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Phylogenetic Relationships of *Amanita* Species Based on ITS1-5.8S rDNA-ITS2 Region Sequences

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To determine the phylogenetic relatedness of *Amanita* species, internal transcribed spacers (ITSs) and the 5.8S ribosomal RNA gene were amplified by polymerase chain reaction and then sequenced according to the dideoxy chain termination method using an automatic DNA sequencer. The ITS region provided sufficient variability for phylogenetic analyses within the species. Analyses of the ITS sequence data by distance and parsimony methods revealed that the *Amanita* species are composed of three distinct groups whose main branch is strongly supported by bootstrap analysis. The Singerian system did not fully correspond to present phylogenetic results based on molecular data. The amyloid nature of the spores was still phylogenetically significant and the type of volva as well as the cap color were proved to be additional important characters in *Amanita* phylogenetics.

Key words: *Amanita*, ITSs, phylogeny, rDNA

The genus *Amanita* belongs to the Amanitaceae and is notorious for its poisonous members. In Europe, it is reported that more than 90% of all deaths resulting from mushroom poisoning have been caused by *A. phalloides* (13). In *Amanita*, there are the most poisonous of all mushrooms, the Death Cap (*A. phalloides*) and the Destroying Angel (*A. virosa*). There are also exquisitely flavored ones like *A. caesarea*, *A. calyptrata*, and *A. velosa*. The rest of *Amanita* come somewhere between these two extremes. *A. muscaria* and *A. pantherina* are hallucinogenic and/or poisonous but normally not fatal; *A. pachycolea* and *A. rubescens* are edible but rather doubtful, and some others are still of unknown edibility (13).

Most members of *Amanita* are mycorrhizal and, as a result, they are most common in the woods or near trees. *Amanita* has its greatest diversity in the temperate zone and about one hundred species are enumerated throughout the world (23). *Amanita* has a membranous, warty, powdery, or cottony universal veil and most *Amanita*, including the most dangerous ones, are also furnished with a partial veil which, upon breaking, often forms a skirt-like ring (annulus) near the top of the stalk. Those species without a partial veil were once placed in a separate genus, *Amanitopsis*. The other genus of agarics consistently equipped with a volva of the universal veil is *Pluteus*, which has pinkish spores and shows strongly and distinctly cyanophilic spores. This genus is phylogenetically close to *Amanita* (12).

Amanita is divided into two subgenera based on whether or not the spores are amyloid. The subgenus *Amanita* has inamyloid spores and the subgenus *Lepidella* has amyloid spores. These two subgenera are in turn subdivided according to the type of volva. If the universal veil is membranous (skin-like), a loose sack or cup is formed at the base of the stalk and the cap is usually bald or adorned with a volval patch. If the universal veil is friable, it manifests itself as a series of concentric scales or rings around the base of the stem. If the universal veil is friable or semi-friable, numerous pieces of tissue form warts, usually deposited on the cap, which are often washed off by rain. When the universal veil is semi-friable and interwoven with the base of the stalk, it will form a collar or free rim but not a true sac. In some Amanitas, such as *A. rubescens* and *A. silvicola*, a distinct volva is not formed at all (1).

In this study, some representative members of *Amanita* were used to infer their phylogenetic relationships and to determine whether they form a monophyletic group or not. Molecular techniques are becoming increasingly important as means to obtain characters for studying taxonomic and phylogenetic relationships of fungi. The analysis of DNA sequence

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data has been especially useful (4, 5, 26). Regions most commonly used for phylogenetic analyses are those coding for nuclear small subunit rRNAs, portions of nuclear rRNA genes and internal transcribed spacers that occur between coding regions for nuclear small and large subunit rRNAs including 5.8S rRNA genes. Noncoding portions of the internal transcribed spacer may have even greater variability than coding regions (26) and have been useful for distinguishing closely related species of fungi (2, 3, 6, 8, 9, 16, 17, 18, 22, 27, 28). For this study, sequence divergences of internal transcribed spacers (ITSs), bounded by 18S and 28S genes of the nuclear ribosomal DNA, were explored as a potential source of characters for phylogenetic studies at the species level of *Amanita*. The primary objective of this study was to sequence ITS regions and analyze phylogenetic relationships within *Amanita*, as well as outgroup isolates of a closely related species, *Pluteus petasatus*.

Materials and Methods

Strains and DNA extraction

The 14 strains used in this study are listed in Table 1. To extract total DNA from Petri dish-grown mycelia (15), cultures were maintained for 5~6 days on malt extract agar (MEA) plates which were covered with a sterile cellophane disc and inoculated with three mycelial plugs per plate. DNA extraction was done by Lecellier and Silar's rapid method for nucleic acids extraction (15). After complete incubation at 24°C, the mycelium was recovered by scraping with a sterile spatula and transferred into an Eppendorf tube. Then 750 μl extraction buffer [100 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 100 mM NaCl and 2% SDS] was added. The sample was vortexed for 30 sec, frozen for 20 sec in liquid nitrogen, and then completely thawed in a 70°C incubator. This process was repeated three or more times until thorough breakage. Extracted DNA was purified with a classical phenol-chloroform extraction and was precipitated with 1 volume of iso-propanol. The pellet was washed twice in 70% ethanol and allowed to air-dry and was resuspended in 50 μl of sterile TE (pH 8.0). Contaminated RNA was removed by treatment with DNase-free RNase *A* at a final concentration of 10 mg/ml for 10 min at 37°C and the remaining DNA was stored at 4°C.

PCR amplification and purification of DNA

Amplifications of two ITSs and their intervening 5.8S gene were performed using NS7 and ITS4 primers (26), *Vent* DNA Polymerase (New England Biolabs), and DynaZyme (Finnzymes Inc.). DNAs of *A. muscaria*, *A. citrina*, *A. hygroscopica* and *A. tenuifolia* were amplified in 20 mM Tris-HCl (pH 8.8), 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 0.1% Triton X-100, 2 mM $MgSO_4$, 0.2 mM of each dATP, dGTP, dCTP, and dTTP, 0.5 μM of each primer, 200 ng of template DNA and 1 unit of the *Vent* DNA Polymerase (New England Biolabs). Total volume was adjusted at 50 μl and 30 cycling reactions were performed in a programmable thermal controller with the following parameters; denature 1 min at 94°C, anneal 1 min at 50°C, extend 1 min 30 sec at 72°C, start the initial step 3 min at 94°C and increase 1 sec per cycle and, upon completion, hold at 4°C.

Reactions of other strains were carried out in 50 μl volume containing 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 0.2 mM

Table 1. *Amanita* species and sources used for phylogenetic analyses, GenBank accession numbers, and size variations of ITS base pairs

Species	Source	Accession numbers	ITS1	ITS2
A. muscaria 1	KCTC 6487 (IFO-8264)	AF085428	229	292
A. muscaria 2	KCTC 6498 (CBS 236.76)	AF085483	230	292
A. citrina 1	KCTC 6488 (IFO-8261)	AF085484	271	272
A. citrina 2	KCTC 6495 (CBS 441.91)	AF085485	280	273
A. rubescens	KCTC 6489 (IFO-8266)	AF085486	282	265
A. aspera	KCTC 6490 (IFO-8262)	AF085487	283	269
A. spissa	KCTC 6491 (IFO-8263)	AF085488	282	266
A. caesarea	KCTC 6493 (CBS 978.69)	AF085489	231	261
A. hygroscopica	KCTC 6686 (ATCC 26843)	AF085490	208	263
A. calyptroderma	KCTC 6494 (CBS 188.67)	AF085491	225	256
A. fulva	KCTC 6850 (CBS 921.72)	AF085492	247	273
A. rhoadsii	KCTC 6689 (ATCC 26845)	AF085493	208	260
A. tenuifolia	KCTC 6687 (ATCC 26764)	AF085494	223	262
Pluteus petasatus	KCTC 6872 (CBS 441.85)	AF085495	280	250

of each dATP, dGTP, dCTP, and dTTP, 0.5 μM of each primer (NS7, ITS4), DNA template and 1 unit of DynaZyme (Finnzymes Inc.). Sample DNAs were amplified using the following cycle parameters; denature 1 min at 94°C, anneal 1 min at 55°C, extend 1 min at 72°C, start the initial step 3 min at 94°C and set the final step 30 min at 72°C with 1 sec increased per cycle and, upon completion, hold at 4°C. PCR products were observed by electrophoresing 5 μl from each tube on 0.5% agarose gel containing EtBr in Tris-acetate EDTA (TAE) buffer. The presence of a single bright band on each lane was checked for a successful amplification. The amplified products were purified from 0.5% agarose gel using the GeneClean II kit (BIO 101). All purified DNAs were stored at -20°C until examined for cloning.

DNA cloning and sequencing

PCR products were cloned into T-vectors to secure high purity of DNA by digesting a Bluescript plasmid (KS+) with *Eco*RV and incubating with *Taq* polymerase (1 unit/μg plasmid/20 μl volume) under standard buffer conditions [50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.1% Triton X-100, 1.5 mM $MgCl_2$] in the presence of 2 mM dTTP for 2 hours at 70°C. After phenol extraction and precipitation, 10 ng of this T vector and 30 ng of the PCR product were mixed with 10× ligase buffer and 1 Weiss unit of T4 DNA ligase (Boehringer Mannheim). The mixture was incubated at 16°C for 4 hours and this ligated mixture was used for the transformation according to the method of Sambrook *et al.* (20). For *Vent* DNA polymerase, 50 ng of amplified DNA and 10 ng of Bluescript plasmid KS (+) digested with *Eco*RV were mixed with 10× ligase buffer and 2 Weiss units of T 4 DNA ligase (Boehringer Mannheim). Transformed DNAs were extracted by the alkaline lysis method and used for DNA sequencing. The dideoxy chain termination method (21) and the ALFexpress DNA sequencer (Pharmacia Biotech) were used for DNA sequencing using internal PCR primers (ITS5, ITS4) designed by White *et al.* (26).

DNA data analyses

The sequence of *Amanita muscaria* 3 accessed from GenBank (accession number: Z54294) was retrieved for comparison and included in phylogenetic analyses of the fourteen strains used in this study. Complete sequences were globally aligned by an alignment algorithm CLUSTAL W (25). The multiple alignment was visually optimized and gaps were treated as missing data to submit to distance and parsimony analyses. For distance analysis, the routine DNADIST in PHYLIP version 3.5 (11) was used to obtain a matrix of Kimura's two parameter distance (14), and then analyzed by the routine NEIGHBOR based on Saitou and Nei's neighbor-joining method (19). For parsimony analysis, the heuristic search option of PAUP version 3. 1.1 (24) was used to compare both branching topologies and branch lengths. The strength of internal branches on resulting trees were statistically tested by the bootstrap analysis (10) of 200 replications.

Results and Discussions

Amplified PCR products were about 950 bp long in size on average and ITS1-5.8S rDNA-ITS2 sequences ranged from 573 bp in *A. rhoadsii* to 658 bp in *A. citrina* 2. Sequence variations were evident in ITS1 with a few regions of complete homology throughout the optimized alignment, whereas sequences were rather conserved in ITS2 with more regions of complete homology interspersed among variable regions. These sequence and length variations provided sufficient characters for phylogenetic analyses (7).

When Hibbett analyzed gilled mushrooms using nuc SSU rDNA and mt SSU rDNA sequences (12), *Pluteus petasatus* was clearly placed outside the Amanitaceae and thus *P. petasatus* was used as an outgroup in phylogenetic analyses of 14 Amanitas in this study. Aligned sequences of ITS regions from compared strains contained 717 nucleotides and had a total of 545 variable sites and 354 informative sites. The distance analysis based on the neighbor-joining method produced a phylogenetic

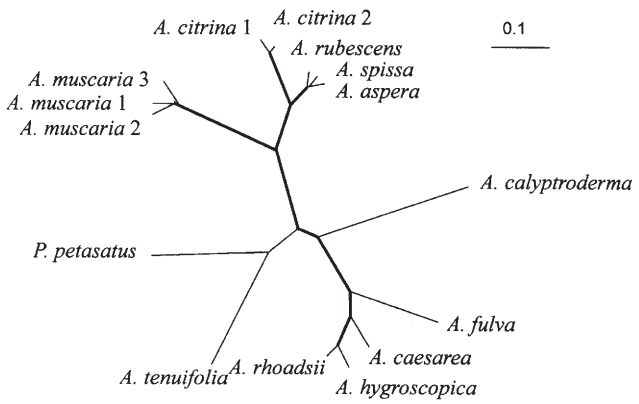

Fig. 1. Phylogenetic tree indicating relationships of *Amanita* species based on sequences of ITS1-5.8S rDNA-ITS2. This tree was created by the neighbor-joining method based on the matrix of Kimura's two parameter distance. This tree was statistically tested by the bootstrap analysis of 200 replications. The bold line indicates branches significantly supported by more than 95%. The scale bar indicates 0.1 unit of computed distance values.

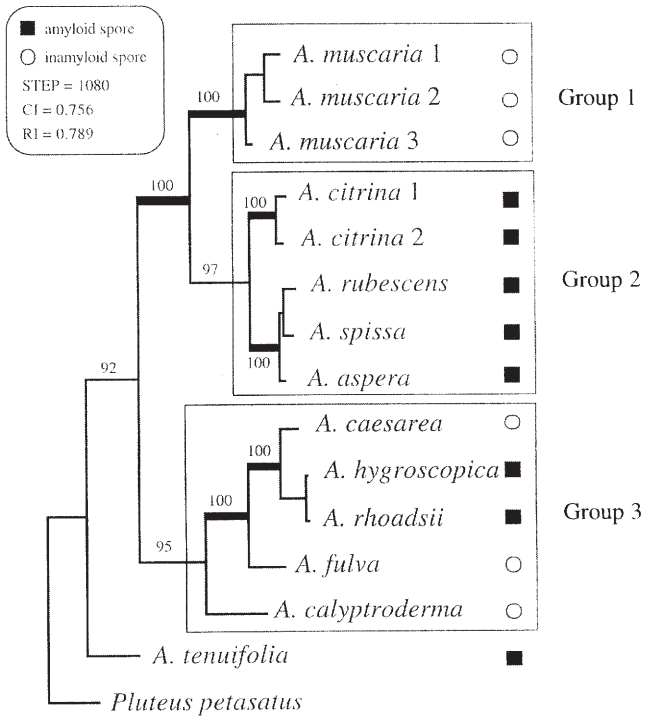

Fig. 2. Phylogenetic tree based on ITS region sequences. A single most parsimonious tree was obtained using the stepwise addition option of the heuristic method of PAUP 3.1.1. Bootstrap values were obtained from 200 replications and given above branches supported by more than 90 %.

tree presented in Fig 1. The parsimony analysis produced a single most parsimonious tree of 1080 steps, with consistency (CI) and retention (RI) indices of 0.756 and 0.789, respectively (Fig. 2). When tree topologies of both Figs. were compared, they were very similar without any significant differences.

Judging from present sequence-based phylogenetic trees, *Amanita* appears to be a monophyletic group. *Amanita* species based on ITS1-5.8S rDNA-ITS2 sequences are clustered into 3 groups, except for *Amanita tenuifolia*. Group 1 includes strains of *A. muscaria*, Group 2 contains *A. citrina, A. rubescens, A. aspera* and *A spissa* and Group 3 consists of *A. caesarea, A. hygroscopica, A. rhoadsii, A. fulva* and *A. calyptroderma (Figs. 1 and 2). Such relationships were supported quite strongly by the bootstrap analysis and matched with, to a certain degree, length variations of ITS1 (Table 1). Unlike gene coding regions, length mutations have been quite common in ITS1 and ITS2 regions (16). Especially the length mutation in ITS1 used to be superior to ITS2. Phylogenetic analyses showed that the majority of Amanita* species were clustered into one major clade by the main branch supported by 92% bootstrap values (Fig. 2), *A. tenuifolia* was the most divergent of the *Amanita* species and, along with *Pluteus petasatus*, was placed right outside the major *Amanita* clade.

The classification of *Amanita* defined by Singer did not fully correlate with phylogenetic analyses of the present sequence data (Fig. 2). According to the Singerian system, Group 1 belongs to the subgenus *Amanita* and Group 2 to the subgenus *Lepidella*, but members of Group 3 belong to both *Amanita* and *Lepidella*. As *Amanita* is divided into two subgenera based on amyloidity (13, 23), the amyloid nature of spores is still important as one of most special characters. Present results suggest that amyloidity alone is not a strong key character to separate subgenera of this genus. In Fig. 2, Group 3 includes species of both amyloid spores and inamyloid spores. In addition to the spore amyloidity, the type of volva is another important character in *Amanita*. Phylogenetic analyses show that the volva type fully correlates with the result of the phylogenetic tree shown in Fig. 2. *Amanita muscaria* of Group 1 has a collarlike or scaly volva and Group 3 has a saclike volva. But in Group 2, a distinct volva is not formed. Another important character, judging from Groups of phylogenetic trees, is the cap color. *A. muscaria* of Group 1 has an orange-red cap, *A. citrina* of Group 2 has a pale greenish-yellow cap and the rest of Group 2 displays a brownish color on the cap. Species of Group 3 have a yellowish or whitish color on the cap. Many other characters once used in classifying *Amanita* species seemed not to be useful in explaining the results of this study.

The importance of morphological characters is still a question in estimating how much they reflect the true phylogeny of *Amanita*. Present molecular data say that some morphological characters used to define subgenera of *Amanita* are subject to homoplasy and are rather inadequate in resolving phylogenetic relationships. But, as the phylogenetic tree is constructed from ITS regions, the inferred tree is a gene tree, which can be different from the species tree (19). To improve taxonomic and phylogenetic interpretations and conclusions, more divergent genes or spacer regions should be searched and compared.

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