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Cyclopeptide toxins of lethal amanitas: Compositions, distribution and phylogenetic implication

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ABSTRACT

Lethal amanitas (*Amanita* sect. *Phalloideae*) are responsible for 90% of all fatal mushroom poisonings. Since 2000, more than ten new lethal *Amanita* species have been discovered and some of them had caused severe mushroom poisonings in China. However, the contents and distribution of cyclopeptides in these lethal mushrooms remain poorly known. In this study, the diversity of major cyclopeptide toxins in seven *Amanita* species from Eastern Asia and three species from Europe and North America were systematically analyzed, and a new approach to inferring phylogenetic relationships using cyclopeptide profile was evaluated for the first time. The results showed that there were diversities of the cyclopeptides among lethal *Amanita* species, and cyclopeptides from *Amanita rimosa* and *Amanita fuligineoides* were reported for the first time. The amounts of amatoxins in East Asian *Amanita* species were significantly higher than those in European and North American species. The analysis of distribution of amatoxins and phallotoxins in various *Amanita* species demonstrated that the content of phallotoxins was higher than that of amatoxins in *Amanita phalloides* and *Amanita virosa*. In contrast, the content of phallotoxins was significantly lower than that of amatoxins in all East Asian lethal *Amanita* species tested. However, the distribution of amatoxins and phallotoxins in different tissues showed the same tendency. Eight cyclopeptides and three unknown compounds were identified using cyclopeptide standards and high-resolution MS. Based on the cyclopeptide profiles, phylogenetic relationships of lethal amanitas were inferred through a dendrogram generated by UPGMA method. The results showed high similarity to the phylogeny established previously based on the multi-locus DNA sequences.

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1. Introduction

The genus *Amanita* is a cosmopolitan genus comprising about 500 described species, which occur worldwide (Kirk et al., 2008). This genus is important to humans as it contains many deadly poisonous species. The poisoning by *Amanita* mushrooms can be classified into three categories: neurotoxicity, nephrotoxicity and hepatotoxicity according to the syndromic classification (Diaz, 2005). Neurotoxic *Amanita* species such as *Amanita muscaria*, *Amanita pantherina* contain ibotenic acid and muscimol, which cause hallucinogenic effects (Benjamin, 1992; Michelot and Melendez-Howell, 2003). Nephrotoxic *Amanita* species such as *Amanita smithiana*, *Amanita proxima*, and *Amanita pseudoporphyria* can induce acute renal failure, the responsible toxin for which is likely to be 2-amino-4,5-hexadienoic acid (Saviuc and Danel, 2006; Kirchmair et al., 2012). Hepatotoxic *Amanita* species, such as *Amanita phalloides*, *Amanita verna* and *Amanita virosa* in Europe and North America, and *Amanita exitialis*, *Amanita fuliginea* and *Amanita subjunquillea* in East Asia, contain cyclopeptides and are responsible for the liver failure and death of humans. These cyclopeptide-containing *Amanita* species account for over 90% of all fatal mushroom poisonings worldwide (Karlson-Stiber and Persson, 2003; Berger and Guss, 2005; Chen et al., 2014).

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The lethal amanitas are a group of cyclopeptide-containing mushrooms of *Amanita* sect. *Phalloideae*, comprising ca. 37 described species worldwide with majority of them distributed in the Northern Hemisphere (Cai et al., 2014). In Europe and North America, the lethal *Amanita* species include *A. phalloides*, *A. verna*, *A. virosa*, *Amanita ocreata, Amanita bisporigera*, *Amanita suballiacea, Amanita tenuifolia,* and *A. phalloides* var. *alba*, which have caused human deaths (Enjalbert et al., 2002; Karlson-Stiber and Persson, 2003; Kaya, et al., 2013). Some of these European and North American poisonous *Amanita* species, such as *A. phalloides* and *A. verna*, were also reported from East Asia (Imazeki et al., 1988; Mao, 2006). Recent comprehensive taxonomic studies demonstrated that these earlier identifications were incorrect (Yang, 2000, 2005). On the other hand, many new lethal *Amanita* taxa, endemic to East Asia, such as *A. subjunquillea* S. Imai (Imai, 1933), *A. fuliginea* Hongo (Hongo, 1953), *A. subjunquillea* var. *alba* Zhu L. Yang (Yang, 1997), *A. exitialis* Zhu L. Yang & T. H. Li (Yang and Li, 2001), *Amanita fuligineoides* P. Zhang & Zhu L. Yang, *Amanita rimosa* P. Zhang & Zhu L. Yang, *Amanita pallidorosea* P. Zhang & Zhu L. Yang (Zhang et al., 2010) and *Amanita subpallidorosea* Hai J. Li (Li et al., 2015) have been described from China and Japan. Recently, 28 phylogenetic species were recognized by multi-locus phylogenetic analyses assisted with morphological studies, 14 of which represented putatively new species (Cai et al., 2014). Many of these Eastern Asian lethal *Amanita* species have caused serious mushroom poisonings in China (Yang and Li, 2001; Chen et al., 2014; Cao et al., 2011; Li et al., 2015).

Up to now, ca. twenty-two cyclopeptide toxins from *Amanita* species have been identified, they are classified into three major groups: amatoxins (bicyclic octapeptides), phallotoxins (bicyclic heptapeptides) and virotoxins (monocyclic heptapeptides) (Wieland, 1986; Li and Oberlies, 2005; Clarke et al., 2012). Amatoxins are responsible for fatal human poisonings through inhibiting RNA polymerase II, subsequently blocking the synthesis of proteins, and eventually leading to cell death (Wieland, 1986). The detailed mode of action and the crystal structure of α-amanitin have been demonstrated (Nguyen et al., 1996; Wang et al., 2006; Kaplan et al., 2008). Phallotoxins and virotoxins do not play a role in human poisonings for they are not absorbed by intestinal cells, although they can cause death of mice and rats within 1–2 h after interperitoneal administration (Karlson-Stiber and Persson, 2003; Li and Oberlies, 2005). The phallotoxins specifically bind to F-actin, thus strongly stabilizing the structure of the assembled filaments (Wieland, 1986).

The contents and compositions of cyclopeptides in leathal amanitas are variable among different species. Amatoxins and phallotoxins were firstly isolated from *A. phalloides*, whereas virotoxins were first isolated in *A. virosa* (Faulstich et al., 1980; Wieland, 1986). In the early years, the quantities of amatoxins and phallotoxins in various cyclopeptide-containing mushrooms including *A. phalloides*, *A. verna*, *A. virosa*, *A. ocreata, A. bisporigera*, *Galerina marginata, Lepiota brunneoincarnata* were determined by various chromatographic methods such as column chromatography, thin-layer chromatography (TLC) or high-performance TLC (HPTLC), coupled with spectrophotometric or colorimetric method for the identification of toxic compounds (Wieland, 1986). Enjalbert et al. (1992) established a high performance liquid chromatographic (HPLC) method combined with UV absorbance, which allows simultaneous determination of up to eight amatoxins and phallotoxins. Later, HPLC method combined with UV absorbance or mass spectrometry was widely applied to various *Amanita* mushrooms, including European and North American species such as *A. phalloides* (Enjalbert et al., 1993a,b, 1996, 1999; Sgambelluri et al., 2014; Kaya et al., 2015), *A. phalloides* var. *alba* (Kaya et al., 2013), *A. bisporigera* (Mcknight et al., 2010) and *A. virosa* (Ahmed et al., 2010; Sgambelluri et al., 2014), and the Eastern Asian species such as *A. exitialis* (Hu et al., 2012), *A. subjunquillea* (Bao et al., 2005), *A. fuliginea* (Chen et al., 2003) and *A. pallidorosea* (Wang et al., 2011). However, most of these studies focused on a small subset of the cyclopeptides, mainly α-, β-amanitin and/or phalloidin. Furthermore, the toxin profiles obtained in different laboratories lacked comparability, largely due to differences among extraction methods of toxins and the conditions of HPLC. In addition, the cyclopeptide toxins of the lethal *A. fuligineoides* and *A. rimosa* mushrooms remain unknown.

The objectives of this study are: (i) to analyze the composition and distribution of major cyclopeptide toxins of the lethal amanitas from Eastern Asia, Europe and North America by means of HPLC, (ii) to identify cyclopeptides in lethal amanitas by mass spectrometry, and (iii) to assess their phylogenetic relationships based on the cyclopeptide profiles.

2. Materials and methods

2.1. Mushroom collection and sample conditions

Twenty-one *Amanita* samples collected from East Asia, Europe and North America were included in this study. The samples determined in this study were deposited in Mycological Herbarium of Hunan Normal University (MHHNU), the Cryptogamic Herbarium of Kunming Institute of Botany, Chinese Academy of Sciences (HKAS) and Mycological Herbarium of Guangdong Institute of Microbiology (GDGM). Information on the collections and their ITS accession number in GenBank are listed in Table 1 and Fig. 1. According to the sample conditions, the tissues used for toxin composition study varied. When available, fully developed, complete *Amanita* carpophores were used, except for *A. bisporigera*, *A. phalloides*, *A. virosa*, *A. fuligineoides* and *A. subjunquillea*, in which cases only small sections of pileus were available. For analysis of toxin composition in different tissues, the carpophores of *A. exitialis*, *A. fuliginea*, *A. pallidorosea* and *A. rimosa* were divided into three parts: pileus, stipe and volva.

2.2. Mushroom identification and phylogenetic analysis based on ITS sequences

All *Amanita* species were identified by both morphological and molecular phylogenetic evidence. DNA extraction, and PCR amplification, sequencing and alignment of ITS sequences followed Zhang et al. (2010) and Cai et al. (2014). The phylogenetic tree was generated with both maximum likelihood (ML) (Felsenstein, 1981) and Bayesian methods (Ronquist and Huelsenbeck, 2003) with the parameters employed in Cai et al. (2014), except that the runs of Bayesian inference were performed for 10 million generations.

2.3. Extraction of the cyclopeptide toxins

All samples were dried to constant weight and then ground by hand with a mortar and pestle. For each sample, 0.05 g ground materials was weighed, dispensed accurately into individual test tube, and extracted with 1 mL of 50% methanol (prepared with doubly distilled water) at 25 °C. The test tubes were placed in a rotary shaker with a radius of 13 mm and shaken at 150 rpm for 12 h. Following centrifugation at 4000 × g for 15 min, the supernatant was decanted and retained while the residue was resuspended in 1 mL of 50% methanol and extracted again as described above. The two supernatants were combined, freeze-dried, dissolved in 1 mL of doubly distilled water, and filtered through a micropore filter membrane (0.22 μm) for HPLC analysis. The extraction was done in triplicate for each sample and the mean value was determined.

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