DNA-BASED IDENTIFICATION OF GASTROINTESTINAL IRRITANT MUSHROOMS IN THE GENUS *CHLOROPHYLLUM*: A FOOD POISONING CASE IN THAILAND

Siriwan Leudang, Sujitra Sikaphan, Sittiporn Parnmen*, Nattaphong Nantachaiphong, Dutsadee Polputpisatkul, Sathaporn Ramchiun, Punthip Teeyapant

Toxicology Center, National Institute of Health, Department of Medical Sciences, Ministry of Public Health, Nonthaburi, 11000, Thailand

ABSTRACT:

Background: Food poisoning caused by mushrooms in local Thai populations has increased annually. Gastrointestinal irritant (GI) mushrooms are the most common cause of food poisoning. In general, poisonous mushrooms are routinely identified based on morphological characteristics; however, standard methods for morphological identification do not always yield satisfactory results. Therefore, the objective of this study was to use the nuclear internal transcribed spacer (ITS) regions and the nuclear large subunit (nuLSU) ribosomal DNA sequences as a species marker for GI mushrooms as well as to identify toxins using a reversed phase LC-MS method.

Methods: Mushroom samples obtained from clinically reported cases during 2014 to 2015 were used in this study. The maximum likelihood and maximum parsimony methods were employed for estimating the phylogenetic trees. Mushroom toxins were identified by liquid chromatography-mass spectrometry.

Results: Based on the Barcode of Life Database (BOLD) revealed the highest identity for all samples tested with scores ranging from 98.06% to 99.86%, while BLAST search yielded 99% to 100% of poisonous mushroom samples to *Chlorophyllum molybdites* and *C. globosum*. Clade characterization was performed by maximum likelihood and maximum parsimony. The combined analyses of ITS and nuLSU revealed a better resolution of the phylogenetic tree with two important clades. Clade I contains member of *C. molybdites*, while all *C. globosum* samples belongs to clade II. Detection of the peptide toxins revealed the presence of amatoxins in *C. globosum.* Alpha-amanitin and beta-amanitin were detected in *C. globosum* sample with the amount of toxins indicated as 0.0059 and 0.0013 mg per gram of mushrooms dry weight, respectively.

Conclusion: DNA-based identification confirmed that the mushrooms ingested by patients were *C. molybdites* and *C. globosum*. Both of these poisonous mushroom species provided new and informative data for future clinical studies in Thailand.

Keywords: Amatoxins; *Chlorophyllum*; Gastrointestinal irritant; Internal transcribed spacer; Large subunit ribosomal DNA

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INTRODUCTION

Wild mushrooms are favorite food for local Thai populations. The most commonly consumed wild mushrooms are *Amanita princeps*, *Amanita hemibapha*, *Astraeus hygrometricus*, *Boletus edulis*, *Cantharellus cinnabarinus*, *Gyroporus cyanescens*, *Heimiella retispora*, *Lactarius hatsudake*, *Lepista nuda*

* *Correspondence to: Sittiporn Parnmen*
E-mail: sittiporn.p@dmsc.mail.go.th

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Table 1 Clinical manifestation of patients and mushroom samples used in this study

Case	Year	Region of Thailand	No. of patients	Latent period	Symptom	Mushroom samples ID
1	2014	South	4	2.30 hr	Intense thirsty, nausea, vomiting, abdominal pain and severe diarrhea	DMSC09255
2	2014	West	4	2 hr	Thirsty, nausea vomiting and severe diarrhea	DMSC09538
3	2014	Northeast	16	1.30 hr	Nausea and vomiting, abdominal pain, fatigue and diarrhea	DMSC11138
4	2015	East	2	1.30 hr	Abdominal pain, nausea, vomiting and diarrhea	DMSC04364, DMSC04365
5	2015	Northeast	5	1.30 hr	Nausea, vomiting, diarrhea and fatigue	DMSC07290, DMSC07291
6	2015	Northeast	8	1-7 hr	Abdominal pain, severe vomiting and watery diarrhea	DMSC 09391
7	2015	Northeast	1	2 hr	Vomiting, diarrhea and fatigue	DMSC13590
8	2015	North	3	2 hr	Spontaneous vomiting, watery diarrhea and fatigue	DMSC14088

and *Russula virescens* [1, 2]. Some mushroom genera include both deadly poisonous species and valued edible species such as *Amanita* and *Russula*. Here is the most common poisonous mushrooms including *Amanita digitosa*, *A. exitialis*, *A. gleocystidiosa*, *A. fuliginea*, *A. pyriformis*, *A. virosa*, *Chlorophyllum molybdites*, *Entoloma* sp., *Inocybe* sp. and *Russula emetica* [3–6]. These poisonous mushrooms are sometimes misidentified as resemble edible species. Mushroom toxicity presents after ingestion of toxic substances. These symptoms can vary from gastrointestinal irritants (GI) to severe cytotoxic effects resulting in death of patients. According to the data provided by the Bureau of Epidemiology (Thailand), the annual mortality rate of mushroom poisoning has been increasing particularly in the rainy season [7]. Mushroom poisoning is an emergency medical situation for physicians. Thus, identification of mushroom samples and their toxic substance is needed for appropriate medical treatments.

Nowadays molecular methods have become important tools for rapid species identification in various groups of fungi [5, 8–11]. The most popular loci are the nuclear internal transcribed spacer (ITS) region and the nuclear large subunit (nuLSU) ribosomal DNA. The ITS region has been chosen as universal barcode marker for fungi [10]. This region clearly showed a barcode gap between intra- and interspecific variation [10]. The nuLSU is less variable than the ITS regions; however, this area is readily amplified from a large group of mushrooms and contain a valuable source of phylogenetic information [5, 9, 11, 12]. In addition, the availability of sequence data in GenBank and the Consortium for the Barcode of Life has constructed the Barcode of Life Database (BOLD) together with an online identification system (IDS), which can be compared and identified the sequences of interests with the online databases [13]. Thus, the aim of this study was to identify gastrointestinal irritant mushrooms in the genus *Chlorophyllum* using the ITS regions and the nuclear large subunit of ribosomal DNA sequences as a species marker based on phylogenetic approaches. We also aimed to diagnose peptide toxins using liquid chromatography-mass spectrometry (LC-MS).

METHODS

Case reports

Details of the case reports of *Chlorophyllum* mushroom poisoning and samples obtained from eight clinically reported cases during 2014 to 2015 were summarized (Table 1). Mushroom samples were harvested by the local epidemiologists and delivered to toxicology center. In some case, the patient brought samples of the mushrooms that they ate to the hospital. A total of 43 patients with typical gastrointestinal syndrome after mushroom ingestion were revealed in the Table 1.

Mushroom samples and molecular methods

Total genomic DNA was isolated using DNeasy™ Plant Mini Kit according to

manufacturer's guide. A dilution of 1:10 of the total genomic DNA was used for PCR amplifications. Samples were PCR amplified and/or sequenced using the ITS1F and ITS4 primers [14, 15] for the nuclear internal transcribed spacer (ITS) region and the LROR, LR5 and LR6 primers [16] for the nuclear large subunit of ribosomal DNA (nuLSU). PCR reactions were conducted on GeneAmp® PCR System 9700 Thermal Cycler (Applied Biosystems®, USA) and reactions were carried out for 34 cycles with PCR profiles of 45 sec at 94°C (denaturation), 45 sec at 52-55°C (annealing) and 1.30 min at 72°C (extension) with final extension of 72°C for 10 min. 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 each primer, 1 µl of genomic DNA template and 9.5 µl nuclease-free water. Amplification products will be cleaned using either QIAquick PCR Purification Kit (QIAGEN) or QIAquick Gel Extraction Kit (QIAGEN) and eluted with 35 µl of elution buffer. DNA sequencing analyses were performed by Macrogen Inc. in Korea.

Molecular identification

Specimens and sequences used for the molecular analysis are showed in Appendix 1. Sequence alignment was done using Geneious Pro 5.4.3 (http://www.geneious.com/) and edited conflicts manually. Nucleotide similarity was performed using the BLAST server in GenBank (http://blast.ncbi.nlm.nih.gov) and the Barcode of Life Database (BOLD) [13]. Phylogenetic trees were performed using maximum likelihood (ML) and maximum parsimony (MP). Maximum likelihood analyses were analyzed in RAxML 7.2.6 using the GTRGAMMA model [17]. Maximum parsimony analyses were performed using PAUP* version 4.0b [18]. The settings for MP were as follows. Outgroup was defined. Heuristic searches setting optimality criterion with parsimony were employed. All characters are of type unordered and have equal weight. Initial MaxTrees setting equaled 100. Branches collapsed (creating polytomies) if maximum branch length is zero. MulTrees option is in effect. The topological constraints were not enforced. Gaps were treated as missing. Starting tree(s) was obtained via stepwise addition. Branch-swapping algorithm was used the tree-bisection-reconnection (TBR) method. Support was then estimated by performing 1000 bootstrap pseudoreplicates. Only clades that received bootstrap support equal or above 70 % under ML and MP were considered as strongly supported. Phylogenetic trees were visualized using the program FigTree (http://tree.bio.ed.ac.uk/ software/FigTree).

Toxins detection

Five gram of mushroom samples were blended and extracted with 20 ml of methanol. The extract was incubated at 60 °C for 10 min, followed by centrifugation at 8000*g* for 5 min. The clear supernatant liquid was decanted to dryness under a stream of nitrogen. Toxins including α-amanitin and β-amanitin (Sigma-Aldrich, USA) used as a references standard library. Toxins were separated using LC methods as described by Chung et al. [19]. The analyses were performed using a reversed phase LC-MS method on Agilent technologies 1100 series LC/MSD system (Agilent, USA).

RESULTS

Molecular identification

Twenty-two new sequences were generated for this study (Appendix 1). Nuclear ITS based-identification using BOLD revealed the highest identity for all samples tested with scores ranging from 98.06% to 99.86%, while BLAST search yielded 99% to 100%. Both databases exhibited identical species identification for *Chlorophyllum globosum* and *C. molybdites*.

Phylogenetic studies of the genus *Chlorophyllum* was carried out using the nuclear ITS and nuLSU sequences. A matrix of 1688 unambiguously aligned nucleotide positions was constructed (763 in nuITS and 925 in nuLSU) and 1124 characters were constant. The topology of the trees from MP and ML analyses did not show any conflict and hence only the ML tree is shown here (Figure 1). Most of mushroom samples are clustered in clades I and II (Figure 1). Clade I comprise only a member of *Chlorophyllum molybdites* (Figure 2E). Morphologically, *C. molybdites* is characterized by pileus umbonate with brown squamules, surface white, cheilocystidia 50−60 × 10−17 µm, broadly clavate and basidiospores 8−9.6(−12) × 5−6.1(−7) µm [average quotient (Q_m) = 1.6±0.13], ellipsoid, smooth, hyaline and thick-walled. Clade II contains a sample of *Chlorophyllum globosum* (Figure 2A). This species is characterized by pileus convex, covered with concentrically arranged patches of brown to dark brown colored squamules, surface pale brown, cheilocystidia 28−35 × 8−10 µm, clavate and basidiospores 8−9.8(−11) × 5−6.5(−8) µm [average quotient (Q_m) = 1.5±0.14], ellipsoid,

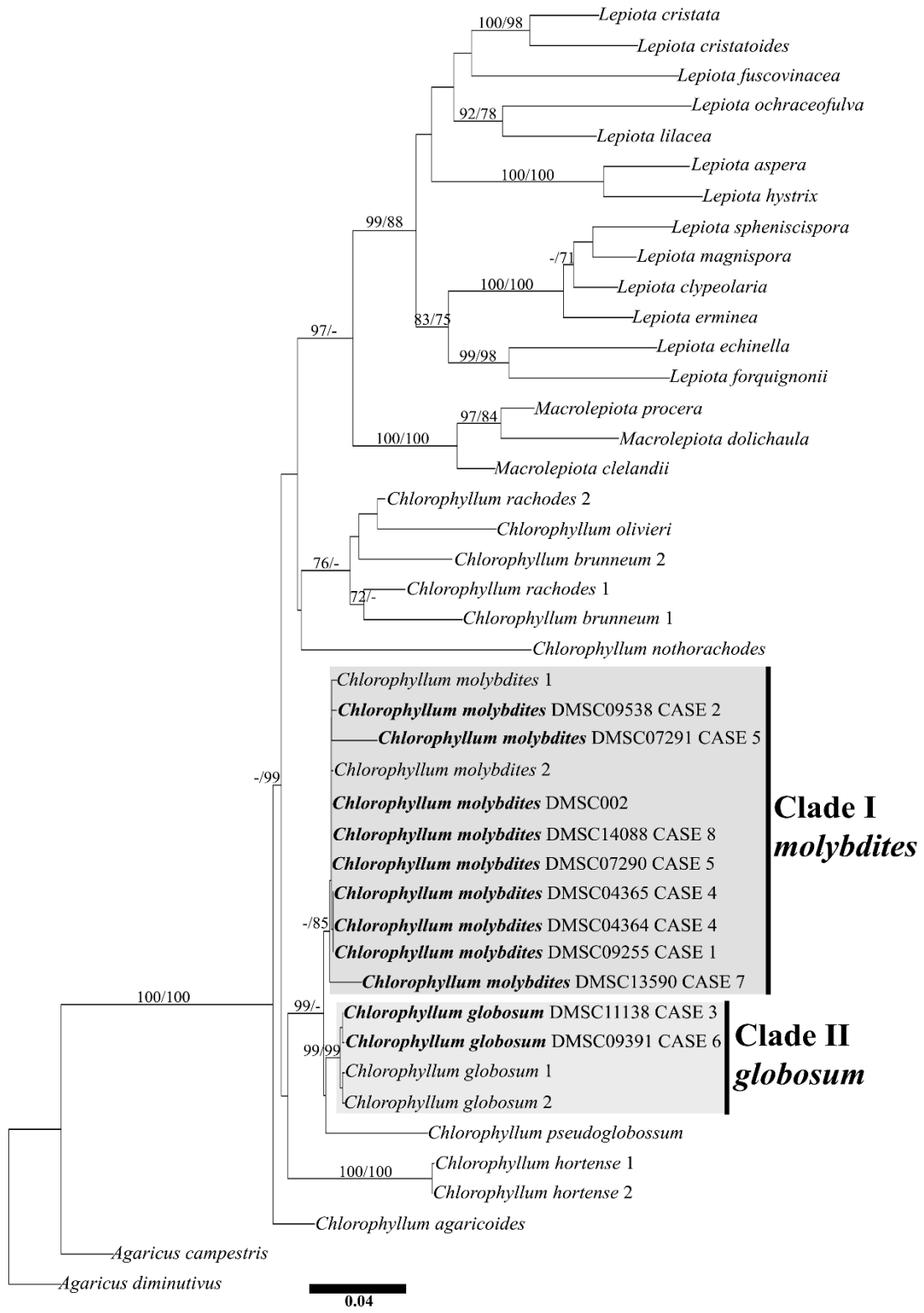

Figure 1 Best-scoring maximum-likelihood tree based on combined data set of the internal transcribed spacer (ITS) and the nuclear large subunit (nuLSU) ribosomal DNA sequences. Bootstrap supports (ML/MP) are given in numbers above branches (ML/MP). Reference sequences were downloaded from GenBank. Sequences derived from mushroom samples (DMSC) are in bold

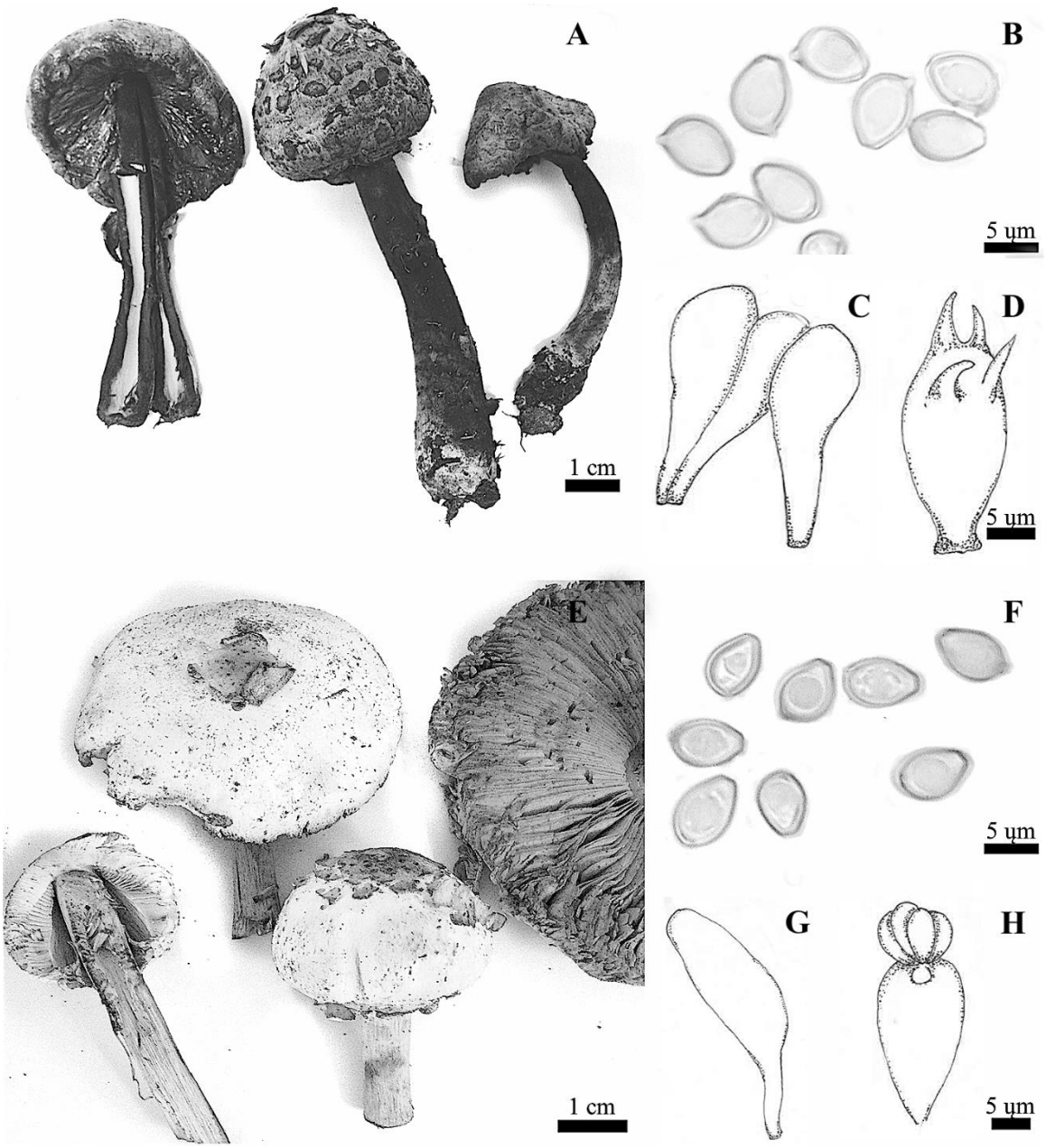

Figure 2 Morphological characteristics of *Chlorophyllum.* (A) *Chlorophyllum globosum* (DMSC09391) with (B) basidiospores, (C) clavate cheilocystidia and (D) 4-spored basidia; (E) *Chlorophyllum molybdites* (DMSC14088) with (F) basidiospores, (G) broadly clavate cheilocystidia and (H) 4-spored basidia

smooth, hyaline and thick-walled.

Toxins detection

The standard reference material and two purified compounds obtained from the mushroom samples were analyzed with MS and the corresponding molecular weights were calculated based on their molecular ion peaks. The molecular weights of these compounds were identical to that of the standard peptide toxins. Of the 11 mushroom samples assayed, only a sample of *C. globosum* contained amatoxins (Figure 2). The MS method generated positive results for alpha-amanitin (*m/z* 919.3, RT = 13.865 min) and beta-amanitin (*m/z* 920.3, RT = 11.046 min) (Figure 3). All alpha-amanitin and beta -amanitin were detected in *C. globosum* sample with the amount of toxins (per gram of mushrooms, dry weight) indicated as 0.0059 and 0.0013 mg, respectively.

DISCUSSION AND CONCLUSION

Based on our Toxicology Center database, we analyzed toxic substances following poisonous

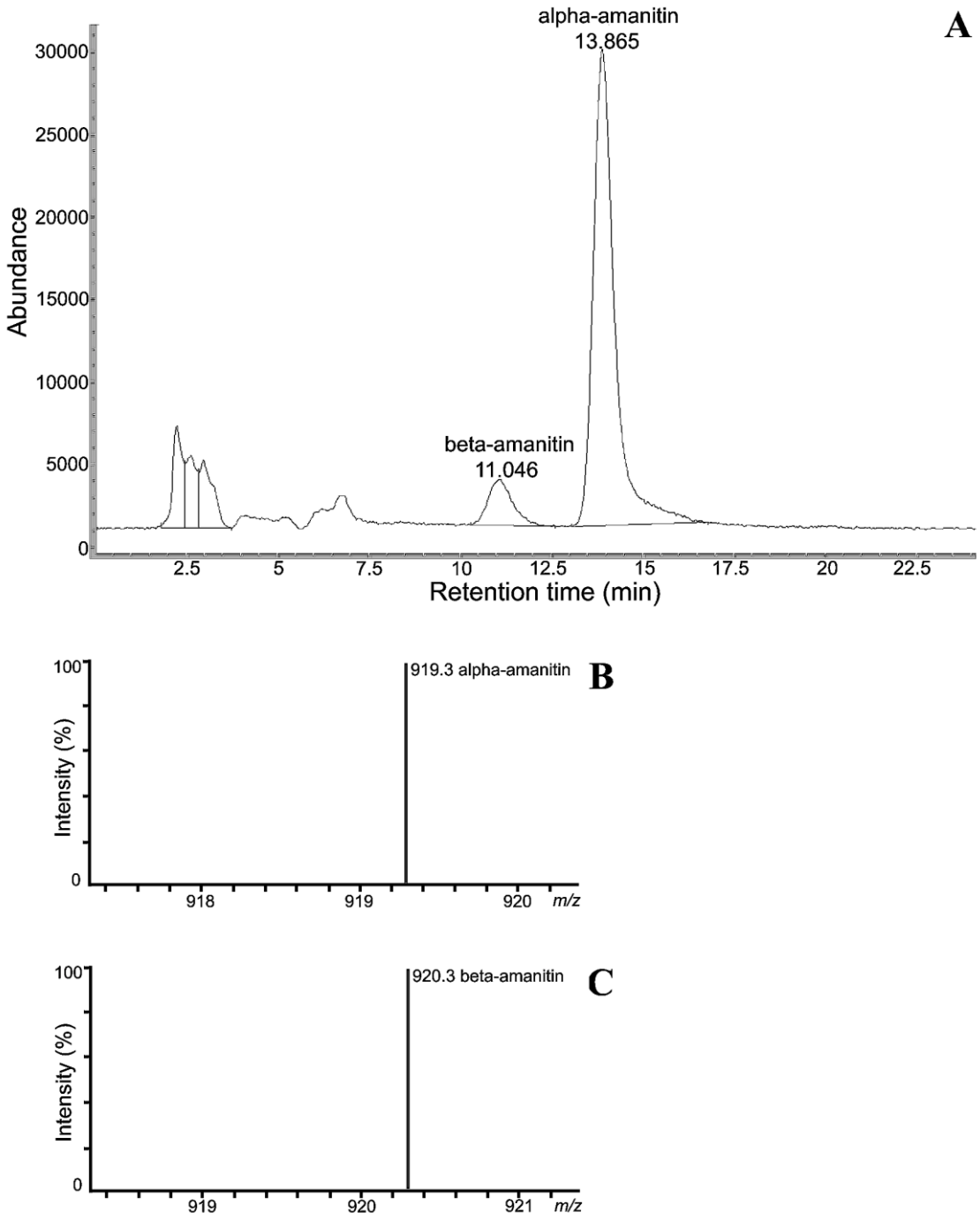

Figure 3 Identification of amanitins using LC-MS. (A) Two-dimensional abundance data and retention time (RT), (B) mass spectrum of alpha-amanitin and (C) mass spectrum of beta-amanitin

mushroom ingestion from the years 2007 to 2014. A total of 220 mushroom samples were analyzed. Most of samples were identified to 76% gastrointestinal irritant (GI) mushrooms, 14% amanitin-containing mushrooms and 10% muscarine-containing mushrooms, respectively. The number of GI mushroom ingestions was found at a very high percentage. Most GI mushroom belongs to the genus *Chlorophyllum*. In this study, we focused on mushroom samples that caused gastrointestinal irritation during a 2-year period. A mushroom samples obtained from eight clinically reported cases were used. The remnant samples of mushrooms harvested by the patients were delivered to our laboratory. Based on their morphology, most samples were primarily identified as *Chlorophyllum molybdites*. This genus is a common mushroom genera in Agaricaceae [20]. Within the genus

Chlorophyllum is containing both edible and poisonous species. In Thailand, there are currently two recognized species including *C. humei*, *C. molybdites* and *C. rhacodes* [2]. The toxic species inhibited is *C. molybdites*, whereas *C. humei* and *C. rhacodes* are no general information for mushroom edibility.

Interestingly, the nuclear ITS sequence-based identification revealed that some mushroom samples ingested are genetically similar to *C. globosum*. Hence, the mushroom samples acquired from 2014 to 2015 were re-examined using the molecular data, peptide toxin analysis and morphological data. We analyzed the combined two molecular loci dataset to infer phylogenetic relationships by using maximum likelihood and maximum parsimony methods. Our results showed that mushroom samples from eight clinically reported cases were separated into two clades including *C. molybdites* and *C. globosum* clades. *Chlorophyllum molybdites* differs from *C. globosum* in having a larger pileus and cheilocystidia. Moreover, based on our findings *C. globosum* contained alpha-amanitin and beta-amanitin with an average level of 0.0059 and 0.0013 mg/g dry weight, respectively. There was no any report on toxic substances occurring in this species. Having ingested the poisonous *C. molybdites*, all patients showed a short latent period of 1 to 2 hours after the meal, nausea, vomiting, severe diarrhea and abdominal pain. The clinical symptoms described in the cases were similar to those of gastrointestinal syndrome revealed by Bresinsky and Besl [21]. In case of *C. globosum*, latent period is longer than above case. There was no death reported in our resulted from the ingestion of *C. globosum*. Although amanitins were detected in *C. globosum*, but there was in low concentration. According to Duffy [22] the amanitins are potently toxic to humans with a lethal dose of alpha-form *ca*. 0.1 mg/kg of body weight.

In conclusion, we suggest that DNA-based identification is particularly suitable for detection and diagnosis of gastrointestinal irritant mushrooms in the genus *Chlorophyllum*. This method can separate the species of *Chlorophyllum* which contain amatoxins. Discovery of *C. globosum* and *C. molybdites* were new for the clinical records of mushroom poisoning in Thailand.

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Appendix 1 Details of samples, their localities, voucher specimens and GenBank accession numbers. New accession numbers obtained in this study are in bold

Taxon	Locality and voucher specimen	GenBank accession number	
		ITS	LSU
Agaricus campestris	USA, PBM2580 (CUW)	DQ486682	DQ110871
Agaricus diminutivus	USA, E.C. Vellinga2360 (UCB)	AF482831	AF482877
Chlorophyllum agaricoides	AFTOL-ID 440	DQ200928	AY700187
Chlorophyllum brunneum 1	USA, E.C. Vellinga 2317 (UCB)	AF482856	-
Chlorophyllum brunneum 2	USA, E.C. Vellinga 2361 (UCB)	-	AF482886
Chlorophyllum globosum 1	Cameroon, D.C. Mossebo	AY243619	-
Chlorophyllum globosum 2	Nigeria, H. Neda N421 (TFM)	AF482842	-
Chlorophyllum globosum	Thailand, DMSC09391	**KU049671**	**KU049678**
Chlorophyllum globosum	Thailand, DMSC11138	**KP229776**	**KU049677**
Chlorophyllum hortense 1	USA, D.E. Hemmes 1365 (SFSU)	AF482843	-
Chlorophyllum hortense 2	China, GDGM57301	HG976895	HG976896
Chlorophyllum molybdites 1	USA, R.W. Kerrigan 1920 (herb. Kerrigan)	AY243618	U85303
Chlorophyllum molybdites 2	USA, DUKE-JJ162	U85309	U85274
Chlorophyllum molybdites	Thailand, DMSC002	**KP229777**	**KU049681**
Chlorophyllum molybdites	Thailand, DMSC09255	**KP229774**	**KU049679**
Chlorophyllum molybdites	Thailand, DMSC09538	**KP229775**	**KU049680**
Chlorophyllum molybdites	Thailand, DMSC04364	**KU049670**	**KU049682**
Chlorophyllum molybdites	Thailand, DMSC04365	**KU049672**	**KU049683**
Chlorophyllum molybdites	Thailand, DMSC07290	**KU049673**	**KU049684**
Chlorophyllum molybdites	Thailand, DMSC07291	**KU049674**	**KU049685**
Chlorophyllum molybdites	Thailand, DMSC13590	**KU049675**	**KU049686**
Chlorophyllum molybdites	Thailand, DMSC14088	**KU049676**	**KU049687**
Chlorophyllum nothorachodes	Australia, H. Lepp1142 (CANB)	AF482855	-
Chlorophyllum olivieri	Netherlands, E.C. Vellinga 2230 (L)	AF482846	AF482887
Chlorophyllum pseudoglobossum	India, CUH AM155	KP642506	KR080484
Chlorophyllum rachodes 1	Netherlands, E.C. Vellinga 2106 (L)	AF482849	-
Chlorophyllum rachodes 2	Germany, M. Enderle (L)	-	AY176345
Lepiota aspera	Netherlands, E.C. Vellinga 2233 (L)	AY176354	AY207219
Lepiota clypeolaria	Germany, E.C. Vellinga 1683 (L)	AY176361	AY176362
Lepiota cristata	USA, E.C. Vellinga 2780 (UC)	GQ203806	DQ457685

Appendix 1 Details of samples, their localities, voucher specimens and GenBank accession numbers. New accession numbers obtained in this study are in bold (cont.)

Taxon	Locality and voucher specimen	GenBank accession number	
		ITS	LSU
Lepiota cristatoides	Netherlands, H.A. Huijser s.n.(herb.Huijser)	AY176363	AY176364
Lepiota echinella	Belgium, H.A. Huijser s.n.(herb.Huijser)	AY176366	AY176367
Lepiota ermine	USA, N. S. Weber 2947 (MICH)	AY176357	AY176358
Lepiota forquignonii	Netherlands, E.C. Vellinga 2284 (L)	AY176370	AY176371
Lepiota fuscovinacea	Netherlands,E.C. Vellinga 2255 (L)	AY176372	AY176373
Lepiota hystrix	France, H.A. Huijser s.n (herb. Huijser)	AY176377	AY176378
Lepiota lilacea	USA, E.C. Vellinga 2451 (UCB)	AY176379	AY176380
Lepiota magnispora	Netherlands, H.A. Huijser	AF391023	AY176381
Lepiota ochraceofulva	Netherlands, E.C. Vellinga 2273 (L)	AY176386	AY176387
Lepiota spheniscispora	USA, E.C. Vellinga 2559	AF391004	AY176404
Macrolepiota clelandii	Australia, K.R. Thiele 2650 (MEL)	AF482838	AF482882
Macrolepiota dolichaula	China, HKAS 38718	DQ221111	DQ411537
Macrolepiota procera	Colombia, NY-EFM539	U85310	AF482880