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Research Article

Four New Species of *Amanita* in Inje County, Korea

Hae Jin Cho[1], Myung Soo Park[1], Hyun Lee[1], Seung-Yoon Oh[1], Yeongseon Jang[2], Jonathan J. Fong[3] and Young Woon Lim[1,*]

[1]*School of Biological Sciences, Seoul National University, Seoul 08826, Korea*
[2]*Division of Wood Chemistry & Microbiology, Korea Forest Research Institute, Seoul 02455, Korea*
[3]*Science Unit, Lingnan University, Tuen Mun, New Territories, Hong Kong*

Abstract *Amanita* (Agaricales, Basidiomycota) is one of the most well-known genera composed of poisonous mushrooms. This genus of almost 500 species is distributed worldwide. Approximately 240 macrofungi were collected through an ongoing survey of indigenous fungi of Mt. Jeombong in Inje County, Korea in 2014. Among these specimens, 25 were identified as members of *Amanita* using macroscopic features. Specimens were identified to the species level by microscopic features and molecular sequence analyses of the internal transcribed spacer and large subunit of nuclear ribosomal RNA. We molecularly identified 13 *Amanita* species, with seven species matching previously recorded species, four species (*A. caesareoides*, *A. griseoturcosa*, *A. imazekii*, and *A. sepiacea*) new to Korea, and two unknown species.

Keywords *Amanita*, Molecular sequence analyses, Mt. Jeombong, New species identification, Poisonous mushrooms

The well-known genus *Amanita* (Agaricales, Basidiomycota) contains both poisonous and edible mushrooms [1]. Species in this genus play important roles in forest ecosystems, as a large majority have a mutualistic association with plants to help effective nutrient uptake [2]. Approximate 500 *Amanita* species have been reported worldwide and are found in a broad range of habitats [3]. *Amanita muscaria* (L.) Lam. is the type species of the genus [4]. Based on morphological features and chemical reaction characters of the fruit body, *Amanita* is divided into two subgenera, including seven sections: *Amanita* Pers. and *Lepidella* (J. E. Gilbert) Veselý emend. Corner & Bas [5]. While this infrageneric classification is not well supported, monophyly of each section, except *Lepidella*, is well supported [5-7].

Nearly 60 *Amanita* species have been described in Korea [8], with most species being reported based on morphological identifications. However, such identification methods are not reliable, and some poisonous *Amanita* species are often confused for edible species [9]. In Korea, 23 deaths due to eating poisonous mushrooms were reported during 2004-2013 according to the Korea Forest Service (http://www.forest.go.kr/). Accurate identification of *Amanita* species is important to reduce these fatal accidents. Molecular phylogenetics is an important tool to help in species identification [6, 10, 11].

Inje County in the Gangwon Province of South Korea is located in the Baekdudaegan Mountains and is known for its rich biodiversity. A total of 523 higher fungi have been reported in Inje County, including 24 *Amanita* species [12]. In 2014, we surveyed Mt. Jeombong in Inje County and identified 25 specimens as *Amanita* species. Specimens were identified to the species level based on morphological features and molecular sequence analysis of the internal transcribed spacer (ITS) and the partial large subunit of nuclear ribosomal RNA (nLSU). Thirteen species were identified, with four species (*A. caesareoides*, *A. griseoturcosa*, *A. imazekii*, and *A. sepiacea*) being new records to Korea and two unknown species. Detailed descriptions are presented for the four newly reported *Amanita* species in Korea.

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***Corresponding author**
E-mail: ywlim@snu.ac.kr

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MATERIALS AND METHODS

Sampling and characterization of morphological features. All specimens were collected at Mt. Jeombong in Inje County (Gangwon Province, Korea) in 2014.

Table 1. *Amanita* specimens examined in this study

Species	Collection No.	Best match (accession No.)				Microscopic feature (μm)	
		ITS	Similarity (%)	nLSU	Similarity (%)	Basidia size	Spore size
A. caesareoides[a,b]	SFC20140912-25	*A. caesareoides* (KP004948)	100	*A. caesarea* (AF024443)	99.5	42.8~55.0 × 11.5~−14.0	8.2~10.8 × 6.4~8.5
	SFC20140822-15						
A. eijii[b]	SFC20140912-04	*A. eijii* (FJ441039)	96.6	*A. cokeri* (HQ539682)	95.6	57.4~86.8 × 12.0~15.2	11.1~13.4 × 7.2~8.5
	SFC20140912-17						
	SFC20141001-14						
A. fulva	SFC20140822-44	*A. fulva* (AB015692)	99.7	*A. fulva* (KF021672)	99.8	45.9~66.7 × 15.3~18.3	9.9~12.0 × 9.6~11.8
	SFC20140730-22						
	SFC20140730-23						
A. griseoturcosa[a,b]	SFC20140822-29	*A. griseoturcosa* (NR 119390)	98.2	*A. cylindrispora* (AY325867)	95.5	39.1~51.2 × 10.2~12.9	9.1~11.8 × 5.9~7.5
A. imazekii[a,b]	SFC20140912-30	*A. imazekii* (AB038764)	100	*A. yuaniana* (AF024488)	94.6	45.8~64.7 × 9.9~14.1	9.8~13.2 × 9.2~11.4
A. manginiana[b]	SFC20140823-10	*A. manginiana* (KJ466378)	98.7	*A. manginiana* (AF024463)	100	-	-
A. pallidorosea[b]	SFC20140822-54	*A. pallidorosea* (KJ739814)	100	*A. pallidorosea* (KJ466444)	99.5	32.7~49.4 × 8.3~13.2	7.3~9.4 × 6.2~7.8
	SFC20140823-08						
A. rubescens	SFC20140822-42	*A. rubescens* (KF245919)	99.7	*A. rubescens* (KF245902)	100	26.8~38.7 × 7.4~10.0	7.2~8.6 × 5.3~6.4
	SFC20140823-07						
	SFC20140912-28						
A. sepiacea[a,b]	SFC20140822-49	*A. sepiacea* (AY436473)	99.8	*A. sepiacea* (AY436501)	99.0	32.3~40.6 × 8.8~11.1	6.7~8.0 × 4.9~6.8
	SFC20140730-17						
	SFC20140730-25						
	SFC20140912-15						
A. subjunquillea	SFC20140912-03	*A. subjunquillea* (KJ466427)	100	*A. subjunquillea* (KF245904)	100	29.4~39.1 × 7.4~11.2	6.7~8.7 × 5.9~8.6
	SFC20140823-11						
A. volvata[b]	SFC20140912-09	*A. volvata* (AB015681)	91.6	*A. volvata* (KF245906)	99.7	42.0~54.5 × 10.2~12.6	9.6~11.8 × 5.3~7.3
Amanita sp. 1	SFC20140912-22	*Amanita* sp. (KJ466417)	85.5	*A. ponderosa* (EF653958)	94.5	-	-
Amanita sp. 2	SFC20140730-10	*A. vaginata* (AB015691)	93.2	*A. angustilamellata* (AF024440)	99.4	-	-

[a]Newly recorded species to Korea.
[b]Newly recorded species to Inje County.

Specimens were dried and deposited in the Seoul National University Fungal Collection (SFC) (Table 1). Specimens identified as *Amanita* were re-examined to determine species based on macro- and microscopic characteristics as described in previous studies [1, 13]. After rehydrating in 3% KOH and staining with Congo red solution, microscopic features such as basidia and spores were observed using a light microscope (Eclipse 80i; Nikon, Tokyo, Japan). The morphological features were characterized in detail with specimens that had confirmed identity based on DNA sequence analyses (described below).

DNA extraction, PCR amplification, and sequencing. Genomic DNA was extracted from a small piece of tissue using a modified cetyltrimethyl ammonium bromide extraction protocol [14]. The ITS region was amplified with ITS1F and ITS4B primers [15], and the nLSU region was amplified with LR0R (http://www.biology.duke.edu/fungi/mycolab/primers.htm) and LR5 primers [16]. PCR amplifications were performed on a thermal cycler (C1000TM; Bio-Rad, Richmond, CA, USA) using the AccuPower PCR Premix (Bioneer Co., Daejeon, Korea) following instructions as outlined in Min *et al.* [17]. PCR products were visualized on a 1% agarose gel and purified using the Expin™ PCR Purification Kit (GeneAll Biotechnology, Seoul, Korea). Sequencing was performed at Macrogen (Seoul, Korea) on an automated DNA sequencer (ABI3700; Applied Biosystems, Foster City, CA, USA) using the aforementioned PCR primers.

Sequence analysis. DNA sequences were proofread using MEGA ver. 5 [18] and aligned with the *Amanita* sequences from the GenBank using Multiple Alignment using Fast Fourier Transform (MAFFT) [19]. Alignments were also checked by eye, and ambiguous positions were adjusted manually. Maximum likelihood (ML) analyses were conducted for the ITS and nLSU datasets, respectively. ML analyses were performed using RAxML ver. 8 [20] with the GTRCAT model of nucleotide substitution and 1,000 bootstrap replicates. For all analyses, *Limacella glioderma* was selected as an outgroup based on a previous study [5]. Intraspecific dissimilarity was calculated using MEGA.

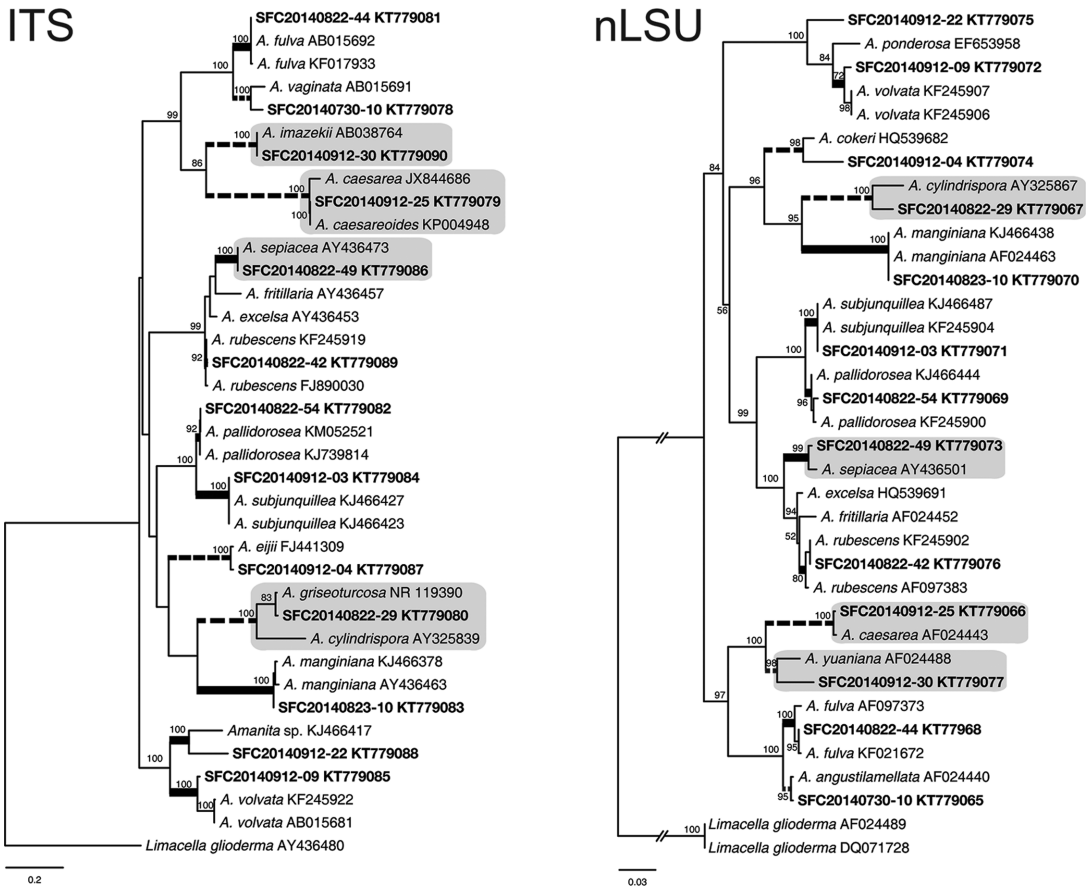

Fig. 1. Phylogenetic trees for *Amanita* species based on maximum likelihood analysis of internal transcribed spacer (ITS) and large subunit of nuclear ribosomal RNA (nLSU) sequences. Bootstrap scores of > 50 are presented at the nodes. The scale bar indicates the number of nucleotide substitutions per site. Thickened branches on the root nodes represent identical results between ITS and nLSU, while dotted lines represent the branches which have incongruent results between the ITS and nLSU. Gray boxes indicate the new species found in Korea.

RESULTS AND DISCUSSION

Approximate 240 specimens were collected from Mt. Jeombong in 2014, with 25 identified as *Amanita*. *Amanita* specimens were divided into 13 taxa according to their macro- and microscopic features. Basidia and spore size were measured for each specimen, and collection numbers were assigned to all *Amanita* specimens examined (Table 1). At the first, on the basis of macro- and microscopic features, six taxa were identified: *A. fulva*, *A. manginiana*, *A. pallidorosea*, *A. rubescens*, *A. sepiacea*, and *A. subjunquillea*. The morphological characteristics of each species were well matched with previous referenced descriptions. Molecular identification using both the ITS and nLSU sequences complemented the morphology-based identification. Sequence similarity and phylogenetic analysis confirmed the identity of six *Amanita* species, in which there was high ITS (over 99.0%) and nLSU (over 99.0%) sequence similarities (Table 1, Fig. 1). However, seven taxa could not be identified at the species level solely based on morphology due to the paucity of distinct traits. In addition, sequence analysis showed some discrepancy of the best match between the ITS and nLSU sequences. Through combining morphological and molecular approaches, five *Amanita* taxa were further determined at the species level: *A. caesareoides*, *A. eijii*, *A. griseoturcosa*, *A. imazekii*, and *A. volvata*. Explanation for the identification of each of the five species follows.

Specimen SFC20140822-15 formed a monophyletic group with *A. caesareoides* for the ITS sequence (100% sequence similarity) but with *A. caesarea* for the nLSU sequence (99.5%). Morphologically, *A. caesareoides* has subglobose to ellipsoid spores [21], while *A. caesarea* has ellipsoid (occasionally elongate) spores. Specimen SFC20140822-15 had subglobose to broadly ellipsoid spores matching *A. caesareoides*. Specimen SFC20140912-04 formed a group with *A. eijii* for the ITS sequence (96.6%) and *A. cokeri* for the nLSU sequence (95.6%). Morphologically, *A. eijii* has white to dirty white basidiocarps that become pinkish to brownish in the center, and *A. cokeri* has white to ivory basidiocarps [22]. Specimen SFC20140912-04 had pinkish to brownish color at the center of basidiocarps, matching *A. eijii*. Specimen SFC20140822-29 formed a monophyletic clade with the type specimen of *A. griseoturcosa* in the ITS phylogenetic tree (98.2%) but *A. cylindrispora* in the nLSU phylogenetic tree (95.5%). Morphologically, these two species are clearly distinguished by their bulb sizes. *A. cylindrispora* has a longer bulb (50~70 mm) compared to the bulb size of *A. griseoturcosa* (38~50 mm) [23]. Our specimen has a shorter bulb, which coincides with *A. griseoturcosa*. Specimen SFC20140912-30 formed a clade with either *A. imazekii* (ITS, 100%) or *A. incarnatifolia* (nLSU, 94.6%), and the specimen formed a clade with *A. murrilliana* (nLSU, 95.0%) and *A. yuaniana* (nLSU, 94.6%). *A. imazekii* has globose to subglobose spores [24], while the other three species have the ellipsoid to elongate spores. Specimen SFC20140912-30 had globose to subglobose spores, identifying it as *A. imazekii*. In the case of specimen SFC20140912-09, its nLSU sequence was highly similar (99.7%) to the sequence of *A. volvata* II (KA12-1194; GenBank: KF245906) of Korea [25]. The low sequence similarity of ITS (91.6%) to *A. volvata* II (KA12-1194, KF245922) and Japanese *A. volvata* (AB015681) [6] raised doubts about its molecular identification. However, morphological characteristics as well as phylogenetic analysis demonstrated that this specimen is indeed *A. volvata* (Fig. 1).

Two immature *Amanita* specimens (SFC20140912-22 and SFC20140730-10) were not confidently identified because morphological features could not be observed and sequence similarity to known species was low (Table 1).

Recently, taxonomic studies based on DNA sequence analysis have increased confidence in identification [17, 26] and showed that Asian fungal species are different from North American and European fungal species with similar morphology [26]. This trend was also found in *Amanita*, with several new species reported from Korea [25, 27]. Many of the previous *Amanita* studies were based solely on morphology, and thus, the true *Amanita* diversity in Korea is unknown. We incorporated molecular phylogenetics in our study to evaluate the diversity of *Amanita* species in Inje County.

Through our surveys of Mt. Jeombong in Inje County, 13 *Amanita* species were identified using morphological and molecular data (Table 1). Seven of these species were recorded previously in Korea (*A. eijii*, *A. fulva*, *A. manginiana*, *A. pallidorosea*, *A. rubescens*, *A. subjunquillea*, and *A. volvata*), and four species are new to Korea (*A. caesareoides*, *A. griseoturcosa*, *A. imazekii*, and *A. sepiacea*) (Fig. 2). Eight of the 13 species are new to the inventory of *Amanita* in Inje County, increasing species diversity from 24 to 32 species. Inje County has high vegetation diversity, so we expected more new records and possibly new species from the area. Below, we provide a detailed description of four new *Amanita* species identified in Korea.

Taxonomy.

Amanita caesareoides Lj. N. Vassiljeva, Notul. Syst. Sect. Cryptogam. Inst. Bot. Acad. Sci. U. S. S. R. 6: 199 (1950) (Table 1, Fig. 2A~2C).

Cap 68 mm, orangish red to yellow from center to margin. Lamellae yellow to bright yellow, crowded. Stipe 127 × 8~13 mm, slightly tapering upward, yellow to bright yellow. The bulb is saccate and lobate, outer surface white, 45 mm long and 25 mm wide. The ring is on the upper part of the stem, yellow, skirt-like, membranous. Basidia 42.8~55.0 × 11.5~14.0 μm, 4-sterigmate. Clamps are present at the bases of basidiole. Spores 8.2~10.8 × 6.4~8.5 μm, Q = 1.12~1.48, subglobose to broadly ellipsoidal, occasionally ellipsoidal.

Specimens examined: Korea, Gangwon Province, Inje County, Gachilbong and Mt. Jeombong, 38°2′28.56″ N, 128°27′58.87″ E, 22 Aug 2014, Young Woon Lim,

Fig. 2. Four new *Amanita* species identified in Korea. A~C, *A. caesareoides* (SFC20140912-25); D~G, *A. griseoturcosa* (SFC20140822-29); H~J, *A. imazekii* (SFC20140912-30); K~M, *A. sepiacea* (SFC20140822-49). a, spores; b, basidia; c, lamella edge cells (scale bars: B, F, I, L = 2 cm, C, G, J, M = 10 μm).

SFC20140822-15; on the ground of mixed woodlands. 37°58′20.76″ N, 128°25′53.15″ E, 12 Sep 2014, Hae Jin Cho, SFC20140912-25 (GenBank accession Nos. KT779079 for ITS and KT779066 for nLSU); on the ground of mixed woodlands.

Remarks: *Amanita caesareoides* is known as the Asian Caesar's mushroom. This species is morphologically similar to *A. caesarea*, *A. hemibapha*, *A. jacksonii*, *A. javanica*, and *A. subjunquillea*. *A. caesareoides* has subglobose to ellipsoid spores, while *A. caesarea*, *A. hemibapha*, *A. jacksonii*, and *A. javanica* have ellipsoid to elongate spores [21] Among them *A. caesarea*, *A. caesareoides*, and *A. jacksonii* have orange red caps, but they have different geographical distributions. *A. caesareoides* is described in eastern Russian, southwestern China, Korea, and Japan; *A. jacksonii* is described in eastern Canada, the eastern U.S., and eastern Mexico. Also, *A. caesarea* is described from regions near the Mediterranean (studies in the Amanitaceae: http://www.amanitaceae.org/).

A. subjunquillea is one of the lethal *Amanita* species that

can be misidentified as *A. caesareoides* due to its similar cap color. *A. caesareoides* has prominently pectinate-striate inward margins from the edge of cap and yellow to bright yellow lamellae with subglobose to ellipsoid spores. *A. subjunquillea* has a fine line at the edge of cap and white to cream lamellae with globose to subglobose spores [9]. *A. caesareoides* and *A. subjunquillea* are clearly separated by ITS and nLSU phylogeny.

Amanita girseoturcosa T. Oda, C. Tanaka & Tsuda, Mycoscience 43: 351-355 (2002) (Table 1, Fig. 2D~2G).
Cap 58~65 mm, grayish-turquoise or turquoise gray to dark turquoise. Lamellae white, 3~4 mm broad, crowded. Stipe 120~150 × 10~12 mm, cylindrical or slightly tapering upward, white, smooth to slightly scaly, stuffed to slightly hollow. Bulb hollow, 38~50 mm long and 22~38 mm wide. Ring on the upper part of the stem, white, skirt-like, membranous. Basidia 39.1~51.2 × 10.2~12.9 µm, 4-sterigmate, and a simple basal septum. Spores 9.1~11.8 × 5.9~7.5 µm, Q = 1.29~1.77, broadly ellipsoidal to elongate, occasionally oval.
Specimens examined: Korea, Gangwon Province, Inje County, Mt. Jeombong, 38°2′28.56″ N, 128°27′58.87″ E, 22 Aug 2014, Young Woon Lim, SFC20140822-29 (GenBank accession Nos. KT779080 for ITS and KT779067 for nLSU); on the ground of mixed woodlands.
Remarks: *Amanita girseoturcosa* is characterized by its turquoise cap. This species was transferred from the section *Phalloideae* to the section *Lepidella* [7].

Amanita imazekii T. Oda, C. Tanaka & Tsuda, Mycologia 93: 1231 (2001) (Table 1, Fig. 2H~2J).
Cap 84 mm, grayish creamy. Lamellae white to pale creamy, crowded. Stipe 190 × 13~16 mm, slightly tapering upward, pruinose to slightly floccose, white. Bulb saccate, white, 54 mm long and 32 mm wide. Ring on the upper part of the stem, white, skirt-like, membranous. Basidia 45.8~64.7 × 9.9~14.1 µm, 4-sterigmate. Clamps present at the bases of basidiole. Spores 9.8~13.2 × 9.2~11.4 µm, Q = 1.01~1.26, globose to broadly ellipsoidal.
Specimens examined: Korea, Gangwon Province, Inje County, Mt. Jeombong, 37°58′20.76″ N, 128°25′53.15″ E, 12 Sep 2014, Hae Jin Cho, SFC20140912-30 (GenBank accession Nos. KT779090 for ITS and KT779077 for nLSU); on the ground of mixed woodlands.
Remarks: This species is closely related with *A. incarnatifolia*, *A. murrilliana*, and *A. yuaniana* in the nLSU sequence analysis. However, *A. imazekii* has spores that range from being globose to subglobose, while the other species have ellipsoid to elongate spores [24].

Amanita sepiacea S. Imai, Bot. Mag., Tokyo 47: 423-432 (1933) (Table 1, Fig. 2K~2M).
Cap 100~150 mm, dark grey to brown to blackish, more dark in the center, dirty white to greyish conical to verrucose warts on cap. Lamellae white and crowded. Stipe 150~215 × 10~20 mm, cylindrical or slightly tapering upward, surface white to dirty white covered with greyish to grey fibrillose squamules. Bulb subglobose to spindle-shaped to turnip-shaped, 25~35 mm wide. Ring on the upper part of the stem, white, skirt-like, membranous. Basidia 32.3~40.6 × 8.8~11.1 µm, 4-sterigmate, and a simple basal septum. Spores 6.7~8.0 × 4.9~6.8 µm, Q = 1.15~1.49, broadly ellipsoidal to ellipsoidal.
Specimens examined: Korea, Gangwon Province, Inje County, Mt. Jeombong, 38°2′28.56″ N, 128°27′58.87″ E, 30 Jul 2014, Hae Jin Cho, SFC20140730-17; on the ground of mixed woodlands. 38°2′26.63″ N, 128°28′19.82″ E, 30 Jul 2014, Hae Jin Cho, SFC20140730-25; on the ground of forest with *Quercus* spp. 22 Aug 2014, Young Woon Lim, SFC20140822-49. 15 Sep 2014, Hae Jin Cho, SFC20140912-15 (Genbank accession Nos. KT779086 for ITS and KT779073 for nLSU).
Remarks: *Amanita sepiacea* has a remarkably large fruit body; the cap grew up to 100~150 mm and stipe rose up to 200 mm. This species is morphologically very similar to *Amanita excelsa* [13] and *Amanita fritillaria* [28]. However, *A. sepiacea* was distinguished in our study from *A. excelsa* and *A. fritillaria* by ITS and nLSU sequence analyses (Fig. 1) [29, 30].

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