RESEARCH ARTICLE

Taxonomical Studies of Three Unrecorded *Entoloma* Species in Korea

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ABSTRACT

Since 2013, more than 100 *Entoloma* (Entolomataceae, Agaricales) collections have been made during field investigations of mushroom flora in Korea. Among these collections, three *Entoloma* species were identified as new records from Korea. Morphological examinations were made based on the macro- and micro-morphological features of the isolates collected during field visits. To ensure the identity of the isolates at the species level, DNA sequences from four gene regions (*rpb2*, ITS, 28S, and mtSSU) were compared. To the best of our knowledge, these are the first records of *E. aprile, E. chytrophilum,* and *E. hirtipes* in Korea. Comprehensive descriptions, photographs, and phylogenetic examinations are presented here.

Keywords: Agaricales, Entolomataceae, Morphology, Molecular phylogeny

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INTRODUCTION

The Entolomataceae Kotl. & Pouzar is a large and highly variable family with a cosmopolitan distribution, including temperate and tropical climates. This family belongs to the order Agaricales that comprises 3 main agaricoid genera: *Rhodocybe* Maire, *Clitopilus* (Fr. Ex Rabenh.) P. Kumm., and *Entoloma* (Fr.) Kumm. *sensu lato*, among which *Entoloma* s.l. is the second largest euagaric genus containing more than 1,500 species distributed in arctic to tropical habitats [1-3]. Based on macroscopic and microscopic features, at least 16 taxa can be defined within *Entoloma* s.l. [4,5]. This genus was traditionally characterized based on the spores that are angular in all views owing to a network of interconnected ridges [6-8].

Since 2013, more than 100 samples of *Entoloma* species were collected during mycological expeditions in Korea. Among these, some species that were frequently found in the field were already recorded in Korea, but 3 distinct species were subjected to re-examination for reliable identification. Here, we describe 3 new records of *Entoloma* species in Korea based on morphology and sequence comparisons using 4 gene regions, comprising *rpb2*, ITS region. 28S, and mtSSU regions of rDNA.

MATERIALS AND METHODS

Sampling and morphological observations

More than 100 samples of *Entoloma* species were collected during mycological expeditions in Korea since 2013. Samples of species not previously reported from Korea were subjected to morphological examinations and phylogenetic analyses. Detailed information concerning the specimens used in this study is shown in Table 1. Dried specimens made were deposited to the Herbarium of Korea National Arboretum (HKNA).

Macro-morphological characters were based on the field notes and color photos of the fresh fruit-bodies. Samples were sectioned and rehydrated in 3% KOH in order to examine micro-morphological characters using an Olympus BX53 microscope (Olympus, Tokyo, Japan) and Jenoptik ProgRes C14 Plus Camera (Jenoptik Corporation, Jena, Germany). Microscopic characters were measured using the ProgRes Capture Pro v. 2.8.8. software (Jenoptik Corporation, Jena, Germany). In the taxonomic descriptions, Q refers to the quotient of basidiospore length divided by basidiospore width [5].

DNA extraction, PCR, and sequencing

For molecular analyses, DNA was extracted from fruit bodies of the collections using a DNeasy Plant mini Kit (Qiagen Inc., Valencia, CA, USA). ITS1/ITS4 [9], LR0R/LR5 [10,11], MS1/MS2 [9], and rpb2-i6f/rpb2-i7r [2] primers were used to amplify the internal transcribed spacer (ITS) including 5.8S rDNA, 28S rDNA, mtSSU, and *rpb2* regions, respectively. The polymerase chain reaction (PCR) products were purified using ExoSAP-IT PCR Cleanup Reagents (Applied Biosystems, Foster City, CA, USA) following the manufacturer's protocols. The resulting products were sequenced by Macrogen Sequencing Service (Macrogen, Seoul, Korea) with the primers used for PCR amplifications.

Phylogenetic analyses of sequence data

All sequences were edited using Chromas v. 2.6.6, BioEdit Sequence Alignment Editor v. 7.0.5.3, and MEGA v. 7.0.26 [12]. The edited sequences were aligned using an online version of MAFFT 7 (http://mafft.cbrc.jp/alignment/server/) [13]. For phylogenetic analyses, available ITS sequences of *Entoloma* species were retrieved from GenBank. The sequence of *Tricholoma vaccinum* (FJ845444) was used as outgroup. Maximum likelihood (ML) analyses were performed using RAxML HPC Black Box v. 8.2.10 [14] using the default option with general time reversible(GTR) substitution model implemented in the CIPRES cluster server (https://www.phylo.org/) at the San Diego Supercomputer Center. Phylogenetic trees obtained from RaxML analyses were viewed in MEGA v. 7 [12]. Tree robustness was evaluated with 1,000 bootstrap replications. The bootstrap support values were indicated in the tree nodes (bootstrap values $\geq$ 70%).

Table 1. *Entoloma* materials used in the DNA sequence analyses.

Species	Specimen No.	Locality	Collection date	GenBank no.			
				ITS	28S	mtSSU	*rpb2*
Entoloma aprile	KA13-0010	Daebong mountain, Gyeongsangnam-do, Korea	14 May 2013	MN088707	MN088712	MN088716	MN095757
E. aprile	KA16-0020	Jeombong mountain, Gangwon-do, Korea	4 May 2016	MN088708	MN088713	MN088717	MN095758
E. chytrophilum	KA15-0373	Nochu Mountain, Gangwon-do, Korea	18 Aug. 2015	MN088709	MN088714	MN088718	MN095759
E. hirtipes	KA13-1522	Odae mountain, Gangwon-do, Korea	22 Oct. 2013	MN088710	MN088715	MN088719	MN095760

RESULTS AND DISCUSSION

Phylogenetic analyses

PCR amplifications and sequencing of 4 gene regions (*rpb2*, ITS, 28S, and mtSSU) were successfully completed for all collections in this study. Sequences were deposited in the GenBank nucleotide database (www.ncbi.nlm.nih.gov), and the accession numbers are listed in Table 1. A GenBank basic local alignment search tool(BLAST) search of sequences from the 4 Korean collections showed > 99% similarity with existing GenBank sequences: KA16-0020 and KA13-0010 with *E. aprile* (AB520845 and AB520846), KA15-0373 with *E. chytrophilum* (KC898430), and KA13-1522 with *E. hirtipes* (JX454867). In the phylogenetic tree based on the ITS region, the sequences obtained in this study clustered within the *Entoloma* clade and formed a single lineage with *E. aprile*, *E. chytrophilum*, and *E. hirtipes* with strong support (BS values: 99/100/100, respectively; Fig. 1). Although the ITS region including the 5.8S rDNA region was sufficient to differentiate *Entoloma* species at the species level, the sequences of *rpb2*, 28S, and mtSSU regions were additionally used to confirm the identity of the species and to resolve their taxonomic positions. The sequences obtained from the 4 gene regions (ITS, 28S, mtSSU and *rpb2*) are provided (Table 1).

Taxonomy

***Entoloma aprile* (Britzelm.) Sacc.**, Syll. fung. (Abellini) 5: 696, 1887 (Fig. 2)

Bas. *Agaricus aprilis* Britzelm. 1885

Description: Pileus 20–30 mm, campanulate to plicate, then convex, expanding with age, centrally depressed, with straight, entire margin, striate, hygrophanous, brown to dark brown. Lamellae adnate, crowded, up to 5 mm broad, moderately thick, white then pink, pinkish brown with serrulate edge. Stipe 25–40×5.5–7.0 mm, cylindrical, white to pale grayish, tapering or thickened toward the base. Basidiospores 8–11×7–9.5 μm, Q = 1.1–1.3, Qav = 1.1, irregularly nodulose-angled, with 5–7 blunt angles in outline. Basidia 4-spored, 30–58 × 11.5–15 μm, clamped. Hymenophoral trama regular, made up of inflated elements, 38–190 × 4.5–9.3 μm. Pileipellis a differentiated cutis of cylindrical hyphae, 10–13.8 μm wide. Stipitipellis a cutis of narrow, cylindrical hyphae 5–11.5 μm wide. Caulocystidia absent. Clamp connections abundant.

Habitat: On soil in broadleaf forest, appearing in early spring.

Collections examined: KOREA. Gyeongsangnam-do, Daebong Mountain, May 14, 2013, KA13-0010; Gangwon-do, Jeombong Mountain, May 4, 2016, KA16-0020.

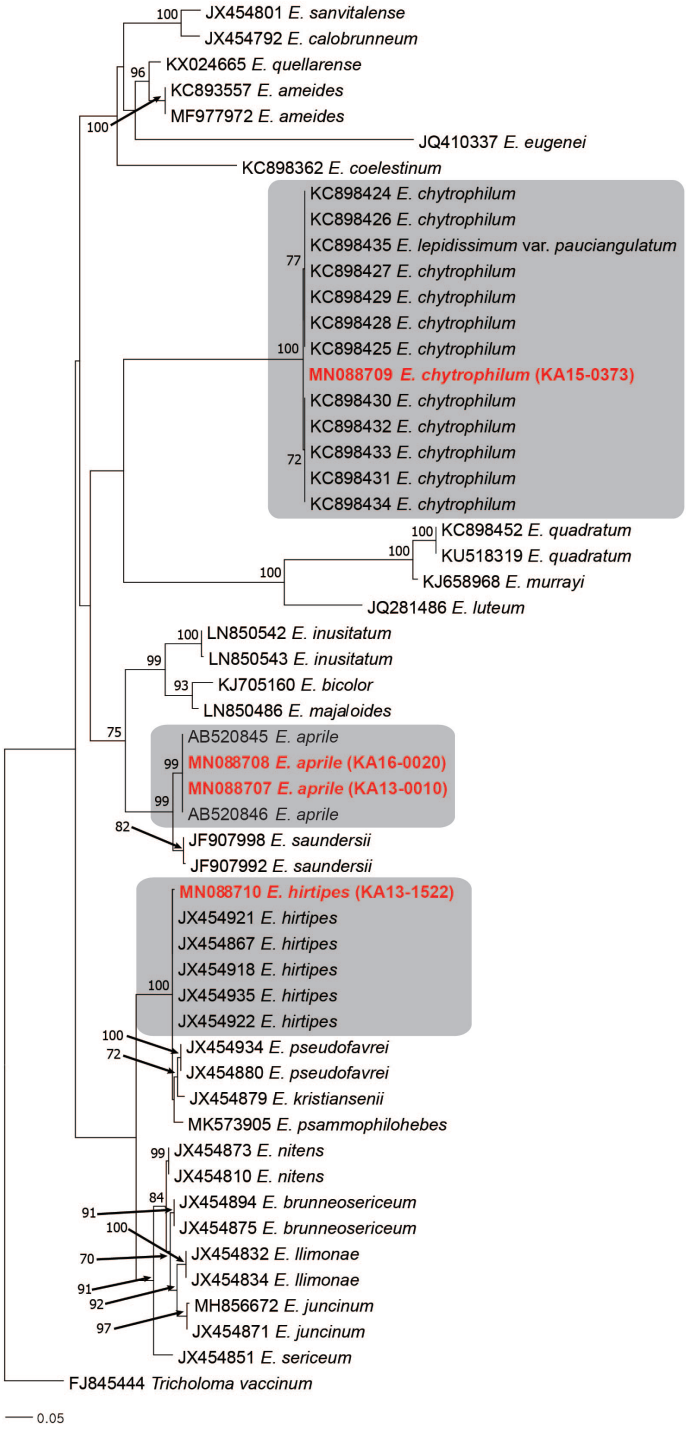

Fig. 1. RAxML tree of *Entoloma* species using internal transcribed spacer (ITS) region sequences. Bootstrapping values higher than 70 % are shown in the branches (1,000 replicates). The scale bar equals the number of nucleotide substitutions per site.

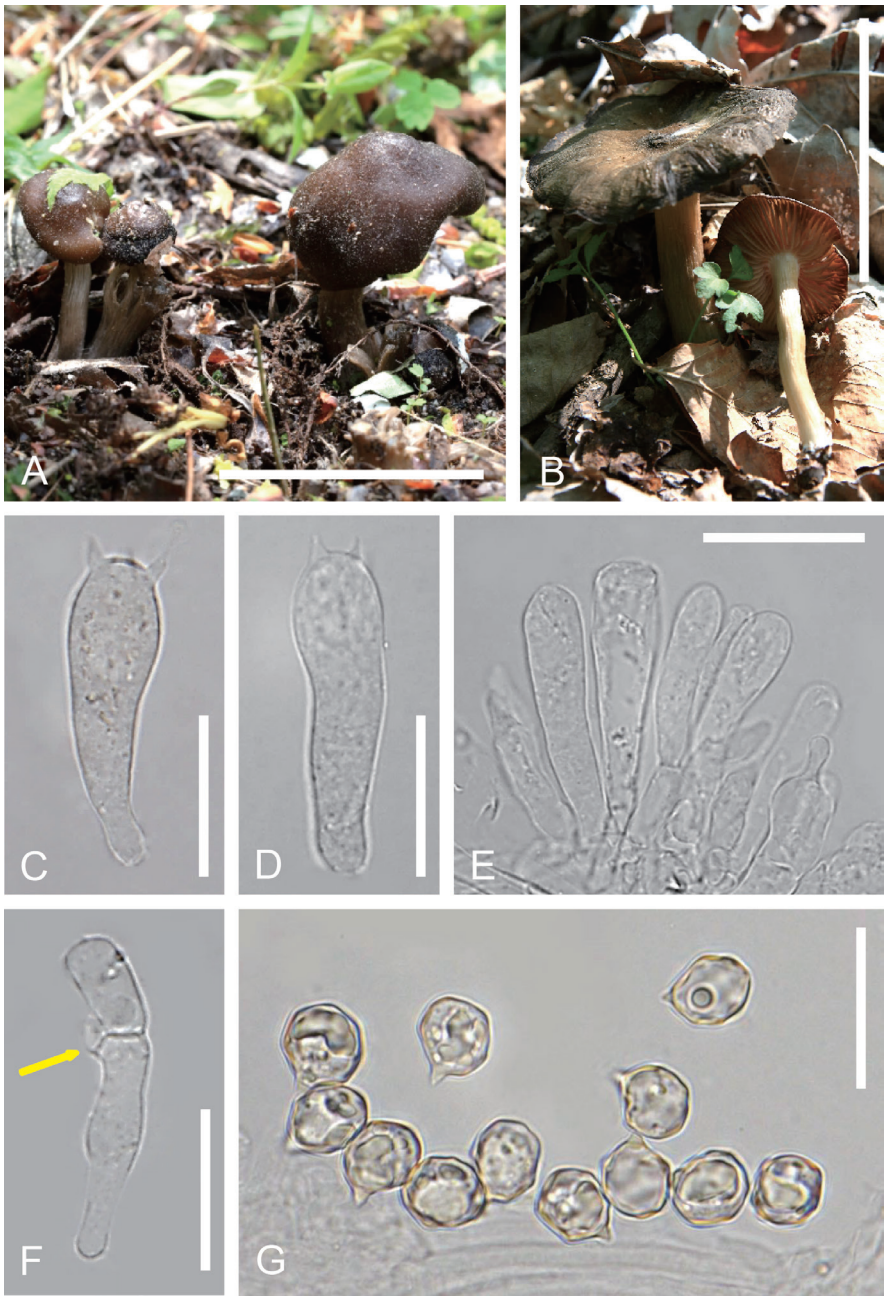

Fig. 2. *Entoloma aprile*. A–B, Natural habit of *E. aprile*; C–E, Basidia; E, Basidioles; F, Clamp-connection; G, Basidiospores. Scale bars A–B = 5 cm; C–G = 20 μm.

Comments: Morozova et al. [15] showed that *E. aprile* belongs to *Rhodopolioid* clade in subgenus *Entoloma* in phylogenetic tree. The rhodopoloioid species are distinguished from other *Entoloma* taxa by their large basidiomata, uniform predominantly brown colors due to simultaneously encrusted-intracellular plasmatic pigment, and nitrous smells [16]. Morphological characteristics of the specimens examined are in good agreement with previous descriptions of *E. aprile* [16,17]. In addition, the Korean collections of *E. aprile* cluster in a highly supported clade with Japanese collections (Fig 1; BS values: 99%). Therefore, these Korean collections were identified as *E. aprile*.

***Entoloma chytrophilum* Wölfel, Noordel. & Dähncke**, in Wölfel & Noordeloos, Öst. Z. Pilzk. 10: 190, 2001 (Fig. 3)

Description: Pileus 5–18 mm broad, plano-convex to concave with depressed center, not hygrophanous, with straight then deflexed undulating margin, radially fibrillose, dark blue, slightly discoloring to bluish violaceus. Lamellae moderately distant, adnate-emarginate with small decurrent tooth, ventricose, white, becoming pinkish, with entire concolorous edge. Stipe 17–38 × 1–2 mm, cylindrical, fibrillose-striate, slightly squamulose in the upper part, concolorous with pileus or grey-blue, with white basal tomentum. Smell strong, fungoid. Basidiospores 7.8–11 × 5.5–7.0 μm, Q = 1.3–1.7, nodulose, heterodiametrical. Basidia 1–4-spored, 35–47.5 × 9–11.5 μm, clavate, clamped. Lamellae edge fertile. Cheilocystidia absent. Pileipellis slightly inflated hyphae 8–18 μm wide with blue intracellular pigment. Clamp connections present.

Habitat: On rotten wood in mixed forests.

Collection examined: KOREA. Gangwon-do, Gangneung-si, Nochu Mountain, August 18, 2015, KA15-0373.

Comments: *Entoloma coelestinum* has been recorded in Korea and the specimen we collected is similar to *E. coelestinum* morphologically. However, basidiospores of *E. coelestinum* are smaller than *E. chytrophilum* (6.9–8.3 × 5.2–6.2 μm vs. 7.8–11 × 5.5–7.0 μm) [15]. In the molecular phylogenetic analysis, the collection used in this study formed a distinct single lineage in the *E. chytrophilum* clade and it formed a sister clade to *E. coelestinum* (Fig. 3). Therefore, morphological and molecular data support its identity as *E. chytrophilum*. Morozova et al. [15] described *E. lepidissimum* var. *pauciangulatum* is now treated as a synonym of *E. chytrophilum*. In RAxML tree of the present study, we also confirmed that *E. lepidissimum* var. *pauciangulatum* clusters together with *E. chytrophilum*. In this study, it was not possible to examine our collection to take a photo under the microscope. Therefore, morphological description and molecular

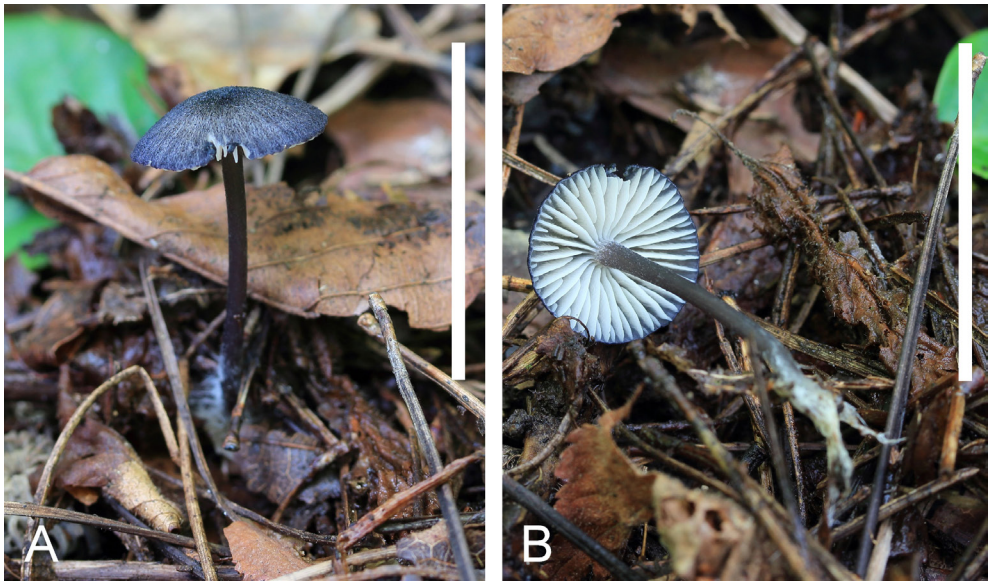

Fig. 3. *Entoloma chytrophilum*. A–B, Natural habitat of *E. chytrophilum*. Scale bars. A–B = 3 cm.

examination were provided here.

***Entoloma hirtipes* (Schumach.) M.M. Moser,** in Gams, Kl. Krypt.-Fl., Bd II b/2, ed. 4 (Stuttgart) 2b/2: 206, 1978 (Fig. 4)

Bas. *Agaricus hirtipes* Schumach. 1803

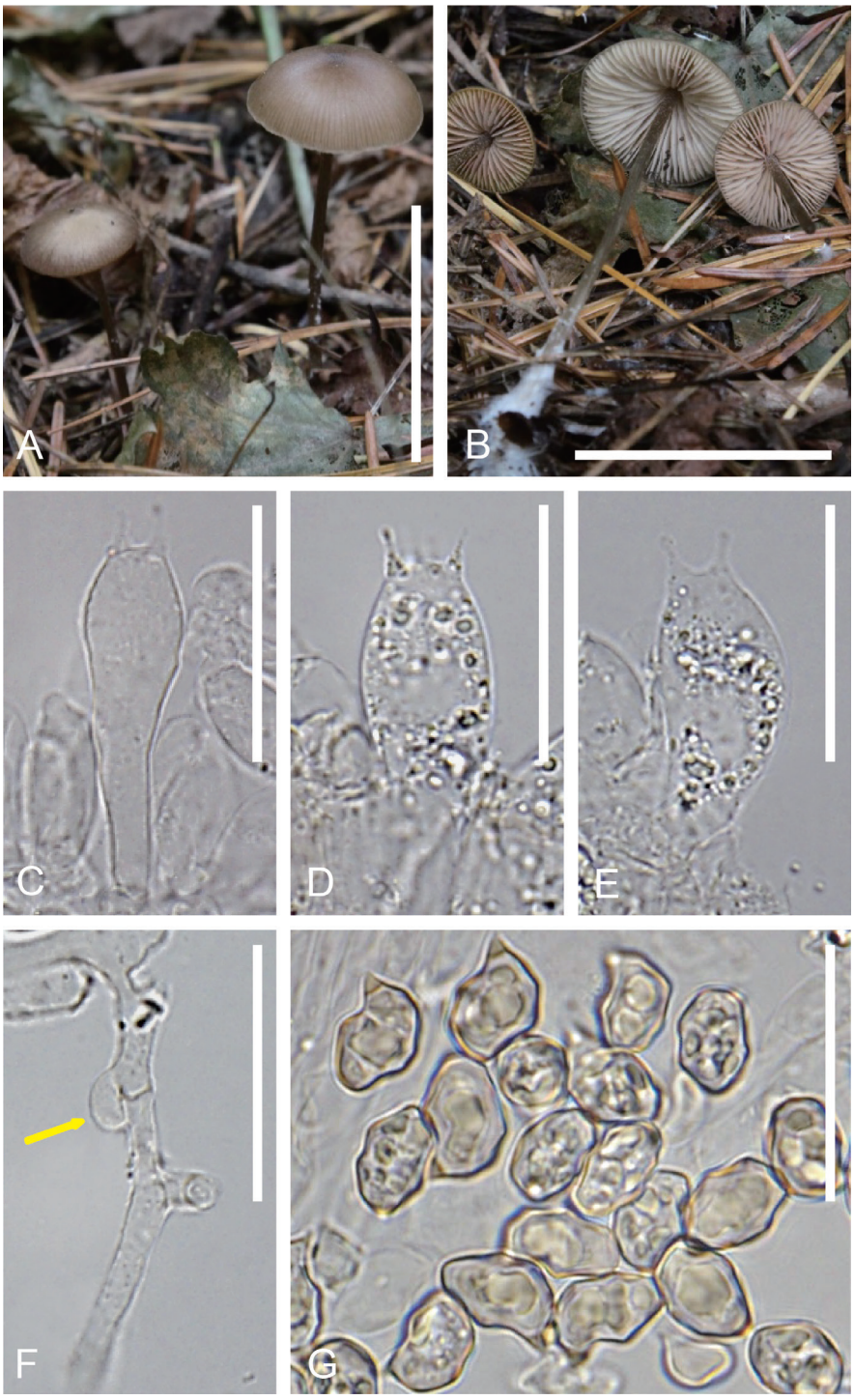

Fig. 4. *Entoloma hirtipes*. A–B, Natural habit of *E. hirtipes*; C–E, Basidia; F, Clamp-connection; G, Basidiospores. Scale bars A–B = 5 cm; C–G = 20 μm.

Description: Pileus 20–50 mm, campanulate to plicate, expanding with central umbo, entire margin, hygrophanous, striate, long convoluted, greyish to brown to blackish to brown, glabrous. Lamellae adnate 3–5 mm broad, ventricose, cream-greyish finally pinkish to brownish, with finely wavy edge. Stipe 40–80 × 2–5 mm, cylindrical, brown to greyish, easily broken. Basidiospores 11–14 × 8–9.5 μm, Q = 1.2–1.5, Qav = 1.41, with 5–7 angles. Basidia 4-spored, 30–41 × 10–13 μm, cylindrical claviform. Pileipellis gray-brownish, 5–10 μm wide. Pileocystidia not observed. Stipitipellis a cutis of cylindrical hyphae 4–8 μm wide. Caulocystidia 35–50 × 4–7 μm, sub-cylindric, numerous, apex often from subcapitulate to capitulate.
Habitat: Widespread in a range of habitats, on soil in broadleaf forest.
Collection examined: KOREA. Gangwon-do, Odae Mountain, October 22, 2013, KA13-1522.
Comments: *Entoloma hirtipes* can be readily distinguished by the much larger basidiospores and mainly in montane or subalpine areas [18]. These characters were similar to the collection examined in this study and detailed morphological features were sufficient to identify this collection as *E. hirtipes*. In RAxML tree (Fig. 1), this collection formed a separate cluster with *E. hirtipes*. Therefore, identity of the Korean collection was confirmed as *E. hirtipes* by morphology and molecular sequence analyses.

CONCLUSION

The present study describes three new records of *Entoloma* species in Korea. These species were identified based on morphological examinations and molecular sequence analyses. To date, only 103 species of *Entoloma* have been recorded in Korea [19-22], indicating that less than 10% of *Entoloma* species occur in Korea. Further studies are needed to increase understanding of this genus in Korea.

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