https://doi.org/10.48048/tis.2023.4301

Characterization of Lethal Toxin-Producing Genes in *Amanita brunneitoxicaria* and PCR-based Detection of Deadly Poisonous Amanitas in the Section *Phalloideae*

Sittiporn Parnmen[1], Nattakarn Nooron[1], Pilailuk Akkapaiboon Okada[1], Sujitra Sikaphan[1], Onanong Pringsulaka[2], Sutheewan Binchai[3] and Achariya Rangsiruji[3,*]

[1]National Institute of Health, Department of Medical Sciences, Ministry of Public Health, Nonthaburi 11000, Thailand
[2]Department of Microbiology, Faculty of Science, Srinakharinwirot University, Bangkok 10110, Thailand
[3]Department of Biology, Faculty of Science, Srinakharinwirot University, Bangkok 10110, Thailand

([*]Corresponding author's e-mail: achariya@g.swu.ac.th)

Received: 20 May 2022, Revised: 29 June 2022, Accepted: 6 July 2022, Published: 18 January 2023

Abstract

A recent discovery of *Amanita brunneitoxicaria* revealed that this mushroom and *A. exitialis* are the main causative agents of fatal mushroom poisoning in Thailand. Poisonous amatoxins present are bicyclic octapeptides encoded by the MSDIN family and macrocyclized by prolyl oligopeptidase B enzyme. Diversity of the MSDIN toxin-associated genes in *A. brunneitoxicaria* however, remains unexplored. Therefore, this study aimed to characterize the MSDIN family members in *A. brunneitoxicaria* in comparison with *A. exitialis* based on whole genome sequencing, followed by gene annotation using bioinformatic tools. Identification of conserved core peptides of the MSDIN family revealed the presence of α-amanitin and β-amanitin as endogenous toxins in *A. brunneitoxicaria*. In addition to the major amatoxins, amanexitide and phallacidin were also found in *A. exitialis*. Phylogenetic analyses of the MSDIN family members showed that an unknown peptide present in *A. brunneitoxicaria* is clustered with phalloidin. On the basis of PCR detection, a modified forward primer was incorporated into the PCR reactions to obtain clear and distinctive DNA fragments of approximately 300 bp from three deadly poisonous mushrooms of *A. brunneitoxicaria*, *A. exitialis* and *A. fuliginea* in the section *Phalloideae*. Hence, they could be distinguished from other edible and less toxic species in the same genus. Such rapid and accurate identification of the target region of the MSDIN family from the lethal mushrooms is relevant as means to reduce mortality.

Keywords: *Amanita*, Cyclic peptide toxin, MSDIN family, Mushroom poisoning, Next-generation sequencing, PCR-based detection, *Phalloideae*, Phylogenetics

Introduction

Amanita is one of well-known genera of macrofungi in the family Amanitaceae (Basidiomycota, Agaricales) which is distributed worldwide. The genus is estimated to have 900 - 1000 species, half of which have been described [1]. It is divided into 7 sections, including *Amanita*, *Amidella*, *Caesareae*, *Lepidella*, *Phalloideae*, *Vaginatae*, and *Validae* based on phenotypic characters [2] as well as molecular phylogenetics (all sections are strongly supported except *Lepidella*) [3]. Both edible and deadly poisonous species are found in the genus *Amanita* (**Figure 1**). Based on Cai *et al.* [4] *ca*. 50 species of lethal amanitas belonging to the section *Phalloideae* have been reported. Within this section, Thongbai *et al.* [5] recently discovered a new species, *A. brunneitoxicaria*, from southern Thailand. According to Ramchiun *et al.* [6], this lethal species was similar in appearance to *A. vaginata* (section *Vaginatae*) which is edible. In additon to *A. brunneitoxicaria*, there are 4 other species of lethal amanitas present in Thailand. These include *A. exitialis*, *A. fuliginea*, *A. fuligineoides* and *A. virosa* of the section *Phalloideae* [7,8]. Among them, *A. brunneitoxicaria* and *A. exitialis* are responsible for most cases of fatal mushroom poisoning in the country [6,8].

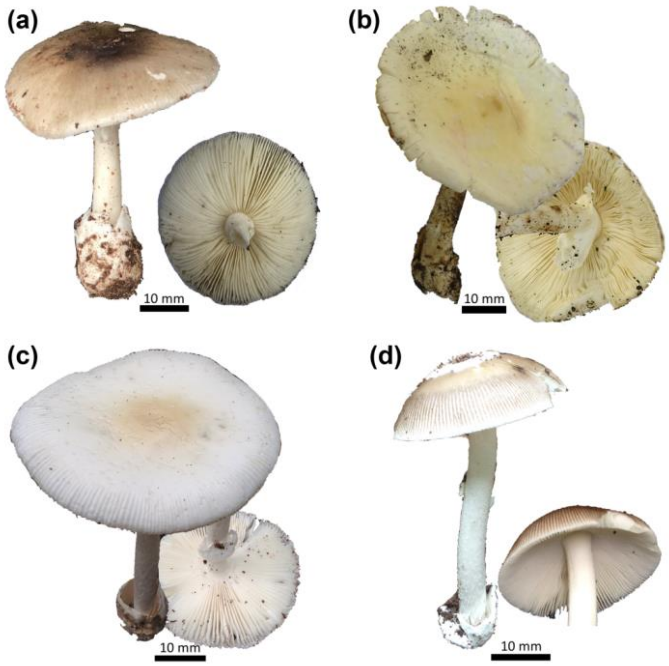

Figure 1 Representatives of *Amanita* mushroom samples: Lethal species (a) *A. brunneitoxicaria*, (b) *A. exitialis*, and edible species (c) *A. princeps*, (d) *A. vaginata*.

Toxic substances such as amatoxins and phallotoxins are present in amanitas as well as in some other distantly related agarics, including *Galerina* (Hymenogastraceae) and *Lepiota* (Agaricaceae) [9,10]. Amatoxins, functioned as specific inhibitors of RNA polymerase II, include α-amanitin and β-amanitin which are responsible for more than 90 % of fatal mushroom poisoning cases worldwide [11,12]. Phallotoxins which include phallacidin and phalloidin, on the other hand, do not appear to be absorbed by the mammalian digestive tract and hence, are not toxic to humans when consumed orally [13]. Having ingested the poisonous amanitas, all patients showed the first latent period within 2 to 12 h of gastrointestinal phase which was characterized by nausea, vomiting, abdominal pain and cramps as well as severe secretory diarrhea. The second latent period with the early signs of liver and renal damage started after 12 to 14 h, and this could eventually lead to death of the patients [6,8].

Ribosomal biosynthesis of amatoxins and phallotoxins involves the production of precursor peptides, followed by macrocyclization [14-16]. These cyclic peptides are encoded by short nucleotide sequences of the MSDIN family. Each precursor peptide of 33 - 37 amino acids consists of 2 conserved regions, an upstream leader peptide (10 amino acids) and a downstream recognition peptide (17 amino acids). In addition, there is a highly variable core peptide (7 - 10 amino acids) which is bounded by 2 conserved proline residues. After translation, the precursor peptides are cleaved by prolyl oligopeptidase B (POPB) enzyme which is a proline-specific peptidase, and subsequently modified by cyclization [13,17,18]. The MSDIN family is however, absent from fungi that do not produce amatoxins or phallotoxins [14,19].

For novice foragers, poisonous species of *Amanita* are often mistaken for edible species due to their phenotypic similarity. Delayed and inappropriate treatment of lethal *Amanita* intoxication can result in severe illnesses and death. This study therefore, focused on characterization of the genes governing the expression of toxic cyclic peptides in *A. brunneitoxicaria* and *A. exitialis* based on next-generation sequencing (NGS) technology and bioinformatic analyses. In addition, an in-house PCR assay was developed for rapid detection of the MSDIN family present in the lethal amanitas of the section *Phalloideae*. The results of which are useful for timely and appropriate medical treatment of mushroom intoxication.

Materials and methods

Mushroom specimens and identification

A total of 30 mushroom specimens, representing 5 sections of the genus *Amanita*, including the sections *Amanita* (*A. digitosa*, *A. mira*), *Caesareae* (*A. hemibapha*, *A. javanica*, *A. princeps*, *A. rubromarginata*), *Phalloideae* (*A. brunneitoxicaria*, *A. exitialis*, *A. fuliginea*), *Vaginatae* (*Amanita* aff. *fuligineodisca*, *A. vaginata*) and *Validae* (*A. porphyria*) were used for analyses (**Table 1**). Of 30 specimens, 10 were mushroom remnants left from food preparation and delivered to the Toxicology Center (NIH, Department of Medical Sciences, Thailand) during 2012 - 2018. Details of mushroom consumption history, geographical distribution and clinical manifestations are shown in **Table 2**. Both clinical and field specimens were primarily identified based on their morphological characters and compared with descriptions provided by amanitaceae.org.

Table 1 Specimens of *Amanita* used in PCR amplification of the MSDIN family.

Taxon	Type of toxin	Edibility	Type of specimen	Number of specimen
Section *Amanita*				
A. digitosa	GI-toxins	Inedible (non-deadly)	Clinical	1
A. digitosa	GI-toxins	Inedible (non-deadly)	Field	1
A. mira	Neurotoxins	Inedible (non-deadly)	Field	2
Section *Caesareae*				
A. hemibapha	N/A	Edible	Field	2
A. javanica	N/A	Edible	Field	2
A. princeps	N/A	Edible	Field	2
A. rubromarginata	Unknown	N/A	Field	2
Section *Phalloideae*				
A. brunneitoxicaria	Hepatoxins	Inedible (deadly)	Clinical	5
A. brunneitoxicaria	Hepatoxins	Inedible (deadly)	Field	1
A. exitialis	Hepatoxins	Inedible (deadly)	Clinical	3
A. exitialis	Hepatoxins	Inedible (deadly)	Field	1
A. fuliginea	Hepatoxins	Inedible (deadly)	Clinical	1
Section *Vaginatae*				
A. aff. *fuligineodisca*	GI-toxins	Inedible (non-deadly)	Field	2
A. vaginata	N/A	Edible	Field	3
Section *Validae*				
A. porphyria	Unknown	Inedible	Field	2
			Total	**30**

Note: N/A = data not available.

Table 2 Details of mushroom consumption history (2012 - 2018), geographical distribution and clinical manifestations.

Case	*Amanita* species (No. of specimen)	Year	Region of Thailand	No. of patient	Death	Symptom
1	*A. digitosa* (1)	2012	Northeast	19	-	Intense thirst, perspiration, nausea and vomiting, headache, abdominal pain, severe diarrhea and fatigue
2	*A. exitialis* (3)	2012	North	8	1	Intense thirst, perspiration, nausea and vomiting, headache, abdominal pain, severe diarrhea, fatigue and cardiac arrest
3	*A. fuliginea* (1)	2012	Northeast	5	1	Severe diarrhea, strangle, choke and death from fulminant hepatic and renal failure
4	*A. brunneitoxicaria* (2)	2017	Northeast	12	1	Nausea, vomiting, abdominal pain and cramps as well as severe secretory diarrhea, liver and renal damage

5	*A. brunneitoxicaria* (2)	2018	Northeast	5	1	Nausea, vomiting, abdominal pain and cramps as well as severe secretory diarrhea, liver and renal damage
6	*A. brunneitoxicaria* (1)	2018	East	1	-	Nausea, vomiting, abdominal pain and cramps as well as severe secretory diarrhea

Genomic DNA extraction and PCR amplification of ITS

Tissues of the mushroom specimens were stored in DNA/RNA Shield (Zymo Research) and kept at -70 °C. Each sample containing 70 mg of a fruiting body was ground in liquid nitrogen. Total genomic DNA was extracted using the DNeasy Plant Mini Kit (QIAGEN, Germany) according to the manufacturer's instructions. All 30 mushroom DNA samples were obtained. The identities of both clinical and field specimens were confirmed by PCR amplification and sequencing of the internal transcribed spacer (ITS) region using the primers ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') [20] and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') [21]. The condition for amplification followed Parnmen *et al.* [8]. PCR products were purified using QIAquick PCR Purification Kit (QIAGEN, Germany), and Sanger sequencing was performed at the Toxicology Center, National Institute of Health.

Library preparation and NGS

High-throughput sequencing data were obtained for 2 clinical specimens of *A. brunneitoxicaria* and *A. exitialis* using the Illumina MiSeq system with QIAseq FX DNA Library Kit (QIAGEN, Germany) constructed with QIAseq index kits (QIAGEN, Germany) following the manufacturer's protocol. DNA concentrations of the samples were measured using fluorescence-based Qubit quantitation assays (Qubit 2.0 Fluorometer, Invitrogen, USA). An aliquot containing 10 ng of the input genomic DNA was used for fragmentation, end repairs and polyadenylation. DNA library was purified using Agencourt AMPure XP beads (Beckman Coulter, USA). The prepared libraries were sequenced on the Illumina MiSeq platform with paired-end 500-cycle mode (MiSeq Reagent Kits v2, Illumina, USA).

Annotation of the MSDIN family

The BBDuk (Decontamination using kmers) program under Geneious Prime version 2020.0.5 (www.geneious.com) was used to remove adapter sequences and low-quality reads. The MSDIN family was explored in the genomic data of 2 lethal amanitas. Gene annotation was performed using Bowtie 2 version 2.3.5.1 [22] and Geneious Prime. Sequences of the MSDIN family members were compared for nucleotide similarities against the GenBank database using BLASTN [23-24]. The obtained amino acid sequences of the MSDIN family members were aligned using blocks substitution matrix (BLOSUM) [25], and a sequence logo was created using WebLogo [26].

Sequence alignments and phylogenetic analyses

Newly generated DNA sequences of the MSDIN family members derived from the samples of *A. brunneitoxicaria* and *A. exitialis* as well as the sequences obtained from a previous study by Luo *et al.* [16] were aligned using MUSCLE program [27]. Phylogenetic analyses were conducted using maximum likelihood (ML) and Bayesian approaches. ML analysis was performed in RAxML-HPC2 on XSEDE 8.2.12 using the GTRGAMMA+I model [28,29]. Branch support was estimated using 1000 bootstrap pseudoreplicates, and clades that received bootstrap values (BS) ≥ 75 % were considered strongly supported. Bayesian analysis was performed using a variant of Markov Chain Monte Carlo method in MrBayes 3.2.2 with GTR+I+G model [30]. Highly supported clades possessed posterior probabilities (PP) ≥ 0.95. Phylogenetic trees were depicted using the program FigTree 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree).

PCR-based detection of lethal amanitas based on the MSDIN family

The target region of the MSDIN family of the lethal amanitas was amplified using a combination of a modified forward primer designated in this study as ABAE-F (5'-ATGTCTGAYRTCAAYRCYRCYCGTCTYC-3') and the reverse primer (5'-CCAAGCCTRAYAWRGTCMACAAC-3') [15]. Bases of the ABAE-F primer were modified based on the original sequence obtained by Hallen *et al.* [14]. Five nucleotides added at the 3' end of the primer were derived from the genomic sequences of *A. brunneitoxicaria* and *A. exitialis* obtained in the present study.

Each PCR reaction of 25 µL contained 9.5 µL of OnePCR (GeneDirex, Korea) reaction mixture with fluorescence dye, 2.5 µL of 10 µM of each primer, 1 µL of genomic DNA template and 9.5 µL of nuclease-free water. Cycling condition included initial denaturation at 94 °C for 4 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 51 °C for 30 s, extension at 72 °C for 30 s, and final extension at 72 °C for 7 min. PCR products were examined using 2 % (w/v) agarose gel electrophoresis.

Results and discussion

Identification of mushroom specimens

This study focused on 2 poisonous species of mushrooms that commonly exist in nature and are the main causes of fatal mushroom poisoning in Thailand. They were the greyish brown cap, *A. brunneitoxicaria*, and the white cap, *A. exitialis*. Morphologically, *A. brunneitoxicaria* (**Figure 1(a)**) is a medium to large agaric mushroom that is characterized by a convex to plane, greyish brown basidiocarp which is darkest at the center, while *A. exitialis* (**Figure 1(b)**) can be distinguished by having a convex to plane, creamy white basidiocarp with pale yellow area at the center. In most of the cases, the patients were mistaken that *A. brunneitoxicaria* and *A. exitialis* were 2 edible species of *A. vaginata* and *A. princeps* [31]. Morphologically, *A. brunneitoxicaria* can be distinguished from *A. vaginata* based on the presence of non-striate pileus margins [6]. From this study, the BLAST search analysis of the obtained ITS sequences (*ca*. 700 bp) of the mushroom specimens revealed 98 - 100 % nucleotide similarities to their corresponding species.

Annotation of the MSDIN family

Total of 1,464,492 and 1,317,588 clean reads were obtained from *A. brunneitoxicaria* and *A. exitialis*, respectively. All reads were assembled using Bowtie 2, and translated via Geneious Prime. Results of the BLAST search are shown in **Table 3**. Ten sequences of the MSDIN family members generated in this study revealed the highest pairwise identity to those of *A. exitialis*. Core peptide sequences, including IWGIGCNP, IWGIGCDP, AWLVDCP and VFSLPVFFP matched the sequences of α-amanitin, β-amanitin, phallacidin and amanexitide, respectively (**Figure 2**). The core peptide sequences of α-amanitin (IWGIGCNP) and β-amanitin (IWGIGCDP) obtained from both *A. brunneitoxicaria* and *A. exitialis* matched the amino acid sequences of the toxic amanitas in the section *Phalloideae*, as well as those of *G. marginata* and *L. brunneoincarnata* [16,17]. These 2 types of amanitins only differ by 1 amino acid residue. The highly toxic amatoxins are rapidly absorbed from the intestine, whereas phallotoxins are poorly absorbed but become poisonous when administered parenterally [11,14,32]. The human lethal dose for α-amanitin was estimated to be 0.1 mg/kg of body weight. On average, the toxic amanitas occurring in Thailand contain 0.05 to 0.60 mg of α-amanitin per gram of mushroom dry weight [8].

Table 3 Identification of the MSDIN family members in *A. brunneitoxicaria* and *A. exitialis* using BLASTN.

Specimen	MSDIN family member	GenBank accession number (this study)	Highest BLAST pairwise similarity (%)	Gene-encoding sequence identification (GenBank accession number)
A. brunneitoxicaria	Alpha-amanitin	MT681655	95.83	*A. exitialis* alpha-amanitin (KF387486)
	Beta-amanitin	MT681657	91.67	*A. exitialis* beta-amanitin (KF387477)
	Unknown peptide	MT681659	85.87	*A. exitialis* phallacidin (KF793337)
A. exitialis	Alpha-amanitin	MT681656	100	*A. exitialis* alpha-amanitin (KF813063)
	Amanexitide	MT681661	98.61	*A. exitialis* amanexitide (KF387479)
	Beta-amanitin	MT681658	98.96	*A. exitialis* beta-amanitin (KF387477)
	Phallacidin	MT681662	100	*A. exitialis* phallacidin (KF813064)
	Unknown peptide 1	MT681660	100	*A. exitialis* MSDIN-like 4 peptide (KF387481)
	Unknown peptide 2	MT681663	100	*A. exitialis* MSDIN-like 5 peptide (KF387482)
	Unknown peptide 3	MT681664	94.79	*A. exitialis* MSDIN-like 6 peptide (KF387483)

Amino acid sequences with unclassified functions, including VWIGYSP, LFFPPDFRPP, FAFVGIPP were found in *A. exitialis*, while ACLVFCP occurred in *A. brunneitoxicaria*. Most of these proprotein sequences possessed 2 unique regions of upstream (MSDINATRLP) and downstream (PCVGD) conserved consensus sequences with invariant proline (P) residues flanking the core peptides. These results were in agreement with Luo *et al.* [33]. Variants of MSDIN such as MSNVN and MSDAN were also present in *A. brunneitoxicaria*.

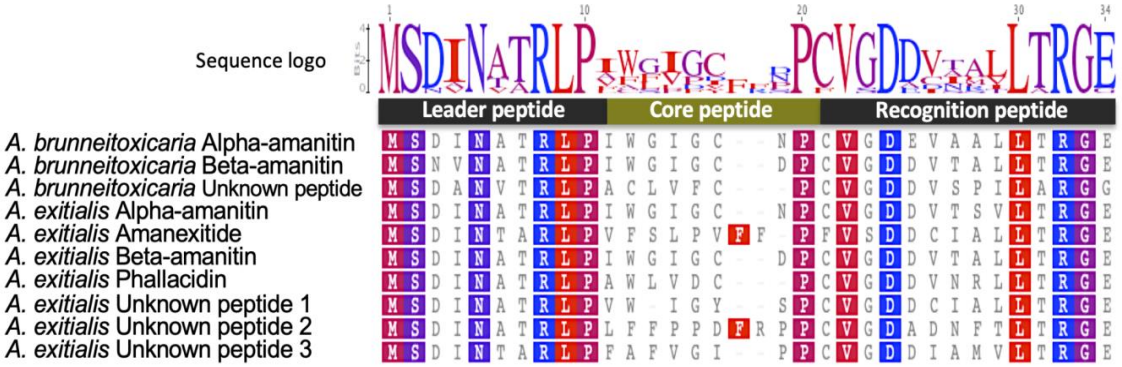

Figure 2 Amino acid sequence alignment of the MSDIN family from *A. brunneitoxicaria* and *A. exitialis*. The height of the amino acid at each position indicates the degree of conservation. Sequence logo was created using WebLogo.

Phylogenetic analyses of the MSDIN family members

In the present study, new DNA sequence data generated were consisted of 10 sequences of the MSDIN family members (**Table 3**). Unambiguous alignment matrix of the MSDIN family members was obtained and used for phylogenetic analyses. Similar tree topologies were produced from the ML and Bayesian approaches and hence, only the ML tree is illustrated with BS ≥ 75 % and PP ≥ 0.95 (**Figure 3**). Five main groups were represented as follows. Group I consists of the specimens containing amanexitide (cyclic nonapeptide) and an unknown peptide of *A. exitialis* 3. The members in groups II and III are mushrooms that produce α-amanitin and β-amanitin, respectively. Groups IV and V on the other hand, contain the phallotoxin-producing mushrooms with 3 unknown peptides present in *A. brunneitoxicaria*, *A. exitialis* 1 and *A. exitialis* 2.

The resulting phylogenetic tree showed that the core amino acid sequence of *A. brunneitoxicaria* unknown peptide (ACLVFCP) is clustered with group V phalloidin (AWLATCP). Three different amino acid substitutions were found at the second (Cys/Trp), fourth (Val/Ala) and fifth (Phe/Thr) positions. Amatoxins as well as phallotoxins are the bicyclic peptides that normally contain the tryptathionine linkage (Trp-Cys cross-bridge) which is involved in the toxin biosynthesis [34]. The core amino acid sequence of *A. brunneitoxicaria* unknown peptide however, revealed the existence of a disulfide bond (Cys-Cys) instead of the tryptathionine linkage.

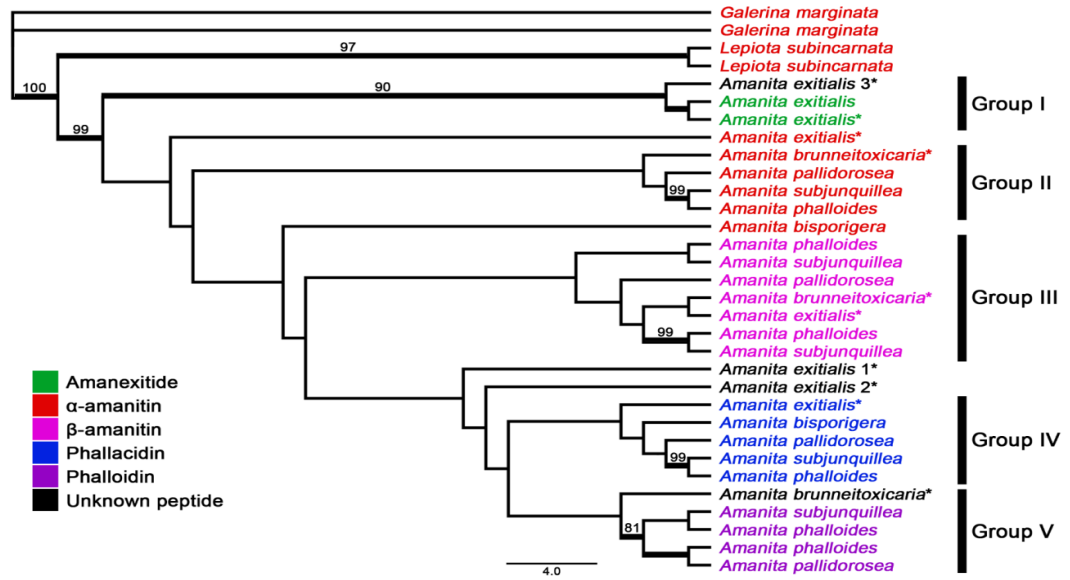

Figure 3 ML phylogeny of MSDIN family members. Numbers above branches are bootstrap values (≥ 75 %) and posterior probabilities (≥ 0.95) are represented as bold branches. Nucleotide sequences obtained in this study are indicated by asterisks.

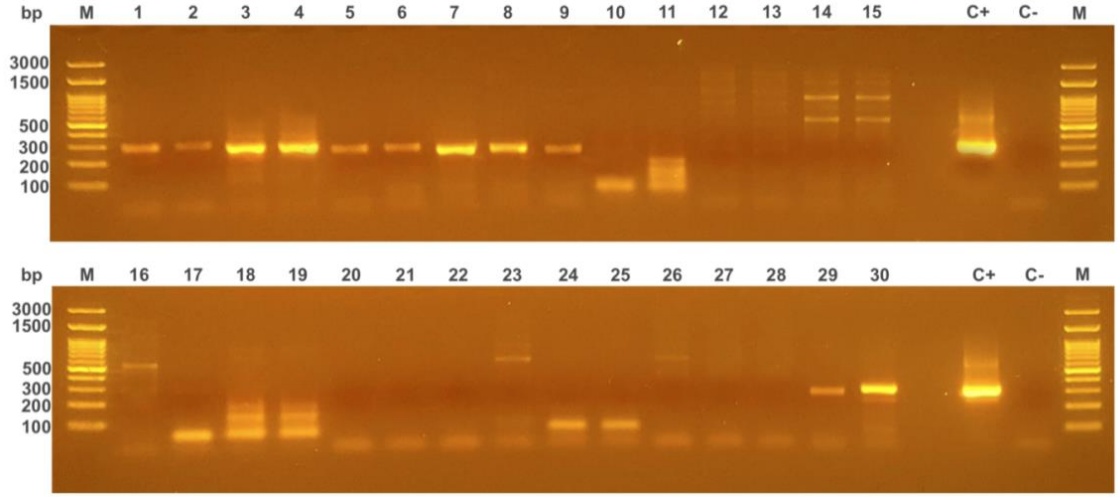

Figure 4 PCR amplification products of the MSDIN family. Lane M: DNA marker, lanes 1 - 5: *A. brunneitoxicaria* (clinical specimens), lanes 6 - 8: *A. exitialis* (clinical specimens), lane 9: *A. fuliginea* (clinical specimen), lanes 10 - 11: *A. digitosa* (clinical & field specimens), lanes 12 - 13: *A.* aff. *fuligineodisca* (field specimens), lanes 14 - 15: *A. hemibapha* (field specimens), lanes 16 - 17: *A. mira* (field specimens), lanes 18 - 19: *A. javanica* (field specimens), lanes 20 - 21: *A. princeps* (field specimens), lanes 22 - 23: *A. porphyria* (field specimens), lanes 24 - 25: *A. rubromarginata* (field specimens), lanes 26 - 28: *A. vaginata* (field specimens), lane 29: *A. brunneitoxicaria* (field specimen), lane 30: *A. exitialis* (field specimen), lane C+: PCR positive control and lane C-: PCR negative control.

PCR-based detection of the MSDIN family

Prior to using the modified forward primer, ABAE-F, the PCR experiments were performed following Li *et al.* [15]. The original forward and reverse primers designed by Hallen *et al.* [14] and Li *et al.* [15] were employed and they produced faint DNA bands of the MSDIN family. The primer ABAE-F was therefore, incorporated into the PCR reactions to obtain clear and distinctive DNA fragments of approximately 300 bp (**Figure 4**). This target region of the MSDIN family was detected from the lethal

mushroom samples in the section *Phalloideae*, including *A. brunneitoxicaria*, *A. exitialis*, *A. fuliginea*. For other sections which included edible and inedible (non-deadly) species of *Amanita*, no PCR product was obtained. Hence, this specific amplification of the MSDIN family could be employed as a molecular marker to distinguish the deadly poisonous amanitas from other species of the same genus.

Evaluation of the MSDIN family based on PCR was accurate for toxin detection without further analysis of DNA sequencing. In terms of the detection sensitivity however, conventional PCR was shown to be 100-fold less sensitive than the newly developed method of hyperbranched rolling circle amplification [35].

Conclusions

Based on the NGS technology the diversity of the toxin-associated genes in *A. brunneitoxicaria* could be elucidated. The MSDIN family present in this species confirmed the ability to produce the lethal toxins, α-amanitin and β-amanitin. The established PCR method based on the MSDIN family could be used to detect 3 deadly poisonous species of *Amanita*, and further applied to other clinical samples obtained as unidentified mushroom remnants. This rapid and accurate identification of the lethal toxin-producing genes in the poisonous mushrooms is certainly important for the patients to receive timely medical treatment and hence, can help reduce mortality.

Acknowledgements

This work was financially supported by the National Institute of Health, Department of Medical Sciences, Ministry of Public Health and the Thailand Science Research and Innovation (TSRI) [grant number 6662/2019].

References

[1] RE Tulloss. *Amanita* - distribution in the Americas with comparison to eastern and southern Asia and notes on spore character variation with latitude and ecology. *Mycotaxon* 2005; **93**, 189-231.
[2] ZL Yang. *Die Amanita-Arten von Südwestchina*. J Cramer, Lehre, Germany, 1997.
[3] M Weiss, ZL Yang and F Oberwinkler. Molecular phylogenetic studies in the genus *Amanita*. *Can. J. Bot.* 1998; **76**, 1170-9.
[4] Q Cai, RE Tulloss, LP Tang, B Tolgor, P Zhang, ZH Chen and ZL Yang. Multi-locus phylogeny of lethal amanitas: Implications for species diversity and historical biogeography. *BMC Evol. Biol.* 2014; **14**, 143.
[5] B Thongbai, SL Miller, M Stadler, K Wittstein, KD Hyde, S Lumyong and O Raspé. Study of three interesting *Amanita* species from Thailand: Morphology, multiple-gene phylogeny and toxin analysis. *PLoS One* 2017; **12**, e0182131.
[6] S Ramchiun, S Sikaphan, S Leudang, D Polputpisatkul, N Nantachaiphong, T Khaentaw, N Nooron, C Uttawichai and S Parnmen. Molecular characterization and liquid chromatography-mass spectrometric multiple reaction monitoring-based detection in case of suspected phalloides syndrome poisoning. *J. Assoc. Med. Sci.* 2019; **52**, 48-55.
[7] K Chaiear, R Limpaiboon, C Meechai and Y Poovorawan. Fatal mushroom poisoning caused by *Amanita virosa* in Thailand. *Southeast Asian J. Trop. Med. Publ. Health* 1999; **30**, 157-60.
[8] S Parnmen, S Sikaphan, S Leudang, T Boonpratuang, A Rangsiruji and K Naksuwankul. Molecular identification of poisonous mushrooms using nuclear ITS region and peptide toxins: A retrospective study on fatal cases in Thailand. *J. Toxicol. Sci.* 2016; **41**, 65-76.
[9] H Luo, HE Hallen-Adams, JS Scott-Craig and JD Walton. Ribosomal biosynthesis of α-amanitin in *Galerina marginata*. *Fungal Genet. Biol.* 2012; **49**, 123-9.
[10] RM Sgambelluri, S Epis, D Sassera, H Luo, ER Angelos and JD Walton. Profiling of amatoxins and phallotoxins in the genus *Lepiota* by liquid chromatography combined with UV absorbance and mass spectrometry. *Toxins* 2014; **6**, 2336-47.
[11] A Bresinsky and H Besl. *A colour atlas of poisonous fungi: A handbook for pharmacists, doctors, and biologists*. Wolfe Publishing, London, 1990.
[12] DA Bushnell, P Cramer and RD Kornberg. Structural basis of transcription: α-amanitin-RNA polymerase II cocrystal at 2.8 Å resolution. *Proc. Natl. Acad. Sci. Unit. States Am.* 2002; **99**, 1218-22.

[13] JA Pulman, KL Childs, RM Sgambelluri and JD Walton. Expansion and diversification of the MSDIN family of cyclic peptide genes in the poisonous agarics *Amanita phalloides* and *A. bisporigera*. *BMC Genom.* 2016; **17**, 1038.
[14] HE Hallen, H Luo, JS Scott-Craig and JD Walton. Gene family encoding the major toxins of lethal *Amanita* mushrooms. *Proc. Natl. Acad. Sci. Unit. States Am.* 2007; **104**, 19097-101.
[15] P Li, W Deng and T Li. The molecular diversity of toxin gene families in lethal *Amanita* mushrooms. *Toxicon* 2014; **83**, 59-68.
[16] H Luo, Q Cai, Y Luli, X Li, R Sinha, HE Hallen-Adams and ZL Yang. The MSDIN family in amanitin-producing mushrooms and evolution of the prolyl oligopeptidase genes. *IMA Fungus* 2018; **9**, 225-42.
[17] H Luo, S-Y Hong, RM Sgambelluri, E Angelos, X Li and JD Walton. Peptide macrocyclization catalyzed by a prolyl oligopeptidase involved in α-amanitin biosynthesis. *Chem. Biol.* 2014; **21**, 1610-7.
[18] CH Zhang, JP Zou, WQ Deng, TH Li and Z Jiang. Molecular cloning and the expression pattern of AePOPB involved in the α-amanitin biosynthesis in *Amanita exitialis* fruiting bodies. *Gene* 2018; **662**, 123-30.
[19] Z He, P Long, F Fang, S Li, P Zhang and Z Chen. Diversity of MSDIN family members in amanitin-producing mushrooms and the phylogeny of the MSDIN and prolyl oligopeptidase genes. *BMC Genom.* 2020; **21**, 440.
[20] M Gardes and TD Bruns. ITS primers with enhanced specificity for basidiomycetes-application to the identification of mycorrhizae and rusts. *Mol. Ecol.* 1993; **2**, 113-8.
[21] TJ White, TD Bruns, SB Lee and JW Taylor. *Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics*. *In*: MA Innis, DH Gelfand, JJ Sninsky and T White (Eds.). PCR Protocols: A guide to methods and applications. Academic Press, New York, 1990, p. 315-22.
[22] B Langmead and SL Salzberg. Fast gapped-read alignment with Bowtie 2. *Nat. Meth.* 2012; **9**, 357-9.
[23] A Morgulis, G Coulouris, Y Raytselis, TL Madden, R Agarwala and AA Schaffer. Database indexing for production MegaBLAST searches. *Bioinformatics* 2008; **24**, 1757-64.
[24] Z Zhang, S Schwartz, L Wagner and W Miller. A greedy algorithm for aligning DNA sequences. *J. Comput. Biol.* 2000; **7**, 203-14.
[25] S Henikoff and JG Henikoff. Performance evaluation of amino acid substitution matrices. *Proteins* 1993; **17**, 49-61.
[26] GE Crooks, G Hon, JM Chandonia and SE Brenner. WebLogo: A sequence logo generator. *Genome Res.* 2004; **14**, 1188-90.
[27] RC Edgar. MUSCLE: Multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res*. 2004; **32**, 1792-7.
[28] MA Miller, W Pfeiffer and T Schwartz. Creating the CIPRES science gateway for inference of large phylogenetic trees. *In*: Proceeding of the Gateway Computing Environments Workshop, New Orleans, United Sates. 2010, p. 1-8.
[29] A Stamatakis. RAxML version 8: A tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* 2014; **30**, 1312-3.
[30] F Ronquist, M Teslenko, PVD Mark, DL Ayres, A Darling, S Höhna, B Larget, L Liu, MA Suchard and JP Huelsenbeck. MrBayes 3.2: Efficient bayesian phylogenetic inference and model choice across a large model space. *Syst. Biol.* 2012; **61**, 539-42.
[31] A Tawatsin, S Parnmen, U Thavara, P Siriyasatien and P Kongtip. Mushroom poisoning in Thailand: Incidence and intoxication to human health. *Med. Res. Arch.* 2018; **6**, 1-12.
[32] P Zhang, ZH Chen, B Xiao, B Tolgor, HY Bao and ZL Yang. Lethal amanitas of East Asia characterized by morphological and molecular data. *Fungal Divers.* 2010; **42**, 119-33.
[33] H Luo, HE Hallen-Adams and JD Walton. Processing of the phalloidin proprotein by prolyl oligopeptidase from the mushroom *Conocybe albipes*. *J. Biol. Chem.* 2009; **284**, 18070-7.
[34] JD Walton, HE Hallen-Adams and H Luo. Ribosomal biosynthesis of the cyclic peptide toxins of *Amanita* mushrooms. *Biopolymers* 2010; **94**, 659-64.
[35] Z He, T Luo, F Fan, P Zhang and Z Chen. Universal identification of lethal amanitas by using Hyperbranched rolling circle amplification based on α-amanitin gene sequences. *Food Chem*. 2019; **298**, 125031.