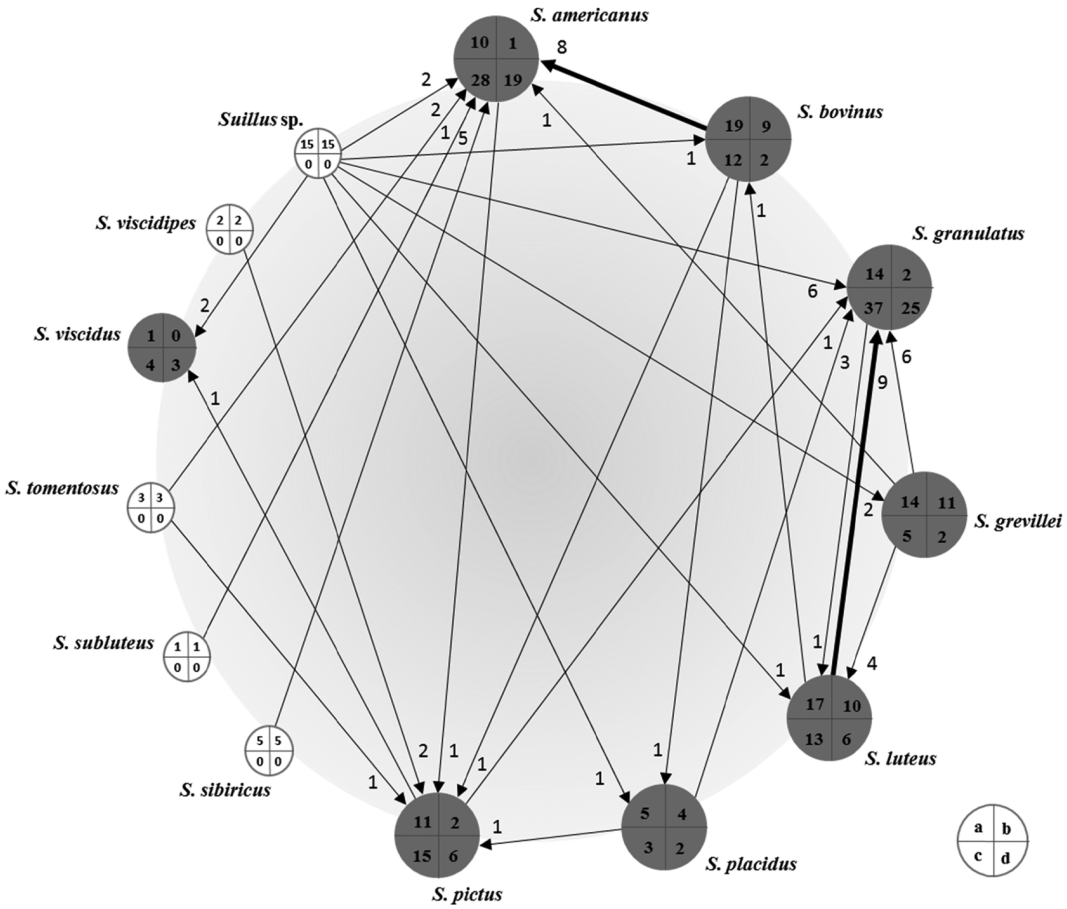

Fig. 2. A flow diagram of *Suillus* species identification. Species represented by a gray circle are verified as present in Korea, while species represented by a white circle were previously recorded in Korea but were absent in this study. Arrows represent a species misidentification, starting at the initial identification and ending at the new identification. a, number of specimens identified based on morphology; b, number of misidentified specimens; c, number of specimens identified based on molecular data; d, number of additional specimens identified from the molecular data.

photographs, or morphological data. Based on our results, we believe that *S. tomentosus*, *S. sibiricus*, and *S. subluteus* should be considered not to occur in Korea until future studies prove otherwise. Another species, *S. viscidipes*, was first reported in Korea in 1993, complete with voucher information, illustrations, and data on microscopic features [15]. With access to the same specimen as in the original report, we were able to sequence its DNA. Based on phylogenetic analyses, this specimen was re-classified as *S. pictus* (Fig. 2). Therefore, we determined that *S. viscidipes* has yet to be reported from South Korea. These results highlight the importance of voucher specimens and/or data used for identification. The availability of voucher specimens and data provide a way to verify or correct the identity of organisms recorded in surveys, especially for fungi with similar morphologies such as in *Suillus* [43].

Out of 117 specimens used in this study, 51 specimens were determined to be misidentified (a 43% misidentification rate). Relatively high misidentification rates were observed in *S. placidus* (80%), *S. grevillei* (78.6%), *S. luteus* (58.8%), and *S. bovinus* (47.3%). Many specimens were re-classified as *S. granulatus* (n = 25) and *S. americanus* (n = 19; Fig. 2). *Suillus granulatus* specimens were primarily misidentified as *S. grevillei* (n = 6), *S. luteus* (n = 9), and *S. placidus* (n = 3); also, in the opposite direction, many *S. grevillei* (n = 6) and *S. luteus* (n = 9) specimens were misclassified as *S. granulatus*. The similarity of these species can be seen in a previous classification; Singer [10] suggested that *S. granulatus*, *S. luteus*, and *S. placidus* belong to same section *Suillus* based on morphological similarity. *Suillus luteus* can be differentiated from *S. granulatus* and *S. placidus* based on the presence of an annulus and the absence of milky droplets [2, 37]. However, these morphological characteristics are unreliable, as they cannot usually be observed in dried samples. Furthermore, in fresh samples, the annulus may be detached from the stem, or the milky droplets may not be produced during the observational period, depending on the ecological environment.

In addition to *S. tomentosus*, *S. sibiricus*, and *S. subluteus*, eight specimens of *S. bovinus* were re-classified as *S. americanus*. *Suillus americanus* possesses several distinguishing features, including a yellow pilus with reddish brown scales,

a stipe covered with glandular dots, and an association with *Pinus strobus* [2]. *Suillus sibiricus* is the species most closely related to *S. americanus*, but can be distinguished by its thicker stipe, gregarious basidiocarps, and annulate features. Although it is challenging to differentiate between the two species, five specimens initially identified as *S. sibiricus* were re-classified in this study as *S. americanus* using a combination of morphological features and ITS sequence analysis. We conclude that the presence of this species in South Korea cannot be verified, as all available records of *S. sibiricus* are unreliable.

We report eight *Suillus* species native to South Korea on the basis of their morphological features and the ITS sequences from voucher specimens. Additionally we provide a simple morphological key for identification and detailed taxonomical descriptions for six species where such information is available.

Taxonomy.

Key to Korean *Suillus* species

1. Cap dry, surface tomentose, reddish brown scales ..... *S. pictus*
1. Cap slimy and glabrous 2
2. Mycorrhizal association with *Larix* 3
2. Mycorrhizal association with *Pinus* 4
3. Cap greyish to brownish, pores greyish ..... *S. viscidus*
3. Cap yellow to brownish orange, pores pale yellow ..... *S. grevillei*
4. Mycorrhizal association with *Pinus strobus* 5
4. Mycorrhizal association with *Pinus densiflora* 6
5. Cap yellowish, light yellow patches along the cap margin ..... *S. americanus*
5. Cap whitish yellow to brown, pores white, pinkish droplets ..... *S. placidus*
6. Stipe with membranous annulus ..... *S. luteus*
6. Stipe without annulus 7
7. Tubes beaded with milky droplets when young ..... *S. granulatus*
7. Tubes not beaded with milky droplets, cap concolorous with stipe ..... *S. bovinus*

***Suillus americanus* (Peck) Snell**, Lloydia 7: 39 (1944) (Fig. 3A).

Basidiocarps: Cap 40~68 mm wide, subconic to convex with incurved margin; a light yellow veil hanging down at the margin; surface of cap yellow to brownish and slimy, sometimes with reddish brown patches. Context mustard yellow or pale apricot color. Stipe 40~75 mm long, 10 mm thick; frequently cylindrical and crooked; scattered with brownish glandular dots; downy surface with membranous ring. Tube yellow or greenish-yellow but brown in age; subdecurrent; pores large and radially arranged; angular in shape; around 1~2 mm wide. Basidia clavate with four sterigmata; 21.7~33 × 5.5~10.3 μm. Basidiospores elliptic and with a smooth surface; 7.4~11.2 × 3~4.2 μm. Pleurocystidia cylindrical to clavate; 33.1~62.2 × 4.3~8.7 μm.

Specimens observed: SFC20120919-28, SFC20120919-30, HCCN23777, HCCN20820.

Habitat in Korea: Mycorrhizal association with *Pinus strobus*; solitary or gregarious; late summer to fall.

Remarks: Mustard yellowish colored cap and pores; cottony patches along the cap margin when young, and presence of epicutis in the cap when old. This species is commonly confused with *S. sibiricus*, but stipe is more slender than *S. sibiricus*.

***Suillus bovinus* (L.) Roussel**, F. Calvados: 34 (1796) (Fig. 3B).

Basidiocarps: Cap 30~100 mm wide, convex with incurved margin when young, and planar when old; glabrate and smooth surface; little viscid, brownish-orange to reddish-brown. Context whitish to yellowish-white, solid, unchanging when cut. Stipe 30~75 mm long, 5~10 mm thick, cylindrical and crooked in the middle or upper part; brownish-yellow or concolorous with the cap; slightly pinstriped and reticulate fibrillose at base. Tubes subdecurrent grayish-yellow or concolorous with the cap; pores radially arranged and irregularly angular in shape. Basidia clavate with four sterigmata; 19.4~25.8 × 5.6~7.9 μm. Basidiospores elliptic and with a smooth surface; 7.2~10.2 × 3~4.4 μm. Pleurocystidia cylindrical to clavate; 28.3~54.4 × 3.7~6.9 μm.

Specimens observed: SFC20121001-04, SFC20130926-03, HCCN20536, KA12-1590.

Habitat in Korea: Mycorrhizal association with *Pinus densiflora*, often solitary or gregarious, summer to fall.

Remarks: Color of stipe and pores similar to that of the cap; sometimes confused with *S. granulatus*, but without secretion of milky droplets. In Korea, this species is strongly associated with *Pinus densiflora* and often found growing with *Gomphidius roseus*.

***Suillus granulatus* (Fr.) Kuntze**, Rev. Gen. Pl. 32: 535 (1898) (Fig. 3C).

Basidiocarps: Cap 40~110 mm wide, hemispherical or convex; surface slimy or glabrous; incurved margin; color variable but typically brownish-orange, light brown, or sometimes pinkish in the center and yellowish-white at the edge when young, but pinkish-brown when mature; wave patterned or spotted with reddish-brown or cinnamon color. Context soft and plump; whitish to yellow and unchanging when cut. Stipe 50~80 mm long, 10~20 mm thick, solid, and scattered with brownish glandular dots; white or yellowish flesh. Tubes subdecurrent, pale yellow or light yellow; pores around 1 mm wide, often beaded with milky droplets when young. Basidia clavate with four sterigmata; 21.2~28.1 × 5.4~7.5 μm. Basidiospores elliptic and with a smooth surface; 6~10.6 × 2.4~3.8 μm. Pleurocystidia cylindrical to clavate; 32.1~54.6 × 4.7~8.7 μm.

Specimens observed: SFC20110818-20, SFC20120814-15, SFC20120922-10, KA12-1193.

Habitat in Korea: Mycorrhizal association with *Pinus densiflora*; solitary or gregarious; summer to fall.

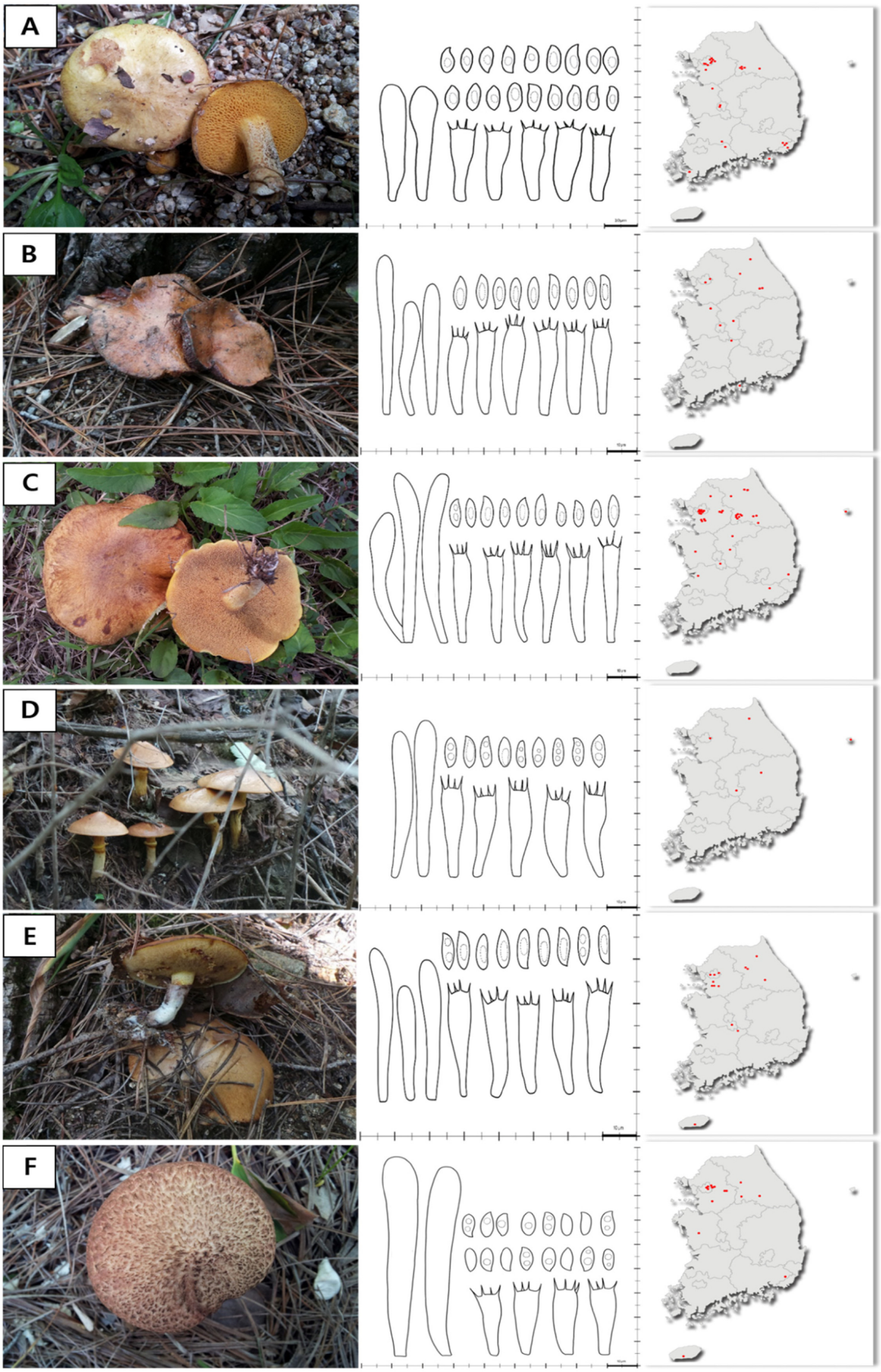

Fig. 3. Fruiting bodies, microscopic features, and geographical distributions of *Suillus* species in South Korea. A, *S. americanus*; B, *S. bovinus*; C, *S. granulatus*; D, *S. grevillei*; E, *S. luteus*; F, *S. pictus*. For the microscopic features (center panels), the basidiospores are above, while pleurocystidia are below and to the left, and basidia below and to the right (scale bars = 10 μm).

Remarks: This species can be distinguished from *S. luteus* by the absence of an annulus, absence of glandular dots, and the secretion of droplets when young.

***Suillus grevillei* (Klotzsch) Singer** Farlowia 2: 259 (1945) (Fig. 3D).
Basidiocarps: Cap 50~100 mm wide, hemispherical or convex to planar; surface viscid and glabrous; lemon yellow or brownish-orange. Context soft and thick; pale yellow. Stipe 75~100 mm long, 10~15 mm thick and viscid; cylindrical and somewhat thickened at the base; yellowish-brown; reticulate above the annulus, and pinstriped and filamentous at base; yellowish annulus. Tubes adnate to decurrent; pale yellow but cinnamon colored when bruised or cut; pores angular, around 1~2 mm wide. Basidia clavate with four sterigmata; 19.5~28.1 × 4.9~8.9 μm. Basidiospores elliptic and smooth surface; 7.8~10.6 × 3.4~5.4 μm. Pleurocystidia cylindrical to clavate; 33.3~53.9 × 4.1~8.1 μm.
Specimens observed: SFC20130917-34, HCCN19126, KA12-1709.
Habitat in Korea: Mycorrhizal association with *Larix*; gregarious and sometimes forming a fairy ring; late summer to fall.
Remarks: This species has a slimy floccose annulus and is commonly associated with *Larix* in Korea.

***Suillus luteus* (Fr.) S. F. Gray**, Nat. Arr. Br. Pl. 1: 646 (1821) (Fig. 3E).
Basidiocarps: Cap 60~100 mm wide, hemispherical or convex to planar; surface viscid and glabrous; shiny when dry; dark brown to reddish-brown; membranous veil hanging from the margin. Context soft, whitish-yellow, and unchanging when cut. Stipe 50~90 mm long, 10~20 mm thick; frequently cylindrical and crooked; pale yellow and scattered with brownish glandular dots above the annulus; typically covered by a purplish-brown veil at base. Tubes 1 mm wide, adnate and small; pale yellow or greyish-yellow; surface usually covered with a partial veil. Basidia clavate with four sterigmata; 20~24 × 4.4~7.7 μm. Basidiospores elliptic and with a smooth surface; 6.4~9 × 2.8~4.6 μm. Pleurocystidia cylindrical to clavate; 24.8~50 × 4.7~9.1 μm.
Specimens observed: SFC20121031-01, SFC20130926-19, HCCN24248, HCCN18972.
Habitat in Korea: Mycorrhizal association with *Pinus*; solitary or gregarious; summer to fall.
Remarks: This species is easily recognized by the purplish-brown annulus and slimy cap surface when moist.

***Suillus pictus* (Peck) A. H. Sm. & Thiers**, Contribution toward a Monograph of North American Species of *Suillus*: 31 (1964) (Fig. 3F).
Basidiocarps: Cap 40~80 mm wide, convex to planar; surface tomentose and dry; inrolled margin; fibrillose veil hanging from the margin; background yellow to orange yellow, mostly forming brownish-red or grayish-brown scales. Context soft and yellow. Tubes decurrent, light yellow; pores large and angular, becomes reddish-brown when cut; covered with membranous veil. Stipe 40~80 mm long, cylindrical or subclavate; 10~20 mm thick; coating similar to the fibrils of the cap; fine soft annulus but evanescent. Context yellow to brownish and soft in the inside. Basidia clavate with four sterigmata; 22.7~30.3 × 5.5~9.2 μm. Basidiospores elliptic and with a smooth surface; 7.2~10.4 × 3~4.8 μm. Pleurocystidia cylindrical to clavate; 32.1~80.4 × 6.3~10.5 μm.
Specimens observed: SFC20120919-31, SFC20120922-06, SFC20120925-11, HCCN23750.
Habitat in Korea: Mycorrhizal association with *Pinus*, solitary or gregarious and sometimes forming a fairy ring, late summer to fall.
Remarks: Surface tomentose with scales present on the cap and stipe.

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