Four Newly Recorded Amanita Species in Korea: Amanita sect. Amanita and sect. Vaginatae

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Abstract  We collected nearly 70 specimens of Amanita species during a diversity study of Korean mushrooms conducted in 2012. In this study, we primarily investigated 23 Amanita specimens belonging to sections Amanita and Vaginatae. Based on sequence data of the internal transcribed spacers and partial large subunit of ribosomal RNA and morphological characteristics, we identified the following 15 phylogenetic species: A. albollavescens, A. ceciliae, A. farnosa, A. fulva, A. griseoflava, A. ibotengutake, A. meliaceps, A. orientiflava, A. pantherina, A. rubrovolvata, A. sinensis, A. subglobosa, A. vaginata, A. cf. vaginata f. alba, and an undescribed Amanita species. In this study, four of the identified Amanita species (A. griseoflava, A. ibotengutake, A. orientiflava, and A. sinensis) were reported for the first time in Korea.

Keywords  Amanita sect. Amanita, Amanita sect. Vaginatae, ITS, Morphology, nLSU, Phylogeny

Amanita Pers. comprises both edible and poisonous mushrooms that exhibit worldwide distribution, usually as mycorrhizal symbionts with plants [1, 2]. Infrageneric classification, which is based mainly on the morphological characteristics of the fruit body, has divided this genus into two subgenera [subgen. Amanita Pers. (having inamyloid spores) and subgen. Lepidella (E. J. Gilbert) Veselý enend. Corner & Bas (having amyloid spores)] and seven sections [Amanita Pers., Caesareae Singer, Vaginatae (Fr.) Quél., Amidella (E. J. Gilbert) Konrad & Maubl., Lepidella (E. J. Gilbert) Corner & Bas, Phalloideae (Fr.) Quél., and Validiae (Fr.) Quél.] [1]. Amanita muscaria (L.) Lam., the type species of the genus, consists of several varieties with different color forms, such as A. muscaria var. muscaria (L.) Lam. (red pileus and white stem and warts), A. muscaria var. alba Peck (white-to-tan pileus, warts, and stem), A. muscaria var. flavivolvata (Singer) D. T. Jenkins (orange-to-red pileus, tannish-yellow warts, and white-to-tan stem), A. muscaria var. formosa Pers. (orange-to-yellow pileus and yellowish-to-tannish warts and stem), A. muscaria var. persicina D. T. Jenkins (melon pileus and tannish-to-yellow warts), and A. muscaria var. regalis (Fr.) Sacc. [now A. regalis (Fr.) Michael] (brown pileus and tannish-to-yellow warts) [3, 4].

While nearly 500 Amanita species have been described, molecular studies are required to confirm and verify morphology-based identifications because some Amanita species are morphologically identical [1, 5, 6]. In addition, some Amanita species represent the most-poisonous higher fungi, which makes accurate identification of morphologically similar species especially important [7, 8]. This situation has prompted many mycologists to investigate the DNA-based phylogenies of Amanita species [1-3, 9-13]. Most reports on Amanita species in Korea only include short morphological descriptions or their sequences have not been deposited in GenBank [7, 14, 15].

We conducted a preliminary investigation of the taxonomy of Amanita species. We collected 70 specimens of Amanita species during a diversity study of Korean mushrooms conducted in 2012 and preserved them in the herbarium of the Korea National Arboretum (KA), Gwangneung Forest, Pocheon-si, Gyeonggi Pref., Korea. For the current study, we selected 23 of these specimens belonging to the
The goal of the present study was to clarify the phylogenetic positions of *Amanita* species collected during the survey conducted in 2012 based on sequence data of the internal transcribed spacers (ITS) and partial large subunit (nLSU) of ribosomal RNA. In addition, we describe four *Amanita* species that have not been previously reported in Korea, and designate them with Korean names.

### MATERIALS AND METHODS

#### Specimens and morphological observations.

The 23 *Amanita* species used in the present study are listed in Table 1 and the dried specimens were deposited in the KA herbarium. Reliable DNA sequence data for other reported *Amanita* species were obtained from GenBank for the phylogenetic analyses. Macro-morphological descriptions were based on field notes and color photographs of basidiomata. Micro-morphological data were obtained from the dried specimens with the aid of a light microscope after sectioning and rehydration in 3% KOH solution. Descriptions of spore shapes are based on the study reported by Bas [16].

#### PCR amplification and sequencing.

DNA was isolated from fresh fruit bodies (approximately 0.1 g) using a modified version of the CTAB procedure reported by Doyle and Doyle [17]. Two primer sets were used for amplification of the ITS regions and partial LSU of ribosomal RNA: ITS5 and ITS4 [18], and LR0R and LR5 [19]. 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. The 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 sec, 52°C for 40 sec, and 72°C for 60 sec, and a final elongation step at 72°C for 8 min. PCR products were purified using an ExoSAP kit (USB, Cleveland, OH, USA). The purified double-stranded PCR fragments were directly sequenced using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer’s instructions. The same primer sets used for amplification of ITS and nLSU were employed for sequencing. Capillary electrophoresis and data collection were performed on an ABI Prism 310 Genetic Analyzer (Applied Biosystems). Sequence data were submitted to GenBank (Table 1).

#### Phylogenetic analyses.

Raw sequences were proofread, edited, and assembled into contigs using PHYDIT 3.2 [20]. DNA sequences were aligned using ClustalX 1.81 [21], and then manually adjusted using PHYDIT. Ambiguously aligned regions were excluded from subsequent analyses.

To determine the phylogenetic positions of the sampled specimens, datasets were analyzed using maximum parsimony (MP) in PAUP version 4.0b10 [22] and Bayesian inference

### Table 1. Information on the *Amanita* species used in this study

| Species | Specimens' | Locality | Collection date | GenBank accession Nos. |
|---------|------------|----------|-----------------|------------------------|
|         |            |          |                 | ITS                    | nLSU       |
| *A. alboflavescens* | KA12-0970 | Gwangneung forest, Pocheon-si, Gyeonggi | 9 Aug 2012 | KF017928 KF021667 |
| *A. ceciliae* | KA12-0758 | Mt. Seodae, Geumsan-gun, Chungnam | 18 Jul 2012 | KF017929 KF021668 |
| *A. farinosa* | KA12-0916 | Gwangneung forest, Pocheon-si, Gyeonggi | 27 Jul 2012 | KF017930 KF021669 |
| *A. fulva* | KA12-0818 | Gwangneung forest, Pocheon-si, Gyeonggi | 26 Jul 2012 | KF017934 KF021673 |
| *A. griseofolia* | KA12-0994 | Gwangneung forest, Pocheon-si, Gyeonggi | 9 Aug 2012 | KF017935 KF021674 |
| *A. ibotengutake* | KA12-1339 | Nari Basin, Ulleung-gun, Gyeongbuk | 5 Sep 2012 | KF017937 KF021676 |
| *Amanita* sp. | KA12-1175 | Mt. Jinak, Geumsan-gun, Chungnam | 21 Aug 2012 | KF017938 KF021677 |
| *A. melleiceps* | KA12-1213 | Mt. Seodae, Geumsan-gun, Chungnam | 29 Aug 2012 | KF017939 KF021678 |
| *A. orientifulva* | KA12-0642 | Mt. Minjuji, Yeongdong-gun, Chungbuk | 17 Jul 2012 | KF017940 KF021679 |
| *A. pantherina* | KA12-1596 | Mt. Geumjeok, Boeun-gun, Chungbuk | 27 Sep 2012 | KF017941 KF021680 |
| *A. rubrovolvata* | KA12-1454 | Gwangneung forest, Pocheon-si, Gyeonggi | 12 Sep 2012 | KF017936 KF021675 |
| *A. sinensis* | KA12-0932 | Gwangneung forest, Pocheon-si, Gyeonggi | 8 Aug 2012 | KF017922 KF021681 |
| *A. subglobosa* | KA12-1225 | Mt. Seodae, Geumsan-gun, Chungnam | 29 Aug 2012 | KF017943 KF021682 |
| *A. vaginata* | KA12-1393 | Nari Basin, Ulleung-gun, Gyeongbuk | 6 Sep 2012 | KF017944 KF021683 |
| *A. rubrovolvata var. alba* | KA12-1023 | Mt. Sokri, Boeun-gun, Chungbuk | 16 Aug 2012 | KF017945 KF021684 |
| *A. rubrovolvata* | KA12-1555 | Mt. Jinak, Geumsan-gun, Chungnam | 25 Sep 2012 | KF017946 KF021685 |
| *A. subglobosa* | KA12-0848 | Gwangneung forest, Pocheon-si, Gyeonggi | 26 Jul 2012 | KF017947 KF021686 |
| *A. vaginata* | KA12-1190 | Mt. Jinak, Geumsan-gun, Chungnam | 28 Aug 2012 | KF017949 KF021688 |

The herbarium of the Korea National Arboretum (KA). Bold-letters indicate the newly recorded *Amanita* species in Korea.
in MrBayes 3.1.2 [23]. Parsimony analysis was performed using a heuristic search with 1,000 random additional replicates and tree bisection-reconnection branch swapping. MP bootstrap support values (MPBSs) for internal nodes were calculated from 1,000 replicates of the MP analysis. Posterior probability (PP) was estimated using the metropolis-coupled Markov-chain Monte Carlo method. Two parallel runs were performed using one cold and three heated chains for 5 million (ITS) and 10 million (nLSU) generations, respectively, starting with a random tree. The trees were sampled every 100 generations. We considered that two independent runs had converged when the average standard deviation (SD) 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 for calculation of a 50% majority consensus tree and for estimation of the PP. PPs below 0.95 were not used for calculation of a 50% majority consensus tree using the burn-in command, and the remaining trees were used for estimation of the PP. PPs below 0.95 were not considered significant, with values below 0.9 being indicated on the resulting phylograms.

RESULTS AND DISCUSSION

Phylogenetic analyses of ITS and nLSU sequences. The ITS dataset included 51 taxa and 601 characters, of which 261 were parsimony-informative. The MP tree was 858 steps long and had a consistency index (CI) of 0.6119, a retention index (RI) of 0.8208, and a homoplasy index (HI) of 0.3881. Bayesian analysis used a GTR + I + G model, and the first 12,500 trees were discarded as burn-in (with a “burninfrac” value of 0.25). For the nLSU dataset, 52 taxa and 567 characters were included, of which 147 characters were parsimony-informative. The MP tree was 451 steps long with CI of 0.5499, RI of 0.8450, and HI of 0.4501. In the Bayesian analysis, the first 35,000 trees using a GTR + I + G model were discarded as burn-in (burninfrac = 0.35).

The specimens used in phylogenetic analyses were divided into two sections—sect. Amanita and sect. Vaginatae—with high support values (Figs. 1 and 2). The phylogenetic positions of Amanita species in our study were generally consistent with the findings reported by Zhang et al. [1] and Moreno et al. [2]. We identified the following 15 phylogenetic species: Amanita alboflavescens Hongo, A. ceciliae (Berk. & Broome) Bas, A. farinosa Schwein, A. fulva Fr., A. griseofolia Zhu L. Yang, A. ibotengutake T. Oda, C. Tanaka & Tsuda, A. melleiceps Hongo, A. orientifulva Zhu L. Yang, M. Weiss, & F. Oberwinkler, A. pantherina (DC.) Krombh., A. rubrovolvata S. Imai, A. subglobosa Zhu L. Yang, A. subglobosa Zhu L. Yang, A. vaginata (Bull.) Lam., A. vaginata f. alba (Sacc.) Romagn., and one undescribed Amanita species. Most of these species formed a group with high support values (MPBS > 70%, PP > 0.95) in the ITS tree (Fig. 1). However, A. pantherina formed a group with moderate support values [MPBS/PP = 64/0.91 in the ITS tree (Fig. 1) and MPBS/PP = 63/0.96 in the nLSU tree (Fig. 2)] and was closely related to A. subglobosa.

Previously reported Amanita species in Korea. The ITS and nLSU sequences of A. subglobosa (HKAS56893 and HKAS58837—deposited in the Cryptogamic Herbarium of the Kunming Institute of Botany, Chinese Academy of Sciences; KA12-0848) and A. pantherina (LEM960325—deposited in the Herbarium, Laboratory of Environmental Mycoscience, Faculty of Agriculture, Kyoto University; KA12-0932, KA12-1225, and KA12-1393) were virtually identical, differing in only one nucleotide. Although the overall morphological features of A. subglobosa and A. pantherina are very similar, according to Yang [24] and Sanmee et al. [25], they can be distinguished based on the existence of clamps at the bases of basidia. Clamps were not found on A. pantherina but were found on A. subglobosa at the bases of basidia, along with a difference in the size of the basidia [24, 25]. Based on the morphological and molecular evidence, we identified specimen KA12-0848 as A. subglobosa, and specimens KA12-0932, KA12-1225, and KA12-1393 as A. pantherina. Amanita ceciliae was closely related to A. griseofolia both morphologically and phylogenetically, with differences in the color of the fruit body and in the thickness and structure of the stipe [26].

Amanita vaginata, which comprises several varieties and formae (Index Fungorum, http://www.indexfungorum.org/Names/Names.asp), belongs to a group of Amanita species with colors ranging from white to gray to brown-capped [27]. Our two specimens, designated as KA12-0665 and KA12-1190, were grouped with A. vaginata CUB: Microbiology MN18 (sequences submitted to GenBank, but unpublished; Department of Microbiology, Chulalongkorn University, Phayathai, Bangkok, Thailand) with strong support in the ITS tree; however, they formed an independent group with weak support in the nLSU tree (Figs. 1 and 2). In our analyses, although the A. vaginata specimens did not represent a monophyletic group, we tentatively assigned these two specimens as members of an A. vaginata complex. One of the specimens, KA12-0962, has morphological similarity to A. vaginata f. alba; however, unfortunately, no reliable sequences of A. vaginata f. alba have been deposited in GenBank. Therefore, we could not confidently conclude that KA12-0962 is A. vaginata f. alba. The previously reported species (i.e., A. alboflavescens, A. farinosa, A. fulva, A. melleiceps, and A. rubrovolvata) were clearly consistent with those of previous taxonomical descriptions (detailed descriptions of each species are available at http://www.amanitaceae.org/), and phylogenetically grouped with the deposited sequences in GenBank (Figs. 1 and 2).

Four newly recorded Amanita species in Korea and an undescribed Amanita species. In the present study, we reported four newly recorded Amanita species that have not been previously reported in Korea as follows: A. griseofolia, A. ibotengutake, A. orientifulva, and A. sinensis. Amanita griseofolia is closely related to A. ceciliae (originally described from Europe) in terms of both morphology and
phylogeny (Figs. 1–3) [1]. However, the original description of *Amanita griseofolia* was originally described from southwestern China [28] and is characterized by a much more robust fruit body, a thicker stipe, and production of more filamentous hyphae in the volval remnants than *A. ceciliae* [1, 26]. In the present study, the differences in these morphological features are consistent with their phylogenetic positions being separated at the trees of the ITS and nLSU sequences (Figs. 1 and 2).

*Amanita ibotenguatake* is a poisonous fungus originally reported from Japan [11]. This species produces the toxic substances ibotenic acid and muscimol, which cause Pantherina syndrome [11]. We confirmed that one of our specimens (KA12-1339—collected from Nari Basin, Ulleung-gun, Korea) could be assigned as *A. ibotenguatake* based on its morphology and phylogeny (Figs. 1~3). In our phylogenetic analyses, *Amanita orientifulva* was closely related to *A. fulva* (Figs. 1 and 2); however, these two species can be
distinguished based on the volval structure and size of basidiocarps, because *A. orientifulva* generally possess larger basidiocarps, as indicated by the phylogenetic analyses of ITS and nLSU sequences (Figs. 1 and 2) [26]. *Amanita sinensis*, which was originally described in a report from China, is found in mixed forests with broad-leaved trees and conifers [24]. Specimen KA12-1555 was grouped with *A. sinensis* LEM960225 in the ITS tree, and its morphological characteristics were identical to those in the original descriptions of *A. sinensis* (Figs. 1 and 2). The undescribed *Amanita* sp. KA12-1175 is included in sect. Vaginatae of the ITS and nLSU trees (Figs. 1 and 2). *Amanita* sp. KA12-1175 possesses distinguishing morphological features of sect. Vaginatae, which can be characterized by the presence of clamp connections at the bases of basidia and the absence of an annulus [28]. In future studies, its morphological features should be carefully compared with those typical of the species belonging to sect. Vaginatae.
Taxonomical remarks on four newly recorded Amanita species in Korea. Here, we have briefly shown the taxonomic remarks on four newly recorded Amanita species in Korea, (e.g., Amanita griseofolia, A. ibotengutake, A. orientifulva, and A. sinensis).

**Amanita griseofolia** Zhu L. Yang, Frontiers in Basidiomycote Mycology: 315 (2004); Section Vaginatae.

**Specimens examined:** Korea, Gyeonggi Pref., Pocheon-si, Gwangneung Forest, collected on 26 Jul 2012 (KA12-0818), 9 Aug 2012 (KA12-0994), and 12 Sep 2012 (KA12-1457) by Han et al.

**Remarks:** Amanita griseofolia is similar to A. ceciliae. KA12-0818 and KA12-0994 were regarded as A. ceciliae by S. K. Han in 2012. However, A. ceciliae differs from A. griseofolia by having a more robust fruit body with a yellow-brown or reddish-brown-to-gray-brown pileus covered with lighter colored volval remnants, white lamellae with white edges, and a thicker stipe [28]. The basidiospores of A. ceciliae are slightly larger than those of A. griseofolia [29].

**Amanita ibotengutake** T. Oda, C. Tanaka, & Tsuda, Mycol. Prog. 1: 360 (2002); Section Amanita.

**Specimen examined:** Korea, Gyeongbuk Pref., Ulleung-gun, Nari Basin, collected on 5 Sep 2012 (KA12-1339) by Han et al.

**Remarks:** Unfortunately, specimen KA12-1339 was immature, therefore, we did not observe basidiospores. However, based on macroscopic features and the results of phylogenetic analyses, we confirmed that our specimen is A. ibotengutake. This species was originally reported from Japan in a mixed forest of Quercus serrata and Pinus densiflora. However, we found this specimen in Nari Basin on Ulleung Island in a mixed forest of Fagus engleriana.
and Acer okamotoanum.

Amanita orientifulva  Zhu L. Yang, M. Weiss, & F. Oberwinkler. Mycologia 96: 643 (2004); Section Vaginatae.

Specimens examined: Korea, Chungbuk Pref., Yeongdong-gun, Mt. Minjuji, collected on 16 Jul 2012 (KA12-0642) by Han et al.; Korea, Chungbuk Pref., Boeun-gun, Mt. Geumjeok, collected on 27 Sep 2012 (KA12-1596) by Han et al.

Remarks: Amanita orientifulva is very similar to A. fulva. Specimens KA12-0642 and KA12-1596 were identified as A. fulva by S. K. Han in 2012. However, they have distinct basidiocarp sizes and volval structures. The basidiocarp of A. orientifulva (pileus diameter ca. 10 cm, stipe ca. 15 × 2 cm) is generally larger than that of A. fulva (pileus diameter ca. 5-7 cm, stipe ca. 9-12 × 1-1.5 cm). The inner layer of the volval limb of A. orientifulva has a more compact arrangement. In addition, this species is characterized by its filamentous hyphae mixed with fewer inflated cells and scattered to locally conspicuous vascular hyphae [26].

Amanita sinensis Zhu L. Yang, Bibl. Mycol. 170: 23 (1997); Section Amanita.

Specimen examined: Korea, Chungnam Pref., Geumsan-gun, Mt. Jinak, collected on 25 Sep 2012 (KA12-1555) by Han et al.

Remarks: In this study, we identified this species based on phylogeny and morphology. Amanita sinensis is recorded in southwestern China, and Oda et al. [30] and Sanmee [25] reported this species in Japan, Nepal, and Northern Thailand. Spore measurements from the protolog are provided for comparative purposes: basidiospores of size 11.1–16.3 × 6.9–10.3 µm (Q = 1.36–1.70, Q = 1.51 ± 0.06, mean ± SD), ellipsoid, inamylloid, colorless, hyaline, thin-walled, and smooth. The length and Q value of basidiospores are somewhat larger for the Korean specimens than for the Chinese specimens, but are similar to those of Japanese and Nepalese specimens.

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