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 *A. rimosa* and *A. 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 *A. phalloides* and *A. 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.

Keywords: *Amanita*, Amatoxins, Phallotoxins, Gene family, Phylogeny, HPLC

Highlights

- The major cyclopeptides in lethal amanitas were identified using cyclopeptide standards and high-resolution MS method.
- The composition and distribution of amatoxins and phallotoxins of the lethal amanitas from Eastern Asia, Europe and North America were compared.
- The dendrogram based on cyclopeptide chromatographic profiles was similar to the phylogenetic tree based on ITS and multi-locus DNA sequences.

1. Introduction

The genus *Amanita* is a cosmopolitan genus comprising about 600 described species, which occur worldwide (Yang et al., 2016). 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 *A. muscaria*, *A. pantherina* contain ibotenic acid and muscimol, which cause hallucinogenic effects (Benjamin, 1992; Michelot and Melendez-Howell, 2003). Nephrotoxic *Amanita* species such as *A. smithiana*, *A. proxima*, and *A. pseudoporphyria* can induce acute renal failure, the responsible toxin for which is likely to be 2-amino-4,5-hexadienoic acid (Saviuc & Danel, 2006; Kirchmair et al., 2012). Hepatotoxic *Amanita* species, such as *A. phalloides*, *A. verna* and *A. virosa* in Europe and North America, and *A. exitialis*, *A. fuliginea* and *A. 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).

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*, *A. ocreata*, *A. bisporigera*, *A. suballiacea*, *A. tenuifolia*, and *A. phalloides* var. *alba*, which have caused human deaths (Enjalbert et al., 2002; Karlson-Stiber and Persson, 2003; Kaya, et al., 2013a). 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), *A. fuligineoides* P. Zhang & Zhu L. Yang, *A. rimosa* P. Zhang & Zhu L. Yang, *A. pallidorozea* P. Zhang & Zhu L. Yang (Zhang et al., 2010) and *A. subpallidorozea* 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 & 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 lethal 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., 1993, 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. pallidorozea* (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.

Table 1. List of samples analyzed in this study.

Scientific Name	Species no.	Geographic origin	Collection time	ITS GenBank accession No.
<i>A. bisporigera</i> G. F. Atk.	MHHNU7224	Hamilton, Canada	2009. 8.26	KU311692
<i>A. exitialis</i> Zhu L. Yang & T. H. Li	MHHNU30297	Guangzhou, Guangdong	2009. 3.21	KT003192
<i>A. exitialis</i>	MHHNU30298	Guangzhou, Guangdong	2009. 3. 22	KR996716
<i>A. exitialis</i>	MHHNU 30937	Shenzhen, Guangdong	2014.3.26	KR996717
<i>A. fuliginea</i> Hongo	MHHNU 30300	Xingtian, Hunan	2009. 6. 10	KT003190
<i>A. fuliginea</i>	MHHNU 30207	Ningxiang, Hunan	2003.6.30	KR996718
<i>A. fuliginea</i> ZP-1453	MHHNU8953	Youxian, Hunan	2014.6.11	KR870315
<i>A. fuligineoides</i> P. Zhang & Zhu L. Yang	HKAS 52316	Mangshan, Hunan	2007.9.6	FJ176721
<i>A. oberwinklerana</i> Zhu L. Yang & Yoshim. Doi	MHHNU 7726	Hupingshan, Hunan	2012.9.8	KU311694
<i>A. oberwinklerana</i>	MHHNU 30819	Guangzhou, Guangdong	2013.5.28	KT003191
<i>A. pallidrosea</i> P. Zhang & Zhu L. Yang	MHHNU 8112	Yanhe, Guizhou	2014.7.18	KU311697
<i>A. pallidrosea</i>	MHHNU 7354	Hupingshan, Hunan	2010.8.31	KU311693
<i>A. phalloides</i> (Fr.) Link.	HKAS 31457	Tubingen, Germany	1994. 9.10	KT239525
<i>A. phalloides</i>	GDGM41101	Lazio, Rome, Italy	2014.10.6	KT003193
<i>A. rimosa</i> P. Zhang & Zhu L. Yang	MHHNU 8041	Ningxiang, Hunan	2014.6.27	KU311696
<i>A. rimosa</i>	MHHNU 7954	Youxian, Hunan	2014.6.11	KU311695
<i>A. subjunquillea</i> S. Imai	MHHNU 7049	Shennongjia, Hubei	2006.7.29	FJ176731
<i>A. subjunquillea</i>	MHHNU 7751	Hupingshan, Hunan	2012.9.10	KR996715
<i>A. virosa</i> (Fr.) Bertillon	HKAS84859	Germany	2009. 9.20	KR862367
<i>A. virosa</i>	HKAS56694	Finland	2009.10.16	JX998030

2. 2. 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 $4000 \times g$ 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 ($0.22 \mu\text{m}$) for HPLC analysis. The extraction was done in triplicate for each sample and the mean value was determined.

Fig. 1. Basidiomata of *Amanita* species **A.** *A. bisporigera* (MHHNU 7224); **B.** *A. exitialis* (MHHNU 30297); **C.** *A. subjunquillea* (MHHNU 7049); **D.** *A. pallidorosea* (MHHNU 8112); **E.** *A. fuliginea* (MHHNU 30300); **F.** *A. fuligineoides* (HKAS 52316); **G.** *A. rimosa* (MHHNU 8041); **H.** *A. oberwinklerana* (MHHNU 7726); **I.** *A. virosa* (photos A, C, F, G, H by Ping Zhang; photos B, D, E by Zuohong Chen; photo I by Zhu L. Yang).

2. 3. Standard samples and linear regression equation

Standard samples of α -amanitin (α -AMA, purchased from Merck-Calbiochem, Merck KGaA, Darmstadt, Germany), β -amanitin (β -AMA, Sigma-Aldrich, St. Louis, MO USA), phallisin (PSC, Sigma-Aldrich), phalloidin (PCD, Sigma-Aldrich) and phalloidin (PHD, Sigma-Aldrich) were dissolved in doubly distilled water to a concentration of 0.1 mg mL^{-1} . A five-point curve was obtained with injections of 5, 10, 15, 20 and 30 μL and the linear regression equation and correlation coefficient were calculated. The correlation regression coefficients of each curves were greater than 0.999. α -AMA, β -AMA, PSC, PCD and PHD in the samples were identified by comparison of retention times and co-injection with standards. Concentrations were calculated using peak areas of reference compounds.

2. 4. HPLC analysis

Cyclopeptides were analyzed by reverse phase HPLC using a Waters Delta 600 HPLC system (Waters Inc., Milford, Massachusetts, USA) coupled with a Waters 2487 UV detector. Separation was carried out on a Sinochrom ODS-BP reverse phase HPLC column ($250 \text{ mm} \times 4.6 \text{ mm}$, particle size $10 \mu\text{m}$; Danlian Elite Analytical Instrument, Dalian, China) at 40°C . The absorbance of the eluates was monitored at 295 nm. The mobile phase and elution profile were analyzed as described by Enjalbert et al. (1992) and Zhang et al. (2005). The mobile phase comprised (A) 0.02 mol L^{-1} aqueous ammonium acetate/acetonitrile (90 : 10 v/v) and (B) 0.02 mol L^{-1} aqueous ammonium acetate/acetonitrile (76 : 24 v/v), both adjusted to pH 5 with glacial acetic acid. All solutions were degassed by sonication prior to use. The elution profile was as follows: (1) 0–15 min, 100–95% A, 0–5% B; (2) 15–35 min, 95–20% A, 5–80% B, then held for 5 min; (3) 40–45 min, 20–0% A, 80–100% B, then held for 5 min; (4) 50–60 min, 0–100% A, 100–0% B. The mobile phase flow rate was 1 mL min^{-1} and the sample injection

volume was 20 μL . The peak eluates were collected for MS analysis.

2. 5. Mass spectrometry analysis (LC-MS)

Liquid chromatography method: Waters Acquity UPLC, Waters BEH C18 LC column, 2.1×100 mm, $1.7 \mu\text{m}$; mobile phase: A, 0.02 mol L^{-1} aqueous ammonium acetate/acetonitrile (90:10 v/v); B, 0.02 mol L^{-1} aqueous ammonium acetate/acetonitrile (76:24 v/v), both adjusted to pH 5 with acetic acid; gradient elution: 0–1 min, 0% B, 1–14 min, 0–100% B, 14–17 min, 100% B, 17–17.1 min, 100–0% B, 17.1–20 min, 0% B; flow rate was 0.25 mL min^{-1} ; injection volume was $5 \mu\text{L}$.

High resolution mass spectrometry method: Waters Xevo G2-QTOF MS/MS; instrument parameters: electrospray positive ionization mode, capillary voltage 3 kV, sample cone voltage 30V, extraction cone voltage 4V, desolvation temperature 300°C , source temperature 100°C , desolvation gas 600 L/h, full scan acquisition mode, scan range 100–1500 (m/z), mass calibration: lockspray with 278.1141 Da and 556.2771 Da of leucine encephalin.

2. 6. Statistical analysis

The concentrations of amatoxins and phallotoxins were expressed as mean $\pm$ SD. To estimate the phylogenetic relationships of the ten *Amanita* species using cyclopeptide profiles, the nineteen main chromatographic peaks were manually scored as binary data for the presence or absence of the toxins, A table containing this binary information was used to calculate Jaccard's pairwise similarity coefficients using the SIMQUAL program (Similarity for Qualitative Data) of NTSYS-pc Version 2.1 (Rohlf, 2000). The phylogenetic tree was performed using the UPGMA (unweighted pair-group method with arithmetic averages) clustering method of NTSYS-pc Version 2.1.

3. Results

3.1 Mushroom identification and phylogenetic analysis based on ITS sequences

The species of *Amanita* were identified by morphological characteristics, and the identification were confirmed by the ITS sequences (Table 1). The resulting tree (Fig. 2) based on ITS sequences strongly resolved the taxa examined into seven lineages comprising nine phylogenetic species. These results are consistent with the results of phylogeny inferred from combined datasets (nrLSU, *rpb2*, *efl-α* and *β-tubulin*) and ITS sequences (Cai et al., 2014).

Fig. 2. Phylogenetic tree generated from maximum likelihood analysis based on ITS sequences. Only maximum likelihood bootstraps and Bayesian posterior probabilities over 50% and 0.90 are reported on the branches.

3.2 Concentrations and distribution of amatoxins and phallotoxins in lethal *Amanita* species

The HPLC profiles of crude extractions of ten *Amanita* species and the mixture of five standard

samples were shown in Fig. 3 and Fig. S1. They indicated that there is a great diversity in major peaks among species. All lethal *Amanita* species belonging to the section *Phalloideae* showed amatoxins and phallotoxins, and in contrast, *A. oberwinklerana* of the section *Lepidella* contained no amatoxins or phallotoxins.

Fig. 3. HPLC profiles of cyclopeptide toxins extracted from lethal *Amanita* species.

Amanita oberwinklerana, supposed to be a lethal amanita, possesses no cyclopeptide toxins.

The compositions of amatoxins (α -amanitin and β -amanitin) and phallotoxins (PSC, PCD and PHD) of lethal *Amanita* species are given in Table 2. The amounts of total toxins ranged from 3.27 to 14.19 mg g⁻¹ dry weight. *A. rimosa* had the highest toxin content, up to fourfold more than that in *A. phalloides*, *A. virosa* and *A. bisporigera*. The amatoxins, which are the only toxins responsible for the death of humans, varied significantly in the species tested. first, the *A. rimosa*, *A. fuligineoides*, *A. fuliginea*, *A. pallidrosea* and *A. subjunquillea* contained more than 5.00 mg g⁻¹ dry weight amatoxins, then the *A. exitialis* and *A. bisporigera* contained amatoxins from 3.00 to 5.00 mg g⁻¹ dry weight, and

finally the *A. phalloides* and *A. virosa* contained only less than 2.00 mg g⁻¹ dry weight.

Table 2. Compositions of amatoxins and phallotoxins in lethal *Amanita* species (mg g⁻¹ dry weight)

Scientific Name and Species no.	Amatoxins (A)			Phallotoxins (P)				Total contents	P/A
	α -AMA	β -AMA	Total	PSC	PCD	PHD	Total		
<i>A. bisporigera</i> MHHNU7224	2.47 $\pm$ 0.06	0.56 $\pm$ 0.04	3.03	trace	0	0.45 $\pm$ 0.03	0.45	3.48	0.15
<i>A. exitialis</i> MHHNU30297	2.20 $\pm$ 0.13	2.16 $\pm$ 0.20	4.36	1.50 $\pm$ 0.01	1.39 $\pm$ 0.01	0	2.89	7.25	0.66
<i>A. exitialis</i> MHHNU30298	2.27 $\pm$ 0.01	2.16 $\pm$ 0.14	4.43	1.13 $\pm$ 0.01	0.77 $\pm$ 0.01	0	1.90	6.33	0.43
<i>A. exitialis</i> MHHNU 30937	1.07 $\pm$ 0.06	0.98 $\pm$ 0.02	2.05	0.55 $\pm$ 0.03	0.67 $\pm$ 0.01	0	1.22	3.27	0.60
<i>A. fuliginea</i> MHHNU 30300	5.14 $\pm$ 0.19	1.20 $\pm$ 0.06	6.34	0.22 $\pm$ 0.01	0.18 $\pm$ 0.01	1.19 $\pm$ 0.10	1.59	7.93	0.25
<i>A. fuliginea</i> MHHNU 30207	4.72 $\pm$ 0.11	1.31 $\pm$ 0.04	6.03	0.26 $\pm$ 0.01	0.14 $\pm$ 0.01	1.07 $\pm$ 0.02	1.47	7.50	0.24
<i>A. fuliginea</i> MHHNU 8953	7.14 $\pm$ 0.50	1.89 $\pm$ 0.12	9.03	0.21 $\pm$ 0.01	1.40 $\pm$ 0.09	1.35 $\pm$ 0.09	2.96	11.99	0.33
<i>A. fuligineoides</i> HKAS 52316	4.72 $\pm$ 0.21	4.13 $\pm$ 0.17	8.85	0	trace	0.25 $\pm$ 0.01	0.25	9.10	0.03
<i>A. pallidrosea</i> MHHNU 8112	4.13 $\pm$ 0.02	1.27 $\pm$ 0.02	5.40	0	trace	2.87 $\pm$ 0.09	2.87	8.27	0.53
<i>A. pallidrosea</i> MHHNU 7354	4.09 $\pm$ 0.16	2.17 $\pm$ 0.12	6.26	0	trace	1.62 $\pm$ 0.10	1.62	7.88	0.26
<i>A. phalloides</i> HKAS 31457	0.50 $\pm$ 0.02	0.94 $\pm$ 0.05	1.44	1.30 $\pm$ 0.02	2.71 $\pm$ 0.06	0.26 $\pm$ 0.01	4.27	5.71	2.97
<i>A. phalloides</i> GDGM41101	0.85 $\pm$ 0.07	0.39 $\pm$ 0.02	1.24	0.79 $\pm$ 0.06	1.80 $\pm$ 0.08	0.22 $\pm$ 0.02	2.81	4.05	2.27
<i>A. rimosa</i> MHHNU 8041	8.08 $\pm$ 0.37	4.17 $\pm$ 0.59	12.25	0	1.05 $\pm$ 0.03	0.89 $\pm$ 0.03	1.94	14.19	0.16
<i>A. rimosa</i> MHHNU 7954	5.34 $\pm$ 0.22	3.44 $\pm$ 0.12	8.78	0	1.74 $\pm$ 0.07	0.72 $\pm$ 0.02	2.46	11.24	0.28
<i>A. subjunquillea</i> MHHNU 7049	1.69 $\pm$ 0.01	1.45 $\pm$ 0.09	3.14	0.60 $\pm$ 0.01	trace	0.25 $\pm$ 0.01	0.85	3.99	0.27
<i>A. subjunquillea</i> MHHNU 7751	5.27 $\pm$ 0.01	3.68 $\pm$ 0.02	8.95	0.33 $\pm$ 0.08	1.35 $\pm$ 0.01	2.04 $\pm$ 0.08	3.72	12.67	0.42
<i>A. virosa</i> HKAS 84859	1.60 $\pm$ 0.09	0	1.60	0.76 $\pm$ 0.02	2.09 $\pm$ 0.01	1.64 $\pm$ 0.02	4.49	6.09	2.81
<i>A. virosa</i> HKAS 56694	1.34 $\pm$ 0.04	0	1.34	trace	1.81 $\pm$ 0.10	0.99 $\pm$ 0.04	2.80	4.14	2.09

The distribution of amatoxins and phallotoxins varied significantly in lethal amanitas, the amatoxin content was higher than phallotoxin content in *A. bisporigera*, *A. exitialis*, *A. fuliginea*, *A. fuligineoides*,

A. fuligineoides, *A. rimosa* and *A. subjunquillea*, the ratios of which ranged from twofold to sevenfold, up to thirty-fivefold in the case of *A. fuligineoides*. Contrarily, phallotoxin content was higher than amatoxin content in *A. phalloides* and *A. virosa*, which contained twofold to threefold more phallotoxins than amatoxins (Table 2).

3.3 Concentration and distribution of amatoxins and phallotoxins in different tissues

Fully developed carpophores of four species (*A. exitialis*, *A. fuliginea*, *A. pallidorozea* and *A. rimosa*) were divided into three sections: pileus, stipe and volva. Each tissue underwent identical extraction and HPLC procedures, and the contents of amatoxins and phallotoxins were shown in Fig. 4. The results indicated that, for all toxins tested, the highest concentrations were found in pileus followed by stipe, and the volva had the lowest amount of toxins.

Fig. 4. The content and distribution of amatoxins and phallotoxins in different tissues of lethal *Amanita* species

Figure 4 also revealed that the distribution of amatoxins and phallotoxins varied in different tissues.

The total amount of amatoxins was higher than that of phallotoxins in pileus, but the ratio of

phallotoxins to amatoxins in stipe and volva was higher than that in pileus, with the volva being the highest. In *A. exitialis*, and *A. fuliginea*, the amount of phallotoxins in the volva was higher than that of amatoxins.

Table 3. Masses and formulae of the main compounds

Peak number	Compounds	True Mass (Da)	True formulae	Observed Masses(Da)	Observed formulae
		(Clarke et al., 2012)	(Clarke et al., 2012)		
1	β -amanitin	919.3382	C ₃₉ H ₅₃ N ₉ O ₁₅ S	920.3458 [M+H ⁺], 958.3026 [M+K ⁺]	C ₃₉ H ₅₃ N ₉ O ₁₅ S
2	α -amanitin	918.3542	C ₃₉ H ₅₄ N ₁₀ O ₁₄ S	919.3602 [M+H ⁺], 957.3159 [M+K ⁺]	C ₃₉ H ₅₄ N ₁₀ O ₁₄ S
5	Unknown	—	—	849.3058 [M+H ⁺], 887.2672 [M+K ⁺]	C ₃₁ H ₄₈ N ₁₀ O ₁₆ S
6	Unknown	—	—	819.2964 [M+H ⁺], 857.2551 [M+K ⁺]	C ₃₅ H ₄₆ N ₈ O ₁₃ S
7	Phallisacin	862.3167	C ₃₇ H ₅₀ N ₈ O ₁₄ S	863.3229 [M+H ⁺], 901.2881 [M+K ⁺]	C ₃₇ H ₅₀ N ₈ O ₁₄ S
8	Amaninamide	902.3593	C ₃₉ H ₅₄ N ₁₀ O ₁₃ S	903.3646 [M+H ⁺], 941.3211 [M+K ⁺]	C ₃₉ H ₅₄ N ₁₀ O ₁₃ S
10	Phallacidin	846.3218	C ₃₇ H ₅₀ N ₈ O ₁₃ S	847.3301 [M+H ⁺], 885.2855 [M+K ⁺]	C ₃₇ H ₅₀ N ₈ O ₁₃ S
13	Phalloidin	788.3163	C ₃₅ H ₄₈ N ₈ O ₁₁ S	789.3251 [M+H ⁺], 827.2801 [M+K ⁺]	C ₃₅ H ₄₈ N ₈ O ₁₁ S
14	Phalloidin II	788.3163	C ₃₅ H ₄₈ N ₈ O ₁₁ S	789.3215 [M+H ⁺], 827.2786 [M+K ⁺]	C ₃₅ H ₄₈ N ₈ O ₁₁ S
16	Viroidin	896.3586	C ₃₈ H ₅₆ N ₈ O ₁₅ S	897.3676 [M+H ⁺], 935.3234 [M+K ⁺]	C ₃₈ H ₅₆ N ₈ O ₁₅ S
17	Unknown	—	—	915.3806 [M+H ⁺], 953.3282[M+K ⁺]	C ₃₈ H ₅₉ N ₈ O ₁₆ S

3.4 Mass spectrometry analysis of the main compounds

Nineteen compounds with clearly distinguishable peaks were identified and purified by multiple HPLC runs using the same column. Over 90% purity was achieved for every compound, and the samples were then analyzed with UPLC-QTOF MS/MS. The results showed that eleven out of nineteen compounds generated effective MS information, and eight compounds did not give strong signal due to lower sample concentrations. The eleven compounds were reported in Table 3 and Fig S2. Peaks 1, 2, 7, 10 had the masses corresponding to that of β -amanitin, α -amanitin, phallisacin, and phallacidin

respectively. Peaks 13 and 14 had the same mass that corresponds to that of phalloidin (see Table 3). Peak 13 had the same elution time of the standard phalloidin, we therefore concluded that peak 13 is phalloidin, while peak 14 is likely to be an analogue of phalloidin, which was named “phalloidin II” here. Peaks 8 and 16 were identified to be amaninamide and viroidin according to the masses of 902.3592 and 896.3585. Peaks 5, 6 and 17 generated accurate masses, but there were no corresponding known compounds.

Fig. 5. Dendrogram of lethal *Amanita* sp. based on toxin profiles

3.5 Phylogenetic relationships based on the cyclopeptide profiles

A dendrogram generated from chromatographic profiles was shown in Fig. 5. The result of the analysis strongly supported two major monophyletic clades (A and B), which corresponded respectively to the lethal amanita group containing cyclopeptides in section *Phalloideae* and to members in section *Lepidella* that contain no cyclopeptides. Within clade A, seven lineages were supported based on the similarity coefficients level of 75%: (1) the *A. fuliginea* lineage (Clade I), (2)

A. phalloides and *A. subjunquillea* lineage (Clade II), (3) *A. pallidorozea* and *A. bisporigera* lineage (Clade III), (4) *A. fuligineoides* lineage (Clade IV), (5) *A. rimosa* lineage (Clade V), (6) *A. exitialis* lineage (Clade VI), and (7) *A. virosa* lineage (Clade VII).

Discussion

Concentration of amatoxins and phallotoxins between lethal *Amanita* species

High performance liquid chromatography (HPLC) technology is the most widely favored method for the qualitative and quantitative analysis of amatoxins and phallotoxins in *Amanita* tissues and clinical serum and urine samples in the past 20 years (Enjalbert et al., 1992, 1993; Defendenti et al., 1998; Mcknight et al., 2010; Hu et al., 2012). The contents of amatoxins and phallotoxins of the *Amanita* species determined by HPLC are shown in Table 4. The amounts of α -amanitin in *A. phalloides*, *A. virosa* and *A. bisporigera* collected from Europe and North America, were 0.67-2.95, 1.39 and 1.70 mg g⁻¹ dry weight respectively (Enjalbert et al., 1993; Mcknight et al., 2010; Sgambelluri et al., 2014; Kaya et al., 2015; Garcia et al., 2015). In this study, we showed that the amounts of α -amanitin in *A. phalloides*, *A. virosa* and *A. bisporigera*, also collected from Europe and North America, were 0.50-0.85, 1.34-1.60 and 2.47 mg g⁻¹ dry weight respectively. Also, our previous studies showed that the amounts of α -amanitin in *A. exitialis* and *A. fuliginea* collected from China were 2.47-5.16 and 9.31 mg g⁻¹ dry weight, respectively (Chen et al., 2003; Hu et al., 2012). In our present study, the amounts of α -amanitin in *A. exitialis* and *A. fuliginea* ranged from 1.07 to 2.27 and 4.72 to 7.14 mg g⁻¹ dry weight respectively. These results indicated that our present finding is in accord with the data in the literature, which lends support to the validity of our methodology.

Table 4. Contents of amatoxins and phallotoxins in *Amanita* species relative to our present study determined by HPLC in literature ^a

Species	Tissue and origin	Amatoxins		Phallotoxins			references
		α -AMA	β -AMA	PSC	PCD	PHD	
<i>A. phalloides</i>	Carpophores (France) ^b	1.54	1.41	1.31	2.32	1.32	Enjalbert et al. 1993
	Carpophores (Turkey) ^c	2.80 $\pm$ 0.13	2.38 $\pm$ 0.06	— ^d	2.12 $\pm$ 0.06	1.32 $\pm$ 0.01	Kaya et al. 2015
	Pileus (Turkey) ^c	2.95 $\pm$ 0.05	2.53 $\pm$ 0.03		2.27 $\pm$ 0.02	1.40 $\pm$ 0.03	Kaya et al. 2015
	Pileus (Portugal) ^c	0.67- 0.78	—	—	—	—	Garcia et al. 2015
	Carpophores (Italy) ^c	1.33	—	—	—	—	Sgambelluri et al., 2014
	Carpophores (USA) ^c	0.88	—	—	—	—	Sgambelluri et al., 2014
<i>A. virosa</i>	Carpophores (Italy) ^c	1.39	0	—	—	—	Sgambelluri et al., 2014
	Pileus (Japan) ^b	6.03	4.53			3.01	Ahmed et al. 2010
<i>A. bisporigera</i>	Pileus (USA) ^c	1.70 $\pm$ 0.68	—	—	2.71 $\pm$ 0.65	11.98 $\pm$ 1.66	Mcknight et al., 2010
<i>A. exitialis</i>	Pileus (China) ^c	2.47 $\pm$ 0.08	2.44 $\pm$ 0.07	—	—	—	Hu et al., 2012
	Pileus (China) ^c	5.16	1.76	—	1.11	0	Chen et al., 2003
<i>A. fuliginea</i>	Pileus (China) ^c	9.31	1.04	—	0.71	1.46	Chen et al., 2003
<i>A. pallidrosea</i>	Carpophores (China) ^c	3.83	—	—	—	—	Cao et al., 2011
<i>A. subjunquillea</i>	Carpophores (China) ^c	2.39	1.65	—	—	0.40	Bao et al., 2005

^a All values are in mg g⁻¹ dry weight, assuming 1 g dry weight = 12.5 g fresh tissue,

^b Fresh tissue, ^c Dry tissue, ^d “—” means the toxin was not detected.

Table 2 and Fig. 2 showed that there was a diversity in compositions and contents of amatoxins and phallotoxins among the lethal *Amanita* species. To our knowledge, this is the first report of the analysis of amatoxins and phallotoxins in *A. rimosa* and *A. fuligineoides*. All lethal *Amanita* species tested contained α -amanitin and β -amanitin except *A. virosa*. No β -amanitin was detected in *A. virosa*, which is consistent with the latest result by means of HPLC (Sgambelluri et al., 2014) and earlier result by means of thin-layer chromatograms (Yocum & Simons, 1977). However, Ahmed *et al.* (2010) reported

that the levels of β -amanitin was comparable to that of α -amanitin in a specimen of *A. virosa* from Japan. We suppose that the species was likely identified inaccurately, as several *Amanita* species that possess white carpophores and are endemic to East Asia, are similar to *A. virosa*; therefore it is difficult to identify by morphologic characters alone.

A survey of the toxin composition of *A. phalloides* indicated that five components, α -amanitin, β -amanitin, PCD, PHD and PSC, are the predominant components, representing 92% of the total toxin content (Enjalbert et al., 1993). Therefore we chose these five components for quantitative analysis in this study. Our results showed that the amounts of total toxins varied significantly in different lethal *Amanita* species, ranged from 3.27 to 14.19 mg g⁻¹ dry weight. Because the responsible toxins for fatal human poisoning are amatoxins, not phallotoxins (Wieland, 1986; Karlson-Stiber & Persson 2003), the evaluation of the amatoxin content of *Amanita* species is more important. The present results (Table 2) showed that the amounts of amatoxins in lethal *Amanita* species ranged from 1.24 to 12.25 mg g⁻¹ dry weight. The amounts of amatoxins in East Asian *Amanita* species, such as *A. fuliginea*, *A. rimosa*, *A. exitialis*, *A. fuligineoides*, *A. pallidrosea* and *A. subjunquillea* are significantly higher than those in European and North America species, such as *A. phalloides*, *A. virosa* and *A. bisporigera*. The average content of amatoxins in East Asian *Amanita* species are about three to eight times the amount in *A. phalloides*, which was responsible for most of mortality in Europe. The data indicate that the East Asian *Amanita* species are more toxic than species from Europe and North America. In fact, all of these East Asian *Amanita* species except *A. fuligineoides* and *A. subjunquillea* were collected from the field near investigated mushroom poisoning accidents. In our investigation of mushroom poisoning cases in southern China from 1994 to 2012, we found that poisoning induced by the ingestion of homogeneous *A. fuliginea* or *A. exitialis* mushrooms could lead to 60-100% of mortality (Chen et al., 2014). One case

of mushroom poisoning caused by *A. pallidorozea* have been reported in China, the mortality rate was 43% in this case (Cao et al., 2011). Another incident caused by *A. pallidorozea* was investigated in Guizhou Province in 2014 by us, in which case 2 patients died with 100% mortality. In 2015, we investigated two cases of mushroom poisoning caused by *A. rimosa* in Hunan and Jiansu provinces, China, which involved 8 patients and 5 deaths, with 62.5% mortality.

Distribution of amatoxins and phallotoxins in different lethal *Amanita* species and tissues

The toxin composition in various carpophore tissues of *Amanita phalloides*, which was subdivided into six parts, namely, cap (pileus), gills, ring, stipe, volva and bulb, were comprehensively and systematically evaluated by Enjalbert et al. (1993, 1996, 1999). The results showed that variation of the toxin concentration in the different tissues was particularly large, and the content of phallotoxins was higher than the content of amatoxins in all tissues, the ratio of phallotoxins to amatoxins (P/A) for the various tissues indicated that bulb and volva contained four times more phallotoxins than amatoxins, whereas cap, gills, ring and stipe group had 1.5 times more phallotoxins than amatoxins. In addition, the environmental factors, mainly the soil type, clearly have an effect on the toxin composition in *A. phalloides*. Our present study indicated that content of phallotoxins was higher than that of amatoxins in *A. phalloides* and *A. virosa*: the result of *A. phalloides* ($P/A = 2.27-2.97$) is consistent with the report by Enjalbert et al. (1993, 1996, 1999), and the result of *A. virosa* ($P/A = 2.09-2.81$) is in line with the report by Yocum & Simons (1977). In contrast, the phallotoxin content was significantly lower than that of the amatoxins in all of other lethal *Amanita* species, and the ratio of phallotoxins to amatoxins (P/A) from 0.15 to 0.66, the P/A being 0.03 in *A. fuligineoides*. That is to say that these *Amanita* species contained about twofold to sevenfold more amatoxins than phallotoxins, even thirty-fivefold in the case of *A. fuligineoides*. Interestingly, all of these lethal *Amanita* species except *A. bisporigera* are

endemic to East Asia. Furthermore, the composition and distribution of amatoxins and phallotoxins in different tissues (pileus, stipe and volva) of *A. exitialis*, *A. fuliginea*, *A. pallidorosea* and *A. rimosa* were analyzed and the results are in agreement with the previous reports in *A. phalloides* (Enjalbert et al., 1993, 1996, 1999; Kaya et al., 2015), in *A. phalloides* var. *alba* (Kaya et al., 2013) and in *A. exitialis* (Hu et al., 2003; Li et al., 2014a).

Cyclopeptides in lethal *Amanita* species

Seven cyclopeptides were affirmatively identified in our present study, of which, β -amanitin, α -amanitin, phallisacin, phallacidin, phalloidin and viroidin were identified by the both of elution time and hi-res mass. A phalloidin analogue (“phalloidin II”) was identified according to the exact molecular weight and the formula. The analogues of phallotoxins were also reported in the previous literature (Clarke et al., 2012; Sgambelluri et al., 2014).

Peak 8 generated an $[M+H]^+$ ion of 903.3646 (m/z) and an $[M+K]^+$ ion of 941.3211 (m/z), which could correspond to either amaninamide or γ -amanitin, as amaninamide and γ -amanitin have the same mass of 902.3592. Amaninamide was firstly reported from *A. virosa* (Buku et al., 1980). In recent years, amaninamide was detected and identified using HPLC-MS/MS method in *A. virosa*, *L. brunneoincarnata* and *A. exitialis* by Sgambelluri et al.(2014) and Deng et al. (2011), respectively. The HPLC profile peak was relatively high, but the two compounds (amaninamide and γ -amanitin) were not detected in *A. phalloides* (Sgambelluri et al.,2014). Also, Clarke et al. (2012) reported that fourteen amatoxins and phallotoxins were identified in *A. phalloides* by means of LC-UV-ToF/MS system and γ -amanitin could be detected, but amaninamide did not exist. These findings indicated that amaninamide mainly exists in *A. virosa* and other *Amanita* species, and γ -amanitin exists in *A. phalloides* and other *Amanita* species. In our present study, the HPLC profiles showed that the

relatively high peak 8 exists in *A. virosa*, *A. fuliginea* and *A. bisporigera*, and only trace of peak 8 exist in *A. phalloides*. Therefore, we can speculate the compound of peak 8 was amaninamide. Also, of the seven affirmative cyclopeptides, viroidin was detected only in *A. virosa*. Virotoxins, which are monocyclic peptides, appear to be restricted to *A. virosa* (Wieland, 1986).

Of the eleven identified compounds, three compounds (peaks 5, 6 and 17) generated accurate masses without corresponding to any reported *Amanita* cyclic peptide toxins. The peak 5 compound with higher concentration and a mass of 848 in *A. exitialis* was identified to be desoxoviroidin by Deng et al. (2011) and Hu & Chen. (2014). In fact, the mass of desoxoviroidin is 880, so the identified desoxoviroidin in *A. exitialis* is incorrect. All twenty-two *Amanita* cyclopeptide toxins were discovered and isolated from *A. phalloides* and *A. virosa* by thin-layer chromatography (TLC) or high-performance TLC (HPTLC) before the 1980s, and no further peptide toxins have been discovered since then. Now, the more sensitive and effective HPLC methods have been developed, and new lethal *Amanita* species are being discovered continuously in recent years in East-Asia. Furthermore, the findings on the molecular diversity of toxin gene families in lethal *Amanita* mushrooms indicated nearly 33.7% of peptides sequences remain unknown (Li et al., 2014b). These three factors provide the possibility for the discovery of new toxins. More than 10 milligrams of compound peak 5 have been prepared from *A. exitialis* in our laboratory; further studies on the structure and function are in progress.

Phylogenetic relationships based on the cyclopeptides of lethal *Amanita* species

The lethal amanitas are a group of cyclopeptide-containing mushrooms included in the genus *Amanita* sect. *Phalloideae*. The relationships of the lethal *Amanita* species based on the morphological and molecular phylogenetic data were elucidated by Zhang et al. (2010) and by Cai et al. (2014).

Twenty eight phylogenetic species were divided into nine major clades based on the multi-locus DNA gene fragment analysis in combination with biochemical and morphological analyses (Cai et al., 2014).

Amanita oberwinklerana was originally described from Japan (Yang and Doi 1999), which was also collected and reported from China (Yang and Li 2001; Yang, 2005, 2015). According to its morphological characteristics, this species was placed in section *Phalloideae* (Yang and Doi 1999; Yang and Li 2001; Yang 2005). However, the results of molecular analyses from both ITS and combined dataset (nrLSU, *rpb2*, *ef1- α* and β -*tubulin*) showed that it appeared to be a member of section *Lepidella* (Zhang et al. 2010; Cai et al. 2014). In our present study, neither amatoxins nor phallotoxins were detected in *A. oberwinklerana*, this biochemical evidence strongly supported that *A. oberwinklerana* should be removed from the section *Phalloideae* and be allocated to the section *Lepidella*. Furthermore, two poisoning case caused by *A. oberwinklerana* were reported by us, which caused acute renal failure (Fu et al., in press), which is in accord with that the mushrooms in the *Lepidella* sections produce primarily acute renal injury (Graeme 2014; Kirchmair et al. 2012).

Vargas et al. (2011) determined the amatoxin and phallotoxin composition of twelve *Amanita* species in Colombia. Eleven of the twelve *Amanita* species analyzed in their report belong to section *Amanita*, *Validae* or *Vaginatae*, and only one species (*A. bisporigera*) belongs to section *Phalloideae*. However, their analysis showed that all of the analyzed species have, surprisingly, α -amanitin in concentrations ranging from 50 ppm to 6000 ppm. Based on our data and other previous literatures, we suppose that the results were inaccurate, because neither amatoxins nor phallotoxins have been detected in *Amanita* species of sections *Amanita*, *Validae*, *Vaginatae* and *Lepidella* (Hallen et al., 2002; Li and Oberlies, 2005; Cai et al., 2014). Furthermore, Hallen et al. (2007) demonstrated that the toxic genes (*AMA1* and *PHA1*) were absent in the *Amanita* species outside of section *Phalloideae*. Also, the

chosen 18 most informative chromatographic peaks for the dendrogram were located in zone between 0 and 5 min, most of the peaks were not amatoxins and phallotoxins. Therefore, the results of the dendrogram in Vargas et al. (2011) could not reflect the relationship between toxic compounds and the taxonomy of the species.

In our present study, we generated a dendrogram by UPGMA method based on the cyclopeptide chromatographic profiles for the first time. The result of the analysis strongly supported that the nine analyzed *Amanita* species in section *Phalloideae* can be divided into seven lineages based on the similarity coefficients level of 75%. Among them, *A. phalloides* and *A. subjunquillea* formed a clade, *A. pallidorozea* and *A. bisporigera* formed another clade. Interestingly, these results are remarkably consistent with the results of phylogenetic analysis based on their ITS sequences and the combined dataset (nrLSU, *rpb2*, *efl-α* and *β-tubulin*) (Cai et al., 2014). The data and prior reports raise the following questions, why is the dendrogram based on the cyclopeptide chromatographic profiles so similar to that based on the DNA sequences? What are the genetic bases of cyclopeptide diversity? And are there any unknown toxins present? Recent studies have demonstrated that the cyclopeptide toxins of *Amanita* mushrooms were synthesized on ribosomes and not by nonribosomal peptide synthetases (NRPSs). The genes encoding the amatoxins and phallotoxins belong to the MSDIN genes family, characterized by highly conserved amino acid sequences flanking a hypervariable toxin region that gives rise to the linear peptides corresponding to the mature toxins (Hallen et al., 2007; Luo et al., 2009; 2010). Analysis of the MSDIN family members identified that there were significant diversities and consensus in the toxin genes among the cyclopeptide-containing species of genus *Amanita* and *Galerina* and clarified that nearly 33.7% of the predicted peptide sequences are for unknown/uncharacterized products (Hallen et al., 2007; Luo et al., 2012; Li et al., 2014b). Also, the

phylogenetic analysis of toxin gene (*a-AMA*) demonstrated that the conservative phylogenetic relationships existed in the same genus, and significant differences were shown at the genus level (Li et al., 2014b). Therefore, these results can well explain the diversity of cyclopeptides and the similarity between the dendrogram based on the cyclopeptides and those based on DNA sequences. Also, the unknown sequences of peptides indicated the existence of new toxins.

Conflict of interest

The authors declare that they have no conflict of interest.

Authors' contributions

Chen ZH conceived and designed the experiments; Tang SS, Zhou Q and Luo T detected and analyzed the cyclopeptide toxins, He ZM carried out the ITS DNA sequences of all *Amanita* species, Zhang P, Yang ZL and Cai Qing provided some *Amanita* materials and identified the species; Chen J performed the LC-MS/MS analysis and Chen ZH wrote the paper. All of the authors approved the final submission. All authors read and approved the final manuscript.

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Supplementary data

Table S1. Data of presence or absence of the nine informative chromatographic peaks in *Amanita* species

Figure S1. HPLC profiles of standard toxins and extraction of ten *Amanita* species

Figure S2. QTOF-MS/MS information of the main compounds

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