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Two Newly Recorded *Entoloma* Species, *E. eugenei* and *E. subaraneosum*, in Korea

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ABSTRACT : Two *Entoloma* species, *E. eugenei* and *E. subaraneosum*, are described here as newly recorded species in Korea. These two species were recently described from far Eastern Asia, Primorsky Territory of Russia, and Jilin province of China, respectively. Here, these species were reported based on internal transcribed spacer and partial large subunit of ribosomal RNA sequences, and macro- and micro-scopic characteristics for the first time in Korea.

KEYWORDS : *Entoloma eugenei*, *Entoloma subaraneosum*, Morphology, Phylogeny, Taxonomy

Entolomataceae Kotl. & Pouzar is the second largest family in agarics, traditionally containing *Clitopilus* (Fr. ex Rabenh.) P. Kumm., *Entoloma* (Fr.) P. Kumm. s.l., and *Rhodocybe* Maire [1, 2]. Among them, genus *Entoloma* s.l. includes more than 1,500 species described in the world with variable morphological characteristics and habitat, and new species are continuously discovered [2-4]. This genus is characterized by medium-sized to fairly large fruit-bodies, pinkish and angular shaped basidiospores, terrestrial, typically convex to plane or uplifted pileus, usually notched lamellae (gills), veil and absence of volva [1]. Recent phylogenetic and morphological studies have reported that *Entoloma* s.l. is a monophyletic group [2, 3].

In Korea, Lee and Lee [5] reported an *Entoloma* species, *E. sinuatum* (Bull.) P. Kumm., for the first time in 1957. Later, Cho [6] provided the taxonomical keys of the 80 species of Korean *Entoloma* species based on modified keys of Hesler (on the basis of microscopic characteristics; Hesler [7]) and Noordeloos (on the basis of habit variable; Noordeloos [8-10]). Over 100 *Entoloma* species have been recorded in Korea [11]. In this paper, we describe two *Entoloma* species, *E. eugenei* and *E. subaraneosum*, as new to Korea based on the results of molecular phylogenetic analyses and morphological investigation. These species were collected from humus locations of Mt. Minjuji (peak elevation 1,242 m), Chungbuk Province, Yeongdon-gun, Korea in 2012.

Macro-morphological characteristics were based on the field notes and color photos of basidiomata. Micro-morphological characteristics were obtained from the dried specimens after sectioning and rehydrating (rehydration according to Largent et al. [12]). Microscopic observations were made using an Olympus BX53 microscope (Olympus Co., Tokyo, Japan) and Jenoptik Prog Res C14 Plus Camera (Jenoptik Co., Jena, Germany). Measurements of microscopic characteristics were made using the Prog Res Capture Pro v.2.8.8. software (Jenoptik Co., Jena, Germany). DNA was isolated from fresh fruit bodies (approximately 0.1 g) using a DNeasy plant mini kit (QIAGEN, Germantown, MD, USA), following the manufacturer's recommendations. The internal transcribed spacer (ITS)

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region and partial large subunit (nLSU) of nuclear ribosomal RNA were amplified using two different primer sets: ITS5 and ITS4 for ITS region [13], and LR0R and LR5 for nLSU [14]. Polymerase chain reaction (PCR) mixtures contained 0.5 pmol of each primer, 0.25 mM dNTPs, 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl2, 2.5 U of Taq DNA polymerase, and 15 ng of template DNA. PCR conditions for ITS and nLSU were as follows: an initial denaturation step at 94°C for 4 min, followed by 34 cycles of 94°C for 40 s, 55°C (ITS) or 52°C (nLSU) for 40 s, and 72°C for 1 min and a final elongation step at 72°C for 8 min. PCR products were purified and sequenced by Macrogen Inc., Seoul, Korea. Sequence data were submitted to GenBank.

Raw sequences were proofread, edited, and assembled using PHYDIT 3.2. [15]. DNA sequences were aligned using ClustalX 1.81 [16], and then manually adjusted using PHYDIT. Ambiguously aligned regions were excluded from subsequent analyses. Maximum parsimony (MP) analysis was performed by heuristic search in PAUP* 4.0 [17] with the following settings: all characteristics were equally weighted, gaps were treated as missing characters, starting trees were obtained by random addition with 1,000 replicates, and tree bisection-reconnection (TBR) branch swapping algorithm. Nodal support for MP was determined by nonparametric bootstrapping, performing 1,000 replicates (MPBS) with a heuristic search consisting of 100 stepwise random addition replicates and TBR branch swapping for each bootstrap replicate. Mr Bayes 3.1 [18] was used for construction of phylogenies under Bayesian inference (BI). Posterior probabilities (PP) were approximated using the metropolis-coupled Markov Chain Monte Carlo method. Two parallel runs were conducted with one cold and three heated chains for 5 million generations, respectively, starting with a random tree. The trees were sampled every 100 generations. We interpreted the convergence of two independent runs had converged when the average standard deviation of the split frequencies dropped below 0.01. The trees obtained before the convergence were discarded using the burn-in command, and the remaining trees were used to calculate a 50% majority consensus tree and to estimate posterior probability (PP). PP values below 0.95 were not considered significant, with values below 0.9 indicated on the resulting phylograms.

As shown in Fig. 1, MP analysis of the ITS sequence data (53 taxa, 683 characters) resulted in seven most-parsimonious trees comprising 1,305 steps [consistency index (CI) = 0.4782, retention index (RI) = 0.5502, homoplasy index (HI) = 0.5218, and 260 were parsimony-informative]. In the Bayesian inference, a set of four chains reached convergence after about 2,000,000 generations, and therefore the first 20,000 trees in each parallel run were discarded by setting the burninfrac command to 0.40; the remaining 60,002 trees (representing 3,000,000 generations) were used for calculation of a 50% majority consensus tree and for determination of PP. The likelihood (ln L) of the best states for cold chains of the two runs was 6738.82 and 6744.96, respectively.

As shown in Fig. 2, MP analysis of the nLSU sequence data (40 taxa, 699 characters) resulted in eight most-parsimonious trees comprising 587 steps [CI = 0.4344, RI = 0.5643, HI = 0.5656, and 144 were parsimony-informative]. In the Bayesian inference, a set of four chains reached convergence after about 2,000,000 generations, and therefore the first 20,000 trees in each parallel run were discarded by setting the burninfrac command to 0.40; the remaining 60,002 trees (representing 3,000,000 generations) were used for calculation of a 50% majority consensus tree and for determination of PP. The likelihood (ln L) of the best states for cold chains of the two runs was 3762.43 and 3768.80, respectively.

The ITS and nLSU sequences data showed that KA12-1131 and KA12-1534 were identical to *E. eugenei* LE 253771 (type material; MPBS/PP = 100/1.0 in ITS tree, MPBS/PP = 100/1.0 in nLSU tree) and *E. subaraneosum* GEGM 28823 (type material; MPBS/PP = 94/0.83 in ITS tree, MPBS/PP = 98/0.98 in nLSU tree), respectively. Morphological characteristics of KA12-1131 and KA12-1534 agreed with the original description of *E. eugenei* and *E. subaraneosum*, respectively, although some microscopic characteristics were slightly different (see below notes of taxonomic description part). Most Korean *Entoloma* species have been identified by morphology alone, and evidence specimens are lacking. In general, morphology alone is considered insufficient for identification in recent fungal taxonomy [2, 3, 19, 20]. Therefore, continual reevaluation of the taxonomic diversity of *Entoloma* species in Korea with molecular data coupled with morphological observations with evidence specimens is necessary.

Recent molecular phylogenetic studies suggested that *Entoloma* s.l. is monophyletic, although several mycologists split this group into smaller genera, such as *Alboleptonia* Largent & R.G. Benedict, *Calliderma* (Romagn.) Largent, *Claudopus* Gillet, Eccilia (Fr.) P. Kumm., *Inoce-*

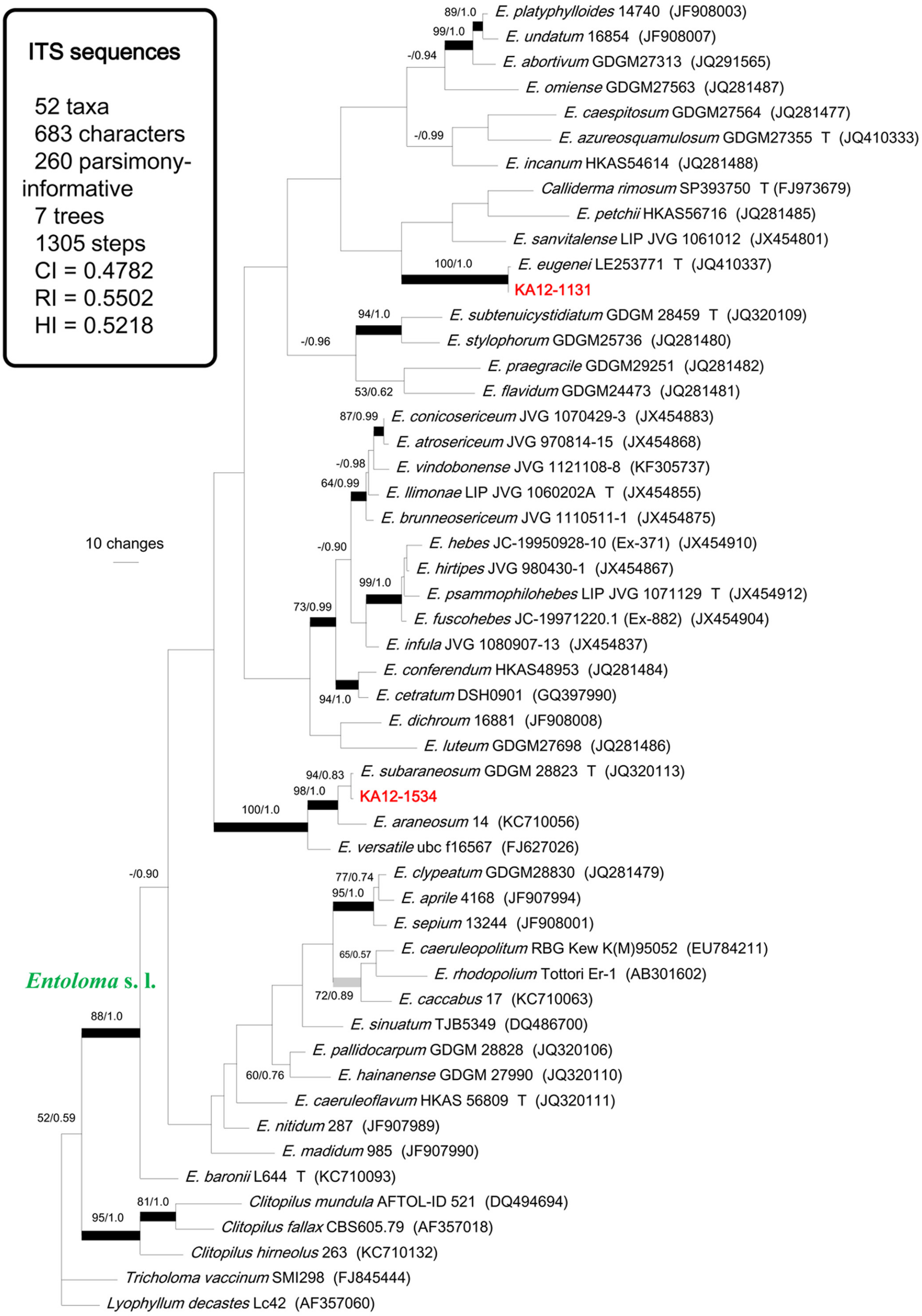

Fig. 1. One of seven most parsimonious trees from a heuristic analysis of internal transcribed spacer (ITS) sequences. Broad black branches indicate maximum parsimony (MPBS) > 60% and Bayesian posterior probabilities (PP) > 0.95 (MPBS/PP). Broad gray branches indicate MPBS and MLBS > 60% and 0.89 < PP < 0.95. Only MPBS > 50 % or PP > 0.89 are shown above or below branches. The symbol 'T' indicated the type materials. CI, consistency index; RI, retention index; HI, homoplasy index.

phalus (Noordel.) P.D. Orton, *Leptonia* (Fr.) P. Kumm., *Nolanea* (Fr.) P. Kumm., and *Pouzarella* Mazzer [2, 3, 21]. Because these smaller genera are not monophyletic [2], the delimitation of these smaller genera should be reevaluated in the future. In our phylogenetic trees, *Calliderma rimosum* Karstedt & Capelari belong to *Entoloma* s.l. (Figs. 1 and 2). This species was recently described from Brazil by Karstedt and Capelari [22] based on mor-

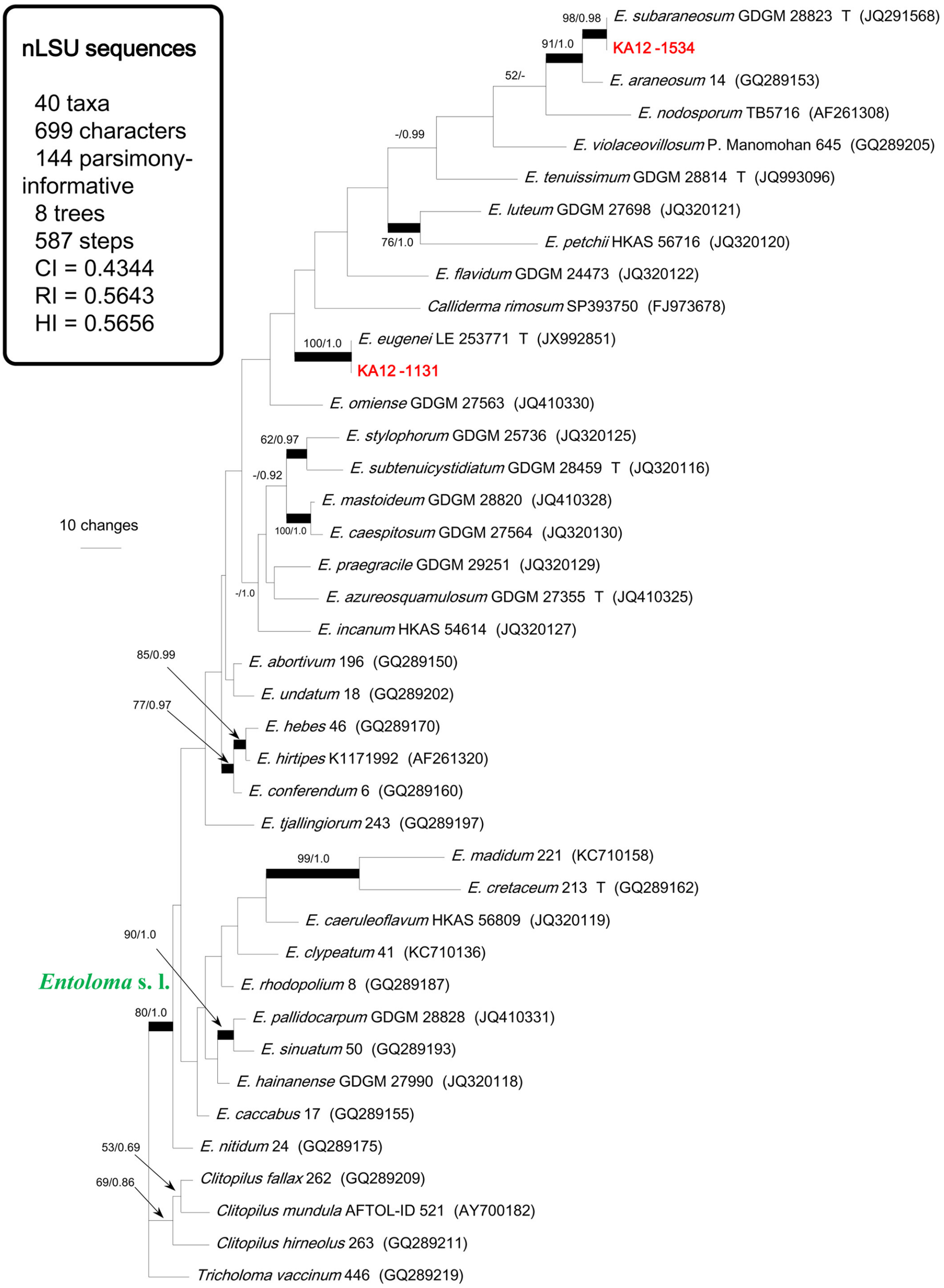

Fig. 2. One of eight most parsimonious trees from a heuristic analysis of partial large subunit (nLSU) sequences. Broad black branches indicate maximum parsimony (MPBS) > 60% and Bayesian posterior probabilities (PP) > 0.95 (MPBS/PP). Only MPBS > 50 % or PP > 0.89 are shown above or below branches. The symbol 'T' indicated the type materials. CI, consistency index; RI, retention index; HI, homoplasy index.

phological characteristics (characterized by the dark brown campanulate pileus with a subvelutinous surface, becoming strongly cracked with age; cheilocystidia cylindrical, clabate, or ventricose; hymeniform pileipellis composed by clavate, ovoid, globose, or ventricose elements with intracellular brownish coagulated pigment). However, they did not perform the phylogenetic analyses with related genera although they provided the ITS and nLSU sequences. He *et al.* [23] also recognized this situation in their phylogenetic studies - genus *Calliderma* is not a monophyletic and it should not be maintained as a separated genus because the eight examined *Calliderma* species were nested in four different clades in *Entoloma* s.l. Therefore, *C. rimosum* should be transferred to genus *Entoloma* in the near future.

Taxonomic description

Entoloma eugenei Noordel. & O.V. Morozova, Mycotaxon 112: 234. 2010. [24]; Fig. 3.

Pileus *ca*. 40 mm broad, hemispherical to plano-convex with incurved margin, velvety, not striate, deep blue with somewhat violet tinge, no change in color when dried. Lamellae adnate-emarginate, subdistant, white to gray whitish, concolorous edge. Stipe *ca.* 85 × 5~8 mm, clavate to cylindrical, entirely squamulose with concolorous squamules, white basal tomentum. Distinguishing odor not detected.

Basidiospores 11.4~13.0 × 7.1~8.0 µm, length/width = 1.5~1.8 (n = 30), heterodiametrical, with 5~7 angles in side view, pale brown to brown in 3% KOH mount. Basidia 31.7~40.0 × 7.5~10.4 µm, length/width = 3.4~4.8 (n = 30), clavate, 4-spored, base clamped. Lamellae edge sterile; trama parallel. Cheilocystidia 30.6~44.4 × 4.6~6.8 µm, length/width = 5.1-8.4 (n = 10), cylindrical, narrowly lageniform, hyaline. Pleurocystidia not observed. Hymenophoral trama regular. Pileipellis a trichoderm of cylindrical hyphae with terminal elements *ca.* 70~95 × 5~10 µm; clamp connections observed at base, abundant.

Habitat: Humus in woodland.

Examined specimen: **Korea**, Chungbuk Province, Yeong-

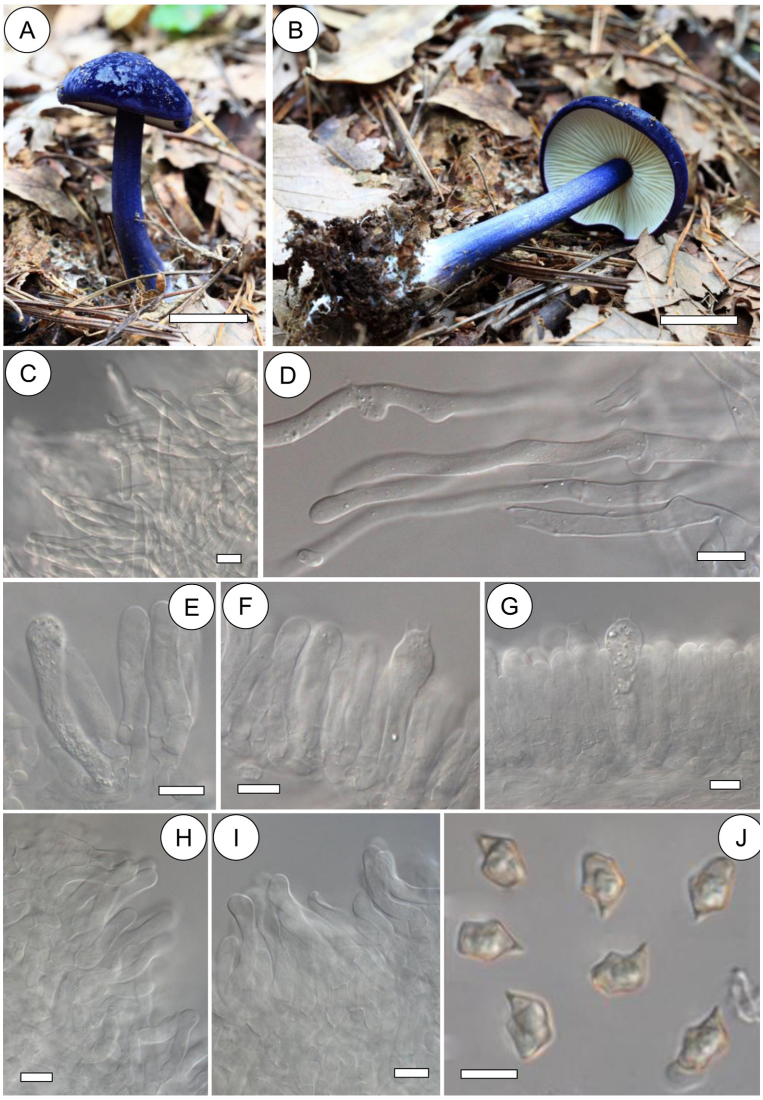

Fig. 3. *Entoloma eugenei* KA12-1131. A~B, Fresh fruit-body; C~D, Pileipellis; E~G, Basidioles and Basidia; H~I, Cheilocystidia; J, Basidiospores (scale bars: A, B = 2 cm, C = 20 µm, D~J = 10 µm).

don-gun, Mt. Minjuji, coll. Han *et al.*, 27 Aug. 2012 (KA 12-1131; GenBank no., ITS: KJ523134, nLSU: KJ523136).

Notes: This species is one of blue species in section *Leptonia*, characterized by the tricholdermal pileipellis with clamp connection [24]. Micro-scopic characteristics of our specimens were slightly differ from those of original description in the size of basidiospores, basidia and terminal elements of pileipellis (original descrition of *E. eugenei*: basidiospores 10.0~12.5 × 6.0~8.0 μm, length/width = 1.3~1.7; basidia 33~44 × 9~12 μm; terminal elements of pileipellis 90~200 × 12~20 μm; refer to Noordeloos and Morozova [24]). This may be caused by a lack of examined specimens, however, our specimen was almost identical to the original description [24] except for these micro-scopic characteristics. In addition, ITS and nLSU sequences of KA12-1131 were identical to the sequences of type material (Figs. 1 and 2). This species could be distinguished from related *Entoloma* species having blue-colored pileus and stipe (e.g. *E. azureosquamulosum* T.H. Li & Xiao Lan He, *E. cyaneum* (Peck) Sacc., *E. dichroum* (Pers.) P. Kumm., *E. panniculus* (Berk.) Sacc., *E. egregium* E. Horak, *E. tjallingiorum* Noordel., etc.) mainly in the size of basidiospores, structure of the pileipellis, and presence of clamp connections in all tissues [21, 24].

Entoloma subaraneosum Xia Lan He & T.H. Li, Fungal Diversity 58: 235. 2013. [23]; Fig. 4.

Pileus *ca.* 10 mm broad, broadly conic to campanulate, without a depression at the disc, completely covered with tomentose to matted fibrillose, not striate, pale brown to brown, no change in color when dried. Lamellae adnate to sinuate, distant, gray whitish to pale brown, concolorous edge. Stipe *ca.* 20 × 1.8 mm, cylindrical, gray

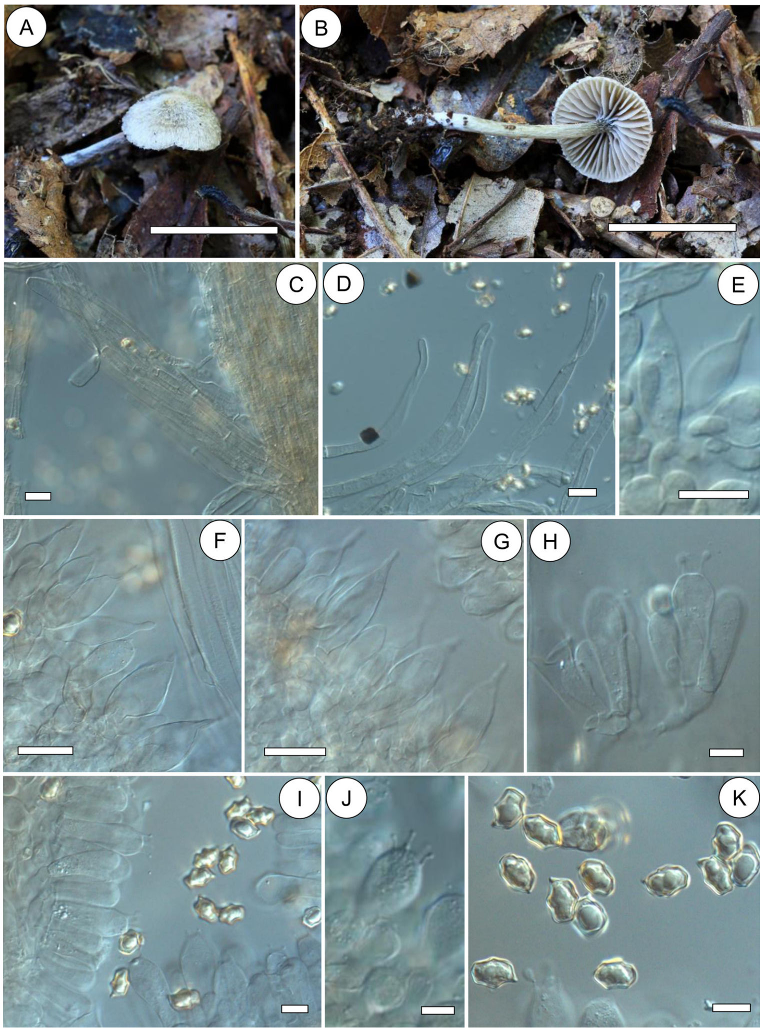

Fig. 4. *Entoloma subaraneosum* KA12-1534. A~B, Fresh fruit-body; C~D, Pileipellis; E~G, Cheilocystidia; H~J, Basidia; K, Basidiospores (scale bars: A, B = 1 cm, C~G = 20 μm, H~K = 10 μm).

whitish to pale brown, white basal tomentum. Distinguishing odor not detected.

Basidiospores 11.6~13.3 × 7.6~8.8 µm, length/width = 1.4~1.6 (n = 30), heterodiametrical, 5~7 angled with pronounced angles in side view, subhyaline to pale brown in 3% KOH mount. Basidia 33.7~40.8 × 11.4~13.6 µm, clavate, 4-spored, base not clamped. Lamellar edge heterogeneous; trama parallel. Cheilocystidia 41.1~63.8 × 11.5~15.7 µm, length/width = 3.2–4.6 (n = 12), abundant, broadly lageniform with a long tapering neck, hyaline. Pleurocystidia not observed. Pileipellis cutis with a trichoderm of cylindrical hyphae, hyaline to yellowish brown in 3% KOH mount, cylindrical to narrowly clavate terminal elements *ca*. 85~180 × 8~15 µm; clamp connections not observed at base.

Habitat: Humus in woodland.

Examined specimen: **Korea**, Chungbuk Province, Yeongdon-gun, Mt. Minjuji, coll. Han *et al*., 24 Sep. 2012 (KA12-1534; GenBank Nos. ITS: KJ523135, nLSU: KJ523 137).

Notes: Our specimen, KA12-1534, has smaller basidia than that of *E. subaraneosum* (42~52 × 13~16 µm). However, except for this characteristic, our specimen was identical to the original description of *E. subaraneosum* and ITS and nLSU sequences [23]. This species is closely related to *E. araneosum* (Quél.) M.M. Moser, phylogenetically and morphologically. However, they are distinguished by the size of basidiospores and structure of terminal elements in pileipellis - *E. araneosum* has slightly larger basidiopores (10~14 × 7~8 µm; length/width = 1.4~1.8) and wider clavate terminal elements in pileipellis (13~27 µm) than those of *E. subaraneosum* [10, 23]. In addition, they could be distinguished by their ITS and nLSU sequences (Figs. 1 and 2).

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