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Molecular phylogeny of Japanese *Amanita* species based on nucleotide sequences of the internal transcribed spacer region of nuclear ribosomal DNA

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The molecular phylogeny of 36 specimens of Japanese *Amanita* species was studied based on nucleotide sequences of the internal transcribed spacer (ITS) region of nuclear ribosomal DNA. The phylogenetic tree obtained supported the traditional classification systems of Bas (1969) and Singer (1986), which are based on morphological characters, in the division of the genus *Amanita* is divided into subgenera *Amanita* and *Lepidella* by the amyloidity of basidiospores. However, at section-level, we suggest that subgenus *Amanita* should be divided into three sections (*Amanita*, *Vaginatae*, and *Caesareae*). Our results also showed the necessity to modify the taxonomic treatments at section-level in the subgenus *Lepidella*. It appears that the establishment of *A. muscaria* and *A. pantherina* from a common ancestral species might be a very recent event, or these might be lower taxa of same species. As for three subspecies of *A. hemibapha* and three varieties of *A. vaginata*, it is necessary to grade up their taxonomical ranks from subspecies/variety to species. A new combination, *A. javanica*, is proposed for *A. hemibapha* subsp. *javanica*.

Key Words——*Amanita*; *Amanita javanica*; classification system; ITS; molecular phylogeny.

The genus *Amanita* Pers. is one of the best known mushroom genera that includes some highly toxic species (Bresinsky and Besl, 1990). Members of this genus are found throughout the world, and about 200 species have been described (Hawksworth et al., 1995). About 50 species have been recognized in Japan (Imazeki and Hongo, 1987). The taxonomy of *Amanita* has traditionally been based on morphological characters of the fruitbody (Bas, 1969; Corner and Bas, 1962; Gilbert, 1940, 1941; Moser, 1983; Singer, 1962, 1975, 1986). Two classification systems (Fig. 1) have been widely accepted: one proposed by Corner and Bas (1962) and later modified by Bas (1969), and the other proposed by Singer (1986). Both systems divide the genus *Amanita* into two subgenera mainly based on amyloidity of the basidiospores: subgenus *Amanita* with inamyloid basidiospores, and subgenus *Lepidella* (Gilb.) Veselý with amyloid basidiospores. However, they differ in the taxonomy at section-level and morphological characters used to delimit the sections (Fig. 1). Further, traditional morphology-based taxonomy often involves confusion in species delimitations, a good example of which can be found in the *Amanita vaginata* group (Bas, 1977).

We made a molecular phylogenetic study of Japanese *Amanita* species in order to rearrange the taxonomic treatments within the genus *Amanita*. First, we analyzed the partial sequences of nuclear ribosomal DNA of 36 *Amanita* specimens obtained in Japan. The sequenced region (ITS region) consists of a portion of 18S rDNA, ITS1, 5.8S rDNA, ITS2 and a portion of 28S rDNA (Van Nues et al., 1994). We constructed a molecular phylogenetic tree of Japanese *Amanita* species based on the sequences. Finally, we rearranged the classification system of the genus *Amanita* and reassessed the relationships of some closely related species and intraspecies.

Materials and Methods

Fungal materials Thirty-six specimens, belonging to 22 species (including three subspecies, five varieties and one forma), were examined (Table 1). These specimens were identified by their morphological characters according to description of Imazeki and Hongo (1987).

Amanita sp. 1 LEM960002 and LEM960017 resembled *A. spissacea* Imai morphologically, but differed in having smaller volval remnants on the pileus and lacking a powdery dark brown volva forming concentric encircling rings on the bulb of the stem that *A. spissacea* has. *Amanita* sp. 2 LEM960194 resembled *A. cokeri* (Gilb. & Kühn.) Gilb. f. *roseotincta* Nagasawa & Hongo, differing in having fewer scales at the lower part of the stem and the upper part of the bulb and a grayish white context.

DNA preparations A minute slice of dried fruit-body (ca. 10 mg) was suspended in 500 μl of extraction buffer (50 mM Tris-HCl pH 8.0, 125 mM EDTA, 100 mM NaCl, 2% (w/v) sodium *N*-dodecanoylsarcosinate, 1% (v/v) 2-mercaptoethanol). DNA was extracted by the method of Nakada et al. (1994).

PCR amplification and DNA sequencing Primers used in PCR amplification were ITS4 and ITS5 (White et al., 1990). The reaction mixture (50 mM KCl, 10 mM Tris-

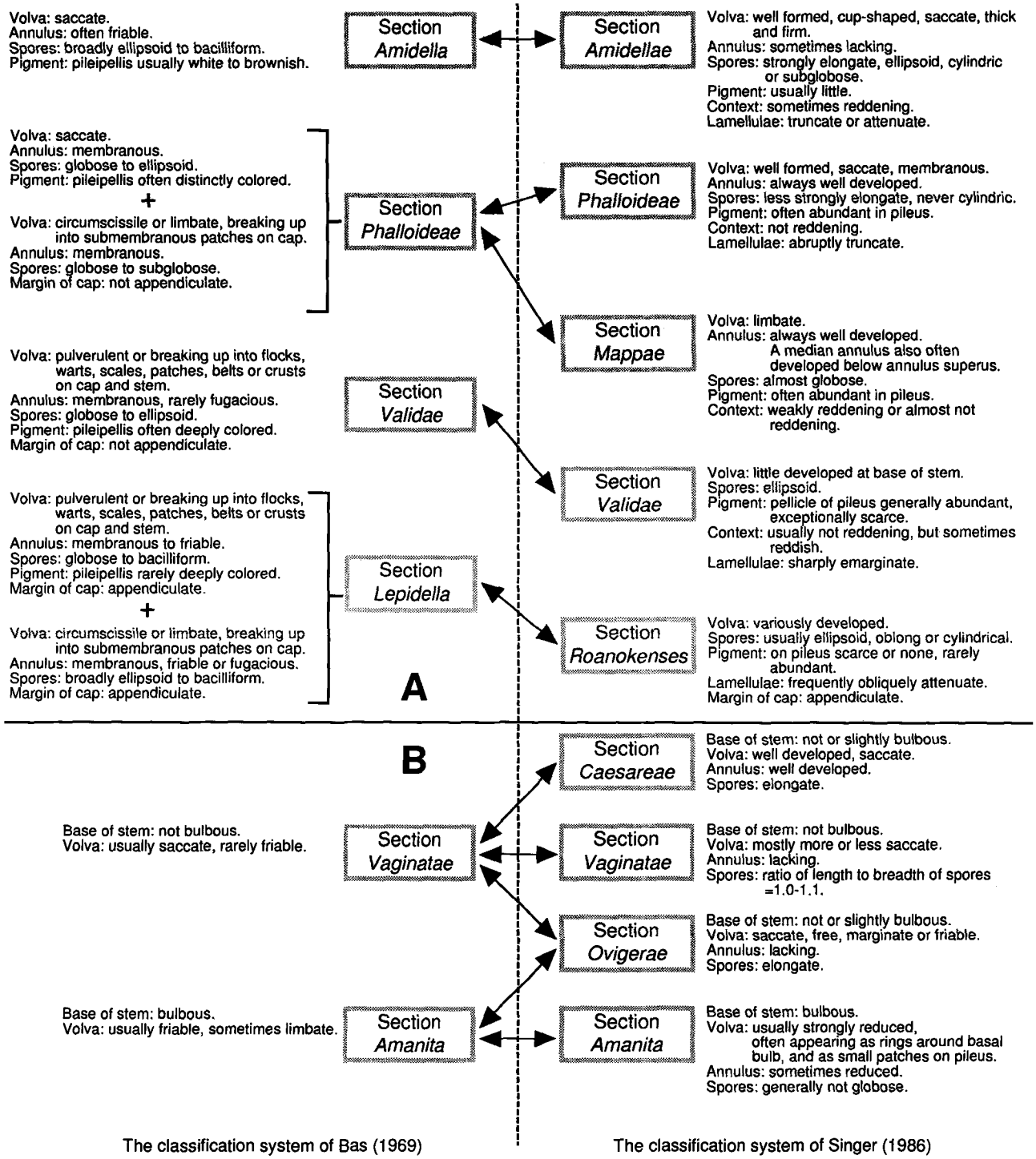

Fig. 1. Comparison of the sections in the classification systems of Bas (1969) and Singer (1986).
A: Subgenus *Lepidella*, B: Subgenus *Amanita*. Sections *Phalloideae* and *Lepidella* of Bas (1969) each consist of two defined groups.

HCl pH 8.0, 50 μM of each dNTPs, 1 mM $MgCl_2$) contained 10 pmol of each primer, 1.5 U of *Taq* DNA polymerase (Takara) and ca. 10 ng of template DNA in a volume of 50 μl. The thermal cycler (TP3000, Takara) was programmed as follows: initial denaturation, 2 min at 95°C; then 35 cycles of 0.5 min at 95°C, 1 min at 50°C, and 1.5 min at 72°C; and final extension at 72°C for 5 min. After electrophoresis in low-melt agarose gel (Sea Plaque GTG Agarose, FMC), the amplified products were excised from the gel. The DNA fragments were cloned into pZErO™-2 (Invitrogen). To avoid artifact DNA sequences caused by errors in DNA polymerization, at least three recombinants were picked up from a batch of transformants, and the homogeneity or majority of

Table 1. Details and nucleotide sequence length (bp) of ITS region of thirty-six specimens of Japanese *Amanita* species.

Taxa[a]	Specimen No.[b]	Locality of collection	Acc. No.[c]	Length (bp)
Subgenus *Amanita*				
Section *Amanita*				
A. pantherina (DC.: Fr.) Krombh.	LEM950167	Kamigamo, Kyoto-shi	AB015701	716
A. pantherina (DC.: Fr.) Krombh.	LEM970678	Toyoura-cho, Hokkaido	AB015701	714
A. pantherina (DC.: Fr.) Krombh.	LEM970680	Niigata-shi, Niigata Pref.	AB015701	712
A. muscaria (L.: Fr.) Pers.	LEM950025	Kaida-mura, Nagano Pref.	AB015700	695
A. muscaria (L.: Fr.) Pers.	LEM950048	Takane-mura, Gifu Pref.	AB015700	695
A. muscaria (L.: Fr.) Pers.	LEM960337	Chino-shi, Nagano Pref.	AB015700	697
A. melleiceps Hongo	LEM970723	Ohita-shi, Ohita Pref.	AB015688	706
A. rubrovolvata Imai	LEM960292b	Takane-mura, Gifu Pref.	AB015689	721
A. sychnopyramis Corner & Bas f. *subannulata* Hongo	LEM960112a	Ohtsu-shi, Shiga Pref.	AB015690	703
Section *Vaginatae* (Fr.) Quél				
A. vaginata (Bull.: Fr.) Vitt. var. *vaginata*	LEM950304a	Kyoto-Gyoen, Kyoto-shi	AB015691	610
A. vaginata (Bull.: Fr.) Vitt. var. *fulva* (Schaeff.) Gill.	LEM960312b	Takane-mura, Gifu Pref.	AB015692	612
A. vaginata (Bull.: Fr.) Vitt. var. *punctata* (Cleland & Cheel) Gilb.	LEM960270	Kyoto-Gyoen, Kyoto-shi	AB015693	625
A. ceciliae (Berk. & Br.) Bas	LEM950069	Kamigamo, Kyoto-shi	AB015694	605
A. hemibapha (Berk. & Br.) Sacc. subsp. *hemibapha*	LEM960078	Kutsuki-mura, Shiga Pref.	AB015699	690
A. hemibapha (Berk. & Br.) Sacc. subsp. *hemibapha*	LEM960355	Oizumi-mura, Yamanashi Pref.	AB015699	690
A. hemibapha (Berk. & Br.) Sacc. subsp. *javanica* Corner & Bas	LEM970513	Tsukuba-shi, Ibaraki Pref.	AB015698	583
A. hemibapha (Berk. & Br.) Sacc. subsp. *javanica* Corner & Bas	LEM970518	Hirakata-shi, Osaka Pref.	AB015698	583
A. hemibapha (Berk. & Br.) Sacc. subsp. *similis* (Boed.) Corner & Bas	LEM960013	Kyoto-Gyoen, Kyoto-shi	AB015697	573
A. hemibapha (Berk. & Br.) Sacc. subsp. *similis* (Boed.) Corner & Bas	LEM960244	Kyoto-Gyoen, Kyoto-shi	AB015697	573
A. longistriata Imai	LEM950067	Kamigamo, Kyoto-shi	AB015678	662
Subgenus *Lepidella* (Gilb.) Veselý *emend.* Corner & Bas				
Section *Phalloideae* (Fr.) Quél.				
A. virosa (Fr.) Bertillon	LEM960310	Takane-mura, Gifu Pref.	AB015676	695
A. pseudoporphyria Hongo	LEM960037a	Midorogaike, Kyoto-shi	AB015702	636
A. porphyria (Alb. & Schw.: Fr.) Secr.	LEM960303	Takane-mura, Gifu Pref.	AB015677	728
A. citrina (Schaeff.) Pers. var. *citrina*	LEM960298	Takane-mura, Gifu Pref.	AB015679	734
A. citrina (Schaeff.) Pes. var. *grisea* (Hongo) Hongo	LEM970501	Syougunzuka, Kyoto-shi	AB015680	732
Section *Amidella* (Gilb.) Konr. & Maubl.				
A. volvata (Peck) Martin	LEM960165	Tatsuno-shi, Hyogo Pref.	AB015681	756
Section *Validae* (Fr.) Quél.				
A. flavipes Imai	LEM960084a	Kutsuki-mura, Shiga Pref.	AB015696	722
A. flavipes Imai	LEM960088a	Kutsuki-mura, Shiga Pref.	AB015696	722
A. rubescens Pers.: Fr.	LEM950063	Kamigamo, Kyoto-shi	AB015682	723
A. spissacea Imai	LEM960187	Tatsuno-shi, Hyogo Pref.	AB015683	704
Amanita sp. 1	LEM960002	Kyoto-Gyoen, Kyoto-shi	AB015695	726
Amanita sp. 1	LEM960017	Kyoto-Gyoen, Kyoto-shi	AB015695	726
Section *Lepidella*				
A. japonica Bas	LEM960167	Tatsuno-shi, Hyogo Pref.	AB015684	719
A. abrupta Peck	LEM960299a	Takane-mura, Gifu Pref.	AB015685	783
A. virgineoides Bas	LEM960205	Matsugasaki, Kyoto-shi	AB015686	756
Amanita sp. 2	LEM960194	Matsugasaki, Kyoto-shi	AB015686	726

a) The classification system follows Bas (1969).
b) LEM: Herbarium, Laboratory of Environmental Mycoscience, Faculty of Agriculture, Kyoto University.
c) The nucleotide sequence data appear in the DDBJ/EMBL/GenBank nucleotide sequence databases.

DNA sequences was confirmed. DNA was sequenced by the dideoxy chain termination method (Sanger et al., 1977) with Thermo Sequenase™ (Amersham) according to the manufacturer's recommendations. The sequence primers used were fluorescent-dye (Cy5)-labeled M13-20 and M13-RV (Pharmacia Biotech). Terminated samples were electrophoresed on ALFred DNA sequencer (Pharmacia Biotech) and sequence data were generated.

Phylogenetic analysis Sequences were aligned using the CLUSTAL W multiple alignment program ver. 1.6 (Thompson et al., 1994). The setting for this run was as follows: fast pairwise alignment parameters (gap penalty = 15, k-tuple size = 4, no. of top diagonals = 10, and window size = 10); multiple alignment parameters (gap opening penalty = 10, gap extension penalty = 4, delay divergent sequences = 40%, and toggle transitions (DNA) = weighted). The sequence alignment has been submitted to TreeBASE, a relational database of phylogenetic information, maintained by the Harvard University Herbaria (URL: http://herbaria.harvard.edu/treebase/). The aligned sequences were analyzed by the neighbor-joining method (Saitou and Nei, 1987), using NEIGHBOR in PHYLIP ver 3.5c package (Felsenstein, 1993). The distance matrix was calculated using DNADIST with Kimura's 2-parameter method and the topology was tested with 1,000 bootstrap trials.

Results

Nucleotide sequences of the amplified ITS region were determined for 36 specimens. ITS regions ranged in length from 573 bp in *A. hemibapha* (Berk. & Br.) Sacc. subsp. *similis* (Boed.) Corner & Bas LEM960013 and LEM960244 to 783 bp in *A. abrupta* Peck LEM960299a (Table 1). Their sequences are deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession numbers AB015676-AB015702. Based on these aligned sequences, we constructed an unrooted molecular phylogenetic tree of Japanese *Amanita* species after 1,000 bootstrap replications. The phylogenetic tree output separated into five groups (Fig. 2).

Group 1 included *A. pantherina* (DC.: Fr.) Krombh., *A. muscaria* (L.: Fr.) Pers., *A. rubrovolvata* Imai, *A. melleiceps* Hongo, and *A. sychnopyramis* Corner & Bas f. *subannulata* Hongo. They have in common inamyloid basidiospores, a striate pileus margin, and a bulbous base of the stem. In Singer's system (1986), they belong to subgenus *Amanita* section *Amanita* or section *Ovigerae* (*A. melleiceps*). In Bas's system (1969), all belong to subgenus *Amanita* section *Amanita*. The longest interspecies genetic distance was 0.1111, between *A. rubrovolvata* LEM960292b and *A. sychnopyramis* f. *subannulata* LEM960112a; and the shortest was 0.0116, between *A. muscaria* LEM960337 and *A. pantherina* LEM970678 or LEM970680. The second shortest was 0.0511, between *A. rubrovolvata* LEM960292b and *A. muscaria* LEM960337. As for *A. pantherina* and *A. muscaria*, we analyzed three specimens each. The shortest intraspecies genetic distance in *A. pantherina* was 0.0000 between LEM970678 and LEM970680, and the longest was 0.0028 between these two and LEM950167. In *A. muscaria*, the shortest was 0.0000 between LEM950025 and LEM950048, and the longest was 0.0146 between these two and LEM960337. These results indicated that *A. muscaria* and *A. pantherina* are closely related phylogenetically.

Group 2 included *A. vaginata* var. *vaginata*, *A. vaginata* var. *fulva* (Schaeff.) Gill., *A. vaginata* var. *punctata* (Cleland & Cheel) Gilb., and *A. ceciliae* (Berk. & Br.) Bas. Their common morphological characters are inamyloid basidiospores, a striate pileus margin, a saccate volva of the stem without a bulbous base or an annulus. These elements belong to section *Vaginatae* in the systems of both Bas (1969) and Singer (1986). The genetic distances among the three varieties of *A. vaginata* were considerably significant. The distance values were 0.2212 between *A. vaginata* var. *vaginata* LEM950304a and *A. vaginata* var. *punctata* LEM960270, 0.1078 between *A. vaginata* var. *vaginata* LEM950304a and *A. vaginata* var. *fulva* LEM960312b, and 0.1967 between *A. vaginata* var. *punctata* LEM960270 and *A. vaginata* var. *fulva* LEM960312b. The distance values were 0.1788 between *A. ceciliae* LEM950069 and *A. vaginata* var. *fulva* LEM960312b, and 0.1684 between *A. ceciliae* LEM950069 and *A. vaginata* var. *vaginata* LEM950304a. Therefore, *A. ceciliae* LEM950069 was closer to *A. vaginata* var. *vaginata* LEM950304a and *A. vaginata* var. *fulva* LEM960312b than was *A. vaginata* var. *punctata* LEM960270.

Group 3 consisted of *A. hemibapha* subsp. *hemibapha*, *A. hemibapha* subsp. *javanica* Corner & Bas, *A. hemibapha* subsp. *similis*, and *A. longistriata* Imai. They are characterized in common by inamyloid basidiospores, a striate pileus margin, a saccate volva, and an annulus of the stem without a bulbous base. These taxa belong to section *Vaginatae* of Bas (1969), but section *Caesareae* of Singer (1986) due to the presence of the annulus. The genetic distance between specimens of each subspecies was negligible. In *A. hemibapha* subsp. *hemibapha*, the distance between LEM960078 and LEM960355 was 0.0000. In *A. hemibapha* subsp. *javanica*, the distance between LEM970513 and LEM970518 was 0.0017. In *A. hemibapha* subsp. *similis*, the distance between LEM960013 and LEM960244 was 0.0000. However, the genetic distances between subspecies were considerably long. The distance between the node of LEM960078 and LEM960355 and the node of LEM970513 and LEM970518 was 0.1263. The distance between the node of LEM970513 and LEM970518 and the node of LEM960013 and LEM960244 was 0.0915.

Group 4 included *A. japonica* Bas, *A. virgineoides* Bas, *A. abrupta*, *A. pseudoporphyria* Hongo, and *Amanita* sp. 2. These taxa have in common amyloid basidiospores and a non-striate pileus margin. All except *A. pseudoporphyria* have an appendiculate margin of the pileus. However, they vary in other morphological characters (Fig. 2). *Amanita pseudoporphyria* belongs to section *Phalloideae* of Bas (1969) and Singer (1986). The others belong to section *Lepidella* of Bas (1969) and

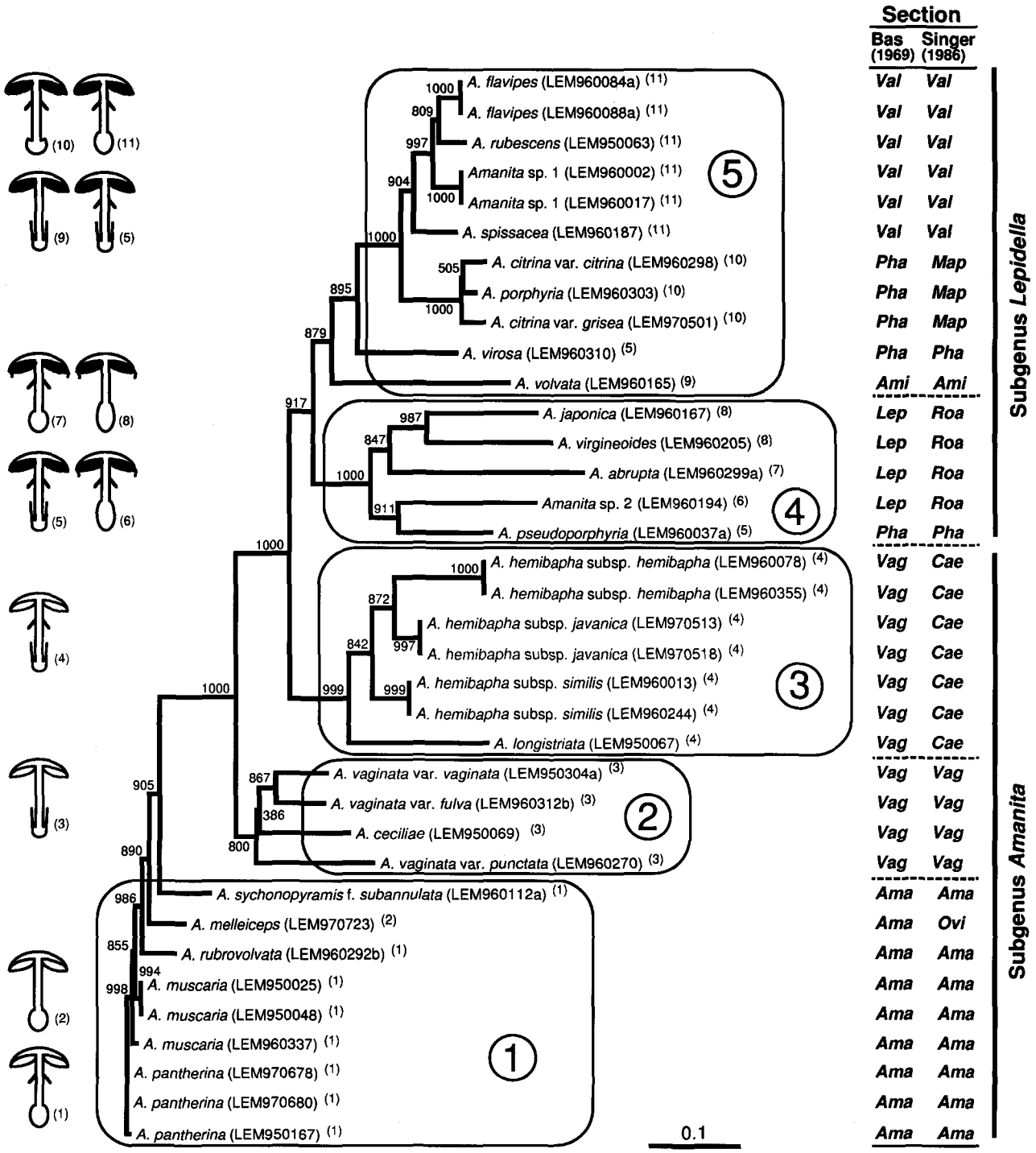

Fig. 2. Unrooted phylogenetic tree of 36 specimens of *Amanita* species based on nucleotide sequences of ITS region. The tree was constructed by the neighbor-joining method. The values on nodes are the confidence levels from 1,000 replicate bootstrap samplings. The distance corresponding to 10 base changes per 100 nucleotide positions is indicated by a bar. The taxonomic ranks are based on the classification systems of Bas (1969) and Singer (1986). The sections are abbreviated as the follows: *Ama, Amanita*; *Vag, Vaginatae*; *Ovi, Ovigerae*; *Cae, Caesareae*; *Val, Validae*; *Pha, Phalloideae*; *Lep, Lepidella*; *Map, Mappae*; *Roa, Roanokenses*; and *Ami, Amidella* (Bas, 1969) or *Amidellae* (Singer, 1986). Mushroom figures show morphological characters of specimens: white gills mean inamyloid basidiospores, and black gills mean amyloid ones. The numbers on mushroom figures correspond to superscripts following specimen names.

section *Roanokenses* of Singer (1986). The genetic distances among specimens were significantly long. The longest was 0.4280 between *Amanita* sp. 2 LEM960194 and *A. abrupta* LEM960299a. The shortest was 0.2562 between *Amanita* sp. 2 LEM960194 and *A. pseudoporphyria* LEM960037a.

Group 5 included *A. flavipes* Imai, *A. rubescens* Pers.: Fr., *Amanita* sp. 1, *A. spissacea* Imai, *A. citrina* (Schaeff.) Pers. var. *citrina*, *A. citrina* var. *grisea* (Hongo) Hongo, *A. porphyria* (Alb. & Schw.: Fr.) Secr., *A. virosa* (Fr.) Bertillon, and *A. volvata* (Peck) Martin. The members of group 5 have amyloid basidiospores and a nonstriate pileus margin in common, but they vary in other morphological characters of the stem, volva, annulus, and pileus (Fig. 2). *Amanita flavipes*, *A. rubescens*, *Amanita* sp. 1, and *A. spissacea*, which belong to section *Validae* of both Bas (1969) and Singer (1986), make one small cluster. *Amanita citrina* var. *citrina*, *A. citrina* var. *grisea*, and *A. porphyria*, which have a limbate volva in common, belong to section *Mappae* of Singer (1986). However, in Bas's system (1969), they belong to section *Phalloideae*. *Amanita virosa* belongs to section *Phalloideae* of both Bas (1969) and Singer (1986). *Amanita volvata* belongs to section *Amidella* of Bas (1969) and section *Amidellae* of Singer (1986). Intraspecies genetic distance between *A. flavipes* LEM960084a and LEM960088a was 0.0014, and that between *Amanita* sp. 1 LEM960002 and LEM960017 was 0.0000. Three morphologically similar specimens, *A. citrina* var. *citrina* LEM960298, *A. citrina* var. *grisea* LEM970501, and *A. porphyria* LEM960303, made one cluster. The genetic distances in this cluster were 0.0383 between *A. citrina* var. *citrina* LEM960298 and *A. porphyria* LEM960303, and 0.0431 between *A. citrina* var. *grisea* LEM970501 and *A. porphyria* LEM960303. The distance between *A. volvata* LEM960165 and the node of the other ten specimens was 0.2239. In group 5, the longest interspecies distance was 0.3549, between *A. volvata* LEM960165 and *A. virosa* LEM960310. The shortest was 0.0383, between *A. citrina* var. *citrina* LEM960298 and *A. porphyria* LEM960303.

Discussion

Reevaluation of the classification systems of Bas (1969) and Singer (1986) based on molecular data

Subgenera *Amanita* and *Lepidella* Groups 1–3 included only taxa of subgenus *Amanita*, and groups 4 and 5 only taxa of subgenus *Lepidella* (Fig. 2). Our molecular phylogeny supported the classifications of Bas (1969) and Singer (1986), in which the genus *Amanita* is divided into two subgenera *Amanita* and *Lepidella* by the amyloidity of basidiospores, which correlated with the striate pileus margin.

Sections of subgenus *Amanita* Singer (1962, 1975, 1986) separated this subgenus into four sections, *Caesareae*, *Vaginatae*, *Ovigerae*, and *Amanita*, based on the shapes of annulus, volva, basidiospores, and so on (Fig. 1). However, Corner and Bas (1962), followed by Bas (1969), considered that there was a long gradating series of intermediates between the exannulate *A. vaginata* (type species of section *Vaginatae* of Singer (1962)) and the annulate *A. caesarea* (type species of section *Caesareae* of Singer (1962)), and united these two sections into one section *Vaginatae*. Furthermore, Corner and Bas (1962) considered that section *Ovigerae*, delimited by an exannulate stem and elongate basidiospores, was artificial and heterogeneous. Therefore, Corner and Bas (1962) and Bas (1969) defined two sections, *Amanita* and *Vaginatae* (Fig. 1). In our molecular phylogenetic study, the members of subgenus *Amanita* examined were clearly separated into three groups (Fig. 2). The members of group 1 have in common a bulbous base of the stem and correspond to section *Amanita* of Bas (1969) or section *Amanita* of Singer (1986), except for *A. melleiceps*, which belongs to section *Ovigerae*. The members of group 2 have in common a saccate volva of the stem without a bulbous base or an annulus and correspond to section *Vaginatae* of Bas (1969) and Singer (1986). The members of group 3 have in common a saccate volva and an annulus of the stem without a bulbous base and correspond to section *Caesareae* of Singer (1986) and *Vaginatae* of Bas (1969) (Fig. 2). Groups 2 and 3 are well separated and have no intermediates in our phylogeny. Therefore, we consider that the annulus is an important taxonomic character for taxa with saccate volva, and section *Caesareae* should be adopted. *A. melleiceps*, which belongs to section *Ovigerae*, was located in group 1 and has a bulbose base as do other species of group 1. We consider that the annulus is not an important taxonomic character for taxa with a bulbous base, and that section *Ovigerae* is not valid.

We propose the following rearrangement of the classification systems of Bas (1969) and Singer (1986), with subdivision of the subgenus *Amanita*.

Section *Amanita*: Stem with a bulbose base. Volva usually friable, sometimes limbate.

Section *Vaginatae* (Fr.) Quél.: Stem without a bulbous base or an annulus. Volva usually saccate, rarely friable.

Section *Caesareae* Sing.: Stem with an annulus and without a bulbous base. Volva usually saccate.

In addition, based on our measurements of basidiospores, the members of group 1 have globose-ellipsoid basidiospores (the average length/breadth ratio of spore in each specimen = 1.04–1.52), the members of group 2 have globose-subglobose basidiospores (the ratio = 1.02–1.05), and the members of group 3 have broadly ellipsoid-ellipsoid basidiospores (the ratio = 1.22–1.43).

Yang (1997) examined *Amanita* species of southwest China morphologically and suggested that subgenus *Amanita* should be separated into three sections, *Amanita*, *Vaginatae*, and *Caesareae*. This suggestion corresponds to our results.

Sections of subgenus *Lepidella* The members of subgenus *Lepidella* examined were separated into groups 4 and 5.

In group 5, *A. citrina* var. *citrina*, *A. citrina* var. *grisea*, and *A. porphyria*, which have a limbate volva, made one small cluster. In the system of Singer (1986),

these taxa belong to section *Mappae*. However, Bas (1969) united these taxa having a limbate volva and the taxa having a saccate volva into section *Phalloideae*. Judging from our results, these taxa should be treated as a group separate from section *Phalloideae*. Furthermore, this small cluster was located closely adjacent to another small cluster of six specimens of section *Validae* without a saccate volva. Therefore, we consider that it is reasonable to treat section *Mappae* of Singer (1986) as a part of section *Validae*. Yang (1997) also proposed the same treatment based on the morphological study of *Amanita* species from southwest China.

Amanita virosa and *A. pseudoporphyria*, both belonging to section *Phalloideae*, separated into group 5 and 4, respectively. Therefore, section *Phalloideae* might be artificial and heterogeneous. As for section *Amidella*, we analyzed only one specimen, *A. volvata* LEM960165. To clarify the validity of section *Phalloideae* and the taxonomic position of section *Amidella*, more species should be examined.

The members of group 4 show wide variation in morphological characters (Fig. 2). In particular *A. pseudoporphyria* is considerably different from other members in having a saccate volva. At this point, it is difficult to define common morphological characters for group 4. However, particular non-protein amino acids and related compounds were identified from *A. abrupta*, *A. virgineoides*, and *A. pseudoporphyria* (Hatanaka et al., 1985; Ohta et al., 1986, 1987). We consider that this chemical character may be useful as a classificational criterion of group 4. These amino acids have also been isolated from *A. gymnopus* Corner & Bas and other related species belonging to section *Lepidella* (=*Roanokenses*) (Hatanaka et al., 1994, 1995).

Phylogenetic analysis at species level

Relationship between *A. muscaria* and *A. pantherina* *Amanita muscaria* and *A. pantherina* are morphologically similar and have been inferred to be closely related on the basis of toxicological similarities (Benedict et al., 1966; Beutler and Der Marderosian, 1981; Chilton and Ott, 1976). However, their relationship has not been addressed at the DNA level. In this study, the relationship between *A. muscaria* and *A. pantherina* was confirmed to be extremely close: the genetic distance between the two species was the shortest interspecies distance in section *Amanita*. Furthermore, for *A. muscaria* LEM960337, *A. pantherina* LEM970678 and LEM970680 were closer than two other specimens of *A. muscaria*. These data suggest that the establishment of *A. pantherina* and *A. muscaria* from a common ancestral species might be a very recent event, or these might be lower taxa of the same species. In North America, *A. muscaria* is separated into six varieties and *A. pantherina* into four varieties based on the color of pileus, distribution patterns, and so on (Jenkins, 1977, 1986). In our research, no genetic difference was detected between the specimens with different colors of pilei. Color variants of *A. muscaria* (LEM950048 with a yellow pileus and LEM950025 with a red pileus) had completely the same sequences. Further research of *A. muscaria* and *A. pantherina* based on DNA analysis is needed to clarify the classification of these fungi. In Japan, *A. muscaria* and *A. pantherina*, which are known as ectomycorrhizal fungi, are found in both conifer and hardwood forests, but *A. muscaria* is found especially near *Betula* trees (Imazeki and Hongo, 1987). The differentiation of these fungi might be related with host-range.

Relationships in the *A. vaginata* group *Amanita vaginata* group includes many apparently closely related taxa at species-level or lower (Bas, 1977). Members of this group are differentiated from each other mainly based on color of pileus. However, the different evaluations of this color have led to proposals of many different taxonomical treatments.

In this study, we analyzed three taxa of the *A. vaginata* group, variously known as *A. vaginata* var. *vaginata*, *A. vaginata* var. *fulva*, and *A. vaginata* var. *punctata* (Gilbert, 1940, 1941; Gillet, 1874; Imazeki and Hongo, 1987) or *A. vaginata*, *A. fulva* (Schaeff.) Fr., and *A. punctata* (Cleland & Cheel) Reid (Fraiture, 1993; Jenkins, 1977, 1986; Reid, 1980). Genetic distances among these taxa are large. Moreover, *A. ceciliae* LEM950069 is located among them. Therefore, we consider that these three taxa of the *A. vaginata* group should be treated as independent species.

Relationships among *A. hemibapha* subspecies Corner and Bas (1962) accepted a rather wide species concept of *A. hemibapha*, recognizing three subspecies within it according to differences in the coloration of the fruitbody and geographical distribution:

A. hemibapha subsp. *hemibapha*: Pileus crimson red, with yellow margin. Stipe yellow with reddish scales. Ring yellow. Distribution: Sri Lanka (as Ceylon).

A. hemibapha subsp. *similis* (Boed.) Corner & Bas (=*A. similis* Boed.): Pileus fuliginous-bistre to brownish olivaceous, with pinkish, yellowish or melleous margin. Stipe bright yellow to pale dingy yellow, with pinkish orange to concolorous scales or fibrils. Ring pinkish orange to grayish. Distribution: Java, Borneo, Singapore and Malaya.

A. hemibapha subsp. *javanica* Corner & Bas: Pileus orange-yellow to ochre yellow, sometimes with reddish brown tinge, with yellow margin. Stipe yellow with orange scales. Ring orange buff. Distribution: Java.

These concepts have been followed in Japan (Imazeki and Hongo, 1987). In this study, we analyzed two Japanese specimens per subspecies. The genetic distances within each subspecies are almost negligible, but genetic distances between subspecies are long, and specimens of intermediate genetic distances have not been found. Moreover, it is apparent that these three subspecies are sympatric in Japan. Based on the above evidence, we suggest that they should be treated as independent species: *Amanita hemibapha* (Berk. & Br.) Sacc., *Amanita similis* Boed., and *Amanita javanica* (Corner & Bas) Oda, Tanaka & Tsuda, comb. nov., respectively.

Amanita javanica (Corner & Bas) Oda, Tanaka & Tsuda, comb. nov.

Basionym: *Amanita hemibapha* (Berk. & Br.) Sacc. subsp. *javanica* Corner & Bas, Persoonia **2**: 297. 1962.

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Literature cited

Bas, C. 1969. Morphology and subdivision of *Amanita* and a monograph of its section *Lepidella*. Persoonia **5**: 285–579.

Bas, C. 1977. Species-concept in *Amanita* sect. *Vaginatae*. In: The species concept in Hymenomycetes. Biblio. Mycol. vol. 61, (ed. by Clémençon, H.), pp. 79–103. J. Cramer, Vaduz.

Benedict, R. G., Tyler, V. E., Jr. and Brady, L. R. 1966. Chemotaxonomic significance of isoxazole derivatives in *Amanita* species. Lloydia **29**: 333–342.

Beutler, J. A. and Der Marderosian, A. H. 1981. Chemical variation in *Amanita*. J. Natural Products **44**: 422–431.

Bresinsky, A. and Besl, H. 1990. A colour atlas of poisonous fungi. Wolfe Publ., London.

Chilton, W. S. and Ott, J. 1976. Toxic metabolites of *Amanita pantherina*, *A. cothurnata*, *A. muscaria* and other *Amanita* species. Lloydia **39**: 150–157.

Corner, E. J. H. and Bas, C. 1962. The genus *Amanita* in Singapore and Malaya. Persoonia **2**: 241–304.

Fraiture, A. 1993. Les amanitopsis d'Europe (Genre *Amanita*, Agaricales, Fungi) Synthèse critique de la littérature. Opera Bot. Belgi. **5**: 1–128.

Felsenstein, J. 1993. PHYLIP: Phylogeny interference package (ver 3.5c). Distributed by the author, Department of Genetics, Univ. Washington, Seattle.

Gilbert, E. J. 1940. Amanitaceae. In: Icon. mycol., vol. 27, pp. 1–200. Comitato Onoranze Bresadoliane, Milano.

Gilbert, E. J. 1941. Amanitaceae. In: Icon. mycol., vol. 27, pp. 201–427, pls. 1–73. Comitato Onoranze Bresadoliane, Milano.

Gillet, C. C. 1874. Les Hyménomycètes. Ch. Thomas, Alençon.

Hatanaka, S. I., Niimura, Y. and Takishima, K. 1985. Non-protein amino acids of unsaturated norleucine-type in *Amanita pseudoporphyria*. Trans. Mycol. Soc. Japan **26**: 61–68.

Hatanata, S. I., Furukawa, J., Aoki, T., Akatsuka, H. and Nagasawa, E. 1994. (2*S*)-2-amino-5-chloro-4-hydroxy-5-hexenoic acid, a new chloroamino acid, and related compounds from *Amanita gymnopus*. Mycoscience **35**: 391–394.

Hatanaka, S. I., Okada, K. and Nagasawa, E. 1995. Isolation and identification of (2*S*)-2-amino-5-chloro-4-hydroxy-5-hexenoic acid from an *Amanita* of the section *Roanokenses* (Amanitaceae). Mycoscience **36**: 395–397.

Hawksworth, D. L., Kirk, P. M., Sutton, B. C. and Pegler, D. N. 1995. Ainsworth & Bisby's dictionary of the fungi, 8th ed., p. 14. CAB International, Wallingford.

Imazeki, R. and Hongo, T. 1987. Colored illustrations of mushrooms of Japan, vol. 1. Hoikusha, Osaka. (In Japanese.)

Jenkins, D. T. 1977. A taxonomic and nomenclatural study of the genus *Amanita* section *Amanita* for North America. Biblio. Mycol., vol. 57. J. Cramer, Vaduz.

Jenkins, D. T. 1986. *Amanita* of North America. Mad River Press, Eureka, California.

Moser, M. 1983. Keys to agarics and boleti (Polyporales, Boletales, Agaricales, Russulales), pp. 224–229. Roger Phillips, London.

Nakada, M., Tanaka, C., Tsunewaki, K. and Tsuda, M. 1994. RFLP analysis for species separation in the genera *Bipolaris* and *Curvularia*. Mycoscience **35**: 271–278.

Ohta, T., Nakajima, S., Sato, Z., Aoki, T., Hatanaka, S. I. and Nozoe, S. 1986. Cyclopropylalanine, an antifungal amino acid of the mushroom *Amanita virgineoides* Bas. Chemistry Letters: 511–512.

Ohta, T., Nakajima, S., Hatanaka, S. I., Yamamoto, M., Shimmen, Y., Nishimura, S., Yamaizumi, Z. and Nozoe, S. 1987. A chlorohydrin amino acid from *Amanita abrupta*. Phytochemistry **26**: 565–566.

Reid, D. A. 1980. A monograph of the Australian species of *Amanita* Pers. ex Hook. (Fungi). Austral. J. Bot. Suppl. **8**: 1–97.

Saitou, N. and Nei, M. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4**: 406–425.

Sanger, F., Nicklen, S. and Coulson, A. R. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**: 5463–5467.

Singer, R. 1962. The Agaricales in modern taxonomy, 2nd ed., pp. 418–430. J. Cramer, Weinheim.

Singer, R. 1975. The Agaricales in modern taxonomy, 3rd ed., pp. 419–430. J. Cramer, Vaduz.

Singer, R. 1986. The Agaricales in modern taxonomy, 4th ed., pp. 442–453. Koeltz Sci., Koenigstein.

Thompson, J. D., Higgins, D. G. and Gibson, T. J. 1994. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22**: 4673–4680.

Van Nues, R. W., Rientjes, J. M. J., Van der Sande, C. A. F. M., Zerp, S. F., Sluiter, C., Venema, J., Planta, R. J. and Raué, H. A. 1994. Separate structural elements within internal transcribed spacer 1 of *Saccharomyces cerevisiae* precursor ribosomal RNA direct the formation of 17S and 26S rRNA. Nucleic Acids Res. **22**: 912–919.

White, T. J., Bruns, T., Lee, S. and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: PCR protocols: A guide to methods and applications, (ed. by Innis, M. A., Gelfand, D. H., Sninsky, J. J. and White, T. J.), pp. 315–322. Academic Press, San Diego.

Yang, Z. L. 1997. Die *Amanita*-Arten von Südwestchina. Biblio. Mycol., vol. 170. J. Cramer, Berlin.