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The molecular diversity of toxin gene families in lethal *Amanita* mushrooms

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ABSTRACT

Mushrooms in lethal *Amanita* species are responsible for a large number of food poisoning cases and deaths. However, the diversity of the toxins in these mushrooms remains largely unknown. This study analyzed the gene families of toxins found in 6 lethal *Amanitae* from Asia and Europe. Fifty-four gene sequences were obtained, accounting for 70.1% of the known MSDIN family members. Of the 54 gene sequences, 20 encode α-amanitin, 5 encode β-amanitin, 16 encode phallacidin, and 13 encode peptides of unknown functions. Bayesian analysis of MSDIN family members identified differences in the number of toxin genes in different toxin families among the *Amanita* species. Ten of the 13 peptides of unknown functions were closely related to known phallotoxins, while the remaining 3 were similar to amatoxins. The *α-AMA* tree indicated that there were significant differences between the *Amanita* and *Galerina* species. However, both the *α-AMA* and *PHA* trees showed that these toxin genes have similar upstream and downstream motif sequences among the *Amanita* species. This study greatly enriches the available diversity information regarding toxin gene families in lethal *Amanita* mushrooms, and could lay a strong foundation for further research about the evolution of *Amanita* toxin genes.

1. Introduction

The genus *Amanita* is rich in species diversity, containing about 500 known species, including some of the most toxic mushrooms found worldwide (Kirk et al., 2008). Based on its morphological and anatomical characteristics, the genus *Amanita* was divided into 2 subgenera: *Amanita* and *Lepidella* (E. –J. Gilbert) Veselý, including 7 subsections: *Amanita*, *Caesareae* Singer, *Vaginatae* (Fr.) Quél.; *Amidella* (E. –J. Gilbert) Konrad & Maubl.; *Lepidella*, *Phalloideae* (Fr.) Quél.; and *Validea* (Fr.) Quél. (Yang, 1997, 2005). The notoriously lethal *Amanita* species were classified into the section *Phalloideae*. These species contained the most powerful toxins, known as cyclopeptides, which are responsible for approximately 90% of the fatalities that result from mushroom poisoning (Bresinsky and Besl, 1990). Amatoxins are considered to be the major toxins responsible for human poisonings, being 10–20 times more toxic than phallotoxins and virotoxins (Li and Oberlies, 2005). In Central Europe and North America, the destroying angels [*Amanita bisporigera* G.F. Atk., *Amanita subalbiacea* (Murrill), *Amanita verna* (Bull. : Fr.) Lam., *Amanita virosa* (Fr.) Bertill., and their allied species] and the death cap (*Amanita phalloides* (Fr.) Link) have caused serious human and animal poisoning issues (Bresinsky and Besl, 1985; Wieland, 1973, 1986). Concurrently, serious mushroom poisoning cases have usually been caused by other *Amanita* species in East Asia, such as

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Table 1
Taxa and genes included in DNA analyses.

Taxa	Species no.	Geographic origin	GenBank accession no.		
			ITS	AMA	PHA
A. bisporigera G.F. Atk.	MSC380551	USA	AY325827	EU196139	EU196142
A. bisporigera	JMP0001	USA	EU819411	_	_
A. exitialis Zhu L. Yang & T. H. Li	GDGM 40051	Guangdong, China	[a]KC755037	[a]KF546278 (AeAMA2)	[a]KF546293 (AePHA1)
A. exitialis	GDGM 43081	Guangdong, China	[a]KF535948	[a]KF546279 (AeAMA3)	[a]KF546294 (AePHA2)
A. exitialis	GDGM 43156	Guangdong, China	[a]KF535949	[a]KF546280 (AeAMA4)	[a]KF546295 (AePHA3)
A. fuliginea Hongo	GDGM26997	Guangdong, China	[a]KF156782	[a]KF546281 (AfAMA2)	[a]KF546296 (AfPHA1)
A. fuliginea	HKAS 77342	Guangdong, China	KF479045	[a]KF546282 (AfAMA3)	[a]KF546297 (AfPHA2)
A. fuligineoides P. Zhang & Zhu L. Yang	GDGM43231	Guangdong, China	[a]KC755030	[a]KF546283 (AfsAMA2)	[a]KF546298 (AfsPHA1)
A.fuligineoides	HKAS 52727	Hunan, China	JX998024	[a]KF546284 (AfsAMA3)	[a]KF546299 (AfsPHA2)
A. manginiana sensu W. F. Chiu	HKAS38460	Yunnan, China	AY436463	_	_
A. pallidorosea P. Zhang & Zhu L. Yang	GDGM43306	Shandong, China	[a]KC755033	[a]KF546285 (ApaAMA2)	[a]KF546300 (ApaPHA2)
A. pallidorosea	GDGM40041	Shandong, China	[a]KF535947	[a]KF546286 (ApaAMA3)	[a]KF546301 (ApaPHA3)
A. pallidorosea	HKAS75786	Gansu, China	JX998037	[a]KF546287 (ApaAMA4)	[a]KF546302 (ApaPHA4)
A. phalloides Secr.	GDGM 40312	Castello, Italy	[a]KC755034	[a]KF546288 (ApAMA1)	[a]KF546303 (ApPHA1)
A.phalloides	GDGM 40043	Lazio, Rome, Italy	[a]KF535946	[a]KF546289 (ApAMA2)	[a]KF546304 (ApPHA2)
A. rimosa P. Zhang & Zhu L. Yang	GDGM40046	Guangdong, China	[a]KC755036	[a]KF546290 (ArAMA2)	[a]KF546305 (ArPHA1)
A. rimosa	GDGM43381	Guangdong, China	[a]KF535945	[a]KF546291 (ArAMA3)	[a]KF546306 (ArPHA2)
A. rimosa	HKAS 77120	Jiangxi, China	KF479044	[a]KF546292 (ArAMA4)	[a]KF546307 (ArPHA3)
A. virosa (Fr.) Bertillon	HKAS 50912	Jilin, China	FJ176737	_	_
A. virosa	HKAS 55298	Czech Republic	FJ755188	_	_
G. marginata	_	USA		JN827311 (GmAMA1-1)	_
G. marginata	_	USA		JN827312 (GmAMA1-2)	_

[a] The sequences were obtained in this study, which have been submitted to GenBank, but unpublished. Others sequences were from the GenBank. The names in the bracket were the abbreviation of the gene names.

by *Amanita exitialis* Zhu L. Yang & T. H. Li (Deng et al., 2011; Yang and Li, 2001), *Amanita subjunquillea* var. *alba* Zhu L. Yang (Kawase et al., 1992), *Amanita fuliginea* Hongo (Zhang et al., 2002), *Amanita pallidorosea* (Cao et al., 2011), *Amanita rimosa* P. Zhang & Zhu L. Yang, and *Amanita fuligineoides* P. Zhang & Zhu L. Yang (Chen et al., 2013; Zhang et al., 2010).

Previous researchers have discussed the distribution features and phylogenetic relationships of lethal *Amanita* species from East Asia, Europe, and North America (Zhang et al., 2010). The results of High Performance Liquid Chromatography (HPLC) showed that *A. exitialis*, *A. fuliginea*, *A. pallidorosea*, and some of the other *Amanita* species produce various toxic peptides, which contain potent toxins (Chen et al., 2003; Deng et al., 2011; Hu et al., 2012; Wang et al., 2011). Studies of *Amanita* toxin genes have reported about 3 *Amanita* species (*A. bisporigera*, *A. phalloides*, and *Amanita ocreata*) and 1 *Galerina* species (*Galerina marginata*). These studies have demonstrated that α-amanitin and phallacidin were synthesized in ribosomes; that the proproteins of α-amanitin and phallacidin were composed of 35 and 34 amino acids, respectively; and that at least 13 MSDIN family members have been found in *A. bisporigera* (Hallen et al., 2007; Luo et al., 2012). However, the toxin gene families of some lethal *Amanita* species in Asia have not been investigated.

This study looks at the toxin genes and MSDIN family members that were cloned from 6 lethal *Amanita* species: *A. exitialis*, *A. fuliginea*, *A. fuligineoides*, *A. pallidorosea*, *A. phalloides*, and *A. rimosa*. Our research details and analyzes the diversities, sequence features, and phylogenetic relationships of MSDIN family members, and the phylogenetic relationships of 2 major toxin-encoding genes (*α-AMA* and *PHA*). Finally, we discuss implications of the results, which are based on the associations between the toxin genes and the *Amanita*'s taxonomy.

2. Materials and methods

2.1. Fungal collections

We collected the fruiting bodies of *A. exitialis*, *A. fuliginea*, *A. fuligineoides*, *A. rimosa* from April 2007–June 2013 in Guangdong, China, and harvested *A. pallidorosea* in August 2011 and 2012 from Shandong, China. The *A. phalloides* (Fr.) Link was collected in November 2007 and September 2010 from Pagliano and Rome, Italy. These collections were deposited in the Fungal Herbarium of the Guangdong Institute of Microbiology (GDGM); the 4 collections (HKAS52727, 75786, 77120, 77342) were deposited in the Cryptogamic Herbarium of Kunming Institute of Botany, at the Chinese Academy of Science (HKAS). The geographic origins are listed in Table 1.

2.2. DNA extraction, PCR amplification, and sequencing of ITS regions

We obtained the total genomic DNA from fresh and dried species, using a modified CTAB procedure (Doyle and Doyle, 1987). The primers ITS1 and ITS4 were used for amplification of the ITS region (White et al., 1990). Polymrease chain reaction (PCR) were performed with an Eppendorf Mastercycler thermal cycler (Eppendorf Inc., Germany) in 25 μL reaction mixtures, containing 1× PCR buffer, 1.5 mM $MgCl_2$, 0.5 mM dNTP mix, and 0.5 μM of each primer, 2 U of Taq polymerase, and 0.5 μL DNA template. Cycling conditions were set as follows: initial denaturation at 94 °C for 4 min, followed by 33 cycles of denaturation at 94 °C for 40 s, annealing at 51 °C for 30 s,