Novel cyclic peptides from lethal *Amanita* species through a genomic approach, and major peptide toxins in the ectomycorrhizal association

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Abstract

Background: Most species in the genus *Amanita* are ectomycorrhizal fungi, and the cyclic peptide toxins that some species produce are notoriously deadly. In total, around 25 of these peptides were found in the fruiting bodies over the past 82 years, and whether any of them are present in the mycorrhizae is unknown. Reportedly, sequenced lethal *Amanita* genomes harbor a significant number of precursor genes of MSDIN family, indicating there could be a much larger capacity for cyclic peptide production in these mushrooms. However, it is largely unknown that to what extent these genes are transcribed, and further, translated into true cyclic peptides.

Method: In this study, three poisonous *Amanita* species, *A. rimosa*, *A. exitialis* and *A. subjunquillea*, were sequenced through PacBio and Illumina techniques. For expression analysis, one strain of *A. subjunquillea* was sequenced through RNA-Seq. A genome-guided approach was adopted to identify cyclic peptides by coupling predicted toxin-biosynthetic genes with mass spectrometry (MS and MS/MS). To investigate whether any of the toxins were express in the microbiome, profiling of known major toxins was conducted on *A. subjunquillea* mycorrhizae via HRMS and gene cloning.

Results: The resultant genomes showed significant potential to produce known and unknown cyclic peptides. Together with our 2 previously sequenced genomes, in total 37 unknown MSDIN genes were discovered. Expression of over 90% of the MSDIN genes was demonstrated in two strains of *A. subjunquillea*. Through the genome-guided approach, 12 MSDIN genes were found to produce true, novel cyclic peptides with no additional posttranslational modifications. When the ectomycorrhizae of *A. subjunquillea* were analyzed by MS, all major toxins were detected. The corresponding MSDINs for these cyclic peptides were successfully cloned directly from the mycorrhizae.

Conclusions: The genome-guided approach provided a speedy method to identify cyclic peptides both in *Amanita* mushrooms and in the ectomycorrhizae. In this study, a significant number of novel MSDIN genes were discovered, most of which were found to be expressed in the tested species. The identification of the 12 novel cyclic peptides strongly suggests that *Amanita* species possess a much larger reservoir of these peptides than previously thought. This is the first report to demonstrate that the cyclic peptides in *Amanita* species are expressed in the mycorrhizal association. All four major toxins, α -amanitin, β -amanitin, phalloidin and phalloidin, are found to be present in the symbiosis, offering new clues to their biological function(s).

Background

The genus *Amanita* (Persoon 1797) is a double-faced twist: on one hand, it harbors some of the best known gourmet mushrooms such as *A. caesarea*, once favored by emperors of the Roman Empire [1]; on the other, it causes over 90% of deadly mushroom poisonings worldwide as the collectors have little knowledge of distinguishing the edible from the deadly [2–4]. Majority of the genus are important ectomycorrhizal fungi that have associations with more than ten families of trees [5]; thus, they are important to forest health. So far toxin studies are based on fruiting bodies and, to the best of our knowledge, there is literally no reports on toxins in *Amanita* mycorrhizae.

Researches on toxins in amanitin-producing (referred as deadly or lethal in this report) *Amanita* mushrooms date back to the 19th century, but due to technical difficulties in extracting, purifying and analyzing the chemical substances, significant advances were not made until the 20th century [6]. Specifically, Lynen purified one of the major toxins, phalloidin, in 1938 [7]. Then, α -amanitin was purified and crystalized three years later [8, 9]. The death cap, *A. phalloides*, became commonly known for its deadly poison in 1951 from death reports [10]. As research progresses, two major types of toxins have been found in dozens of lethal *Amanita* species, i.e., amatoxins and phallotoxins. The amatoxins function as a highly specific, efficient inhibitor of eukaryotic RNA polymerase II, and the phallotoxins effectively prevent depolymerization of actin fibers [6]. It has been shown that amatoxins and phallotoxins in *Amanita* are synthesized on ribosomes, which represents the first ribosomal cyclic peptide discovered in Kingdom Fungi [11]. They are encoded by MSDIN gene family as precursor peptides of 34–37 amino acids. The precursors are then cleaved and macrocyclized into 7–10 amino acid cyclic peptides by a specialized prolyl oligopeptidase, POPB [12, 13]. It is a bifunctional enzyme catalyzing both hydrolysis of peptide bond and transpeptidation. To achieve mature state of the toxins, many of these resultant cyclic peptides need to undergo further posttranslational modifications, mainly including multiple hydroxylations, sulfoxidation, epimerization, and formation of a cross-bridge between Trp and Cys, in which order these reactions occur is unknown. The MSDIN genes encoding amatoxins, such as α - and β -amanitins, and phallotoxins, such as phalloidin and phalloidin, are readily found in the sequenced genomes of lethal *Amanita* species [11, 14]. In addition, there are 20 to 30 other unknown MSDIN genes discovered in each of these genomes. In the cases of *A. bisporigera* and *A. phalloides*, roughly half of the unknown MSDIN genes are found to be expressed at the transcription level, and the results of mass spectrometry indicate that two of the genes can produce corresponding cyclic peptides [15]. In the past 10 years, only a few novel cyclic peptides have been discovered, including amanexitide in *A. exitialis* [16], and CylF and CylE in *A. phalloides* [15]. In total, roughly 25 cyclic

peptides are known to human knowledge [1, 17]. In contrast, newly sequenced genomes of lethal *Amanita* species show that the MSDIN genes far outnumber the known cyclic peptides, indicating there could be a much larger potential for novel cyclic peptides in these mushrooms. However, it is largely unknown whether these MSDINs translate into true cyclic peptides.

Conventional methods for peptide characterization in *Amanita* mainly use TLC, HPLC, hydrolysis, and NMR techniques, which in general need significant amount of the subjects examined [1, 6, 18, 19]. Suggested by HPLC analyses in many laboratories, the most significant peptide production in *Amanita* species is attributed to the major toxins, i.e., α -amanita, β -amanitin, phalloidin and phalloidin [13, 20, 21]. Noticeably, a number of less significant peaks were present in the vicinity of these known peptides, many of which are of insufficient amount for further analyses via conventional methods. In light of this, a more sensitive method would be well appreciated.

Functionally speaking, cyclic peptides produced by *Amanita* species are more diverse than commonly known, as they are apparently more than toxins. Antamanide, a cyclic decapeptide, protects mice from phalloidin toxicity. Through competition, the molecule effectively inhibits the uptake of phalloidin by OATP1B1 transporter of the liver cells [22]; the same transporter is used by other phallotoxins [23]. Newer finding suggests the peptide is a novel inhibitor of the mitochondrial permeability transition pore [24]. Cycloamanides (CylA, CylB, CylC and CylD), antamanide, and their synthetic derivatives display immunosuppressive activity in both in vivo and in vitro assays [25–27]. In *A. phalloides*, researchers suspected that CylE and CylF are related to cycloamanides due to sequence similarities [6]. These examples have placed many of the cyclic peptides outside the toxin category, indicating that they may have diverse functions in *Amanita* mushrooms.

In this study, two lethal *Amanita* species, *A. rimosa* and *A. exitialis*, and an independent strain of *A. subjunquillea* were sequenced and MSDIN genes mined. Together with our two previous sequenced species, *A. pallidiorosea* and *A. subjunquillea*, five sets of MSDIN genes were analyzed using combined methods. Cyclic peptides were predicted by directly using amino acid sequences in the core regions of the MSDIN genes. Modifications after cyclization, such as hydroxylation, sulfoxidation and cross-bridge formation, were also taken into consideration. Possible molecular formulas with and without further modifications were generated for all candidate peptides. Through high resolution MS, masses matching putative cyclic peptides were mined. Further amino acid composition was elucidated based on MS/MS results, which were analyzed manually and via multiple peptide analyzing platforms. Mass spectrometry was also applied to plant root tips associated with *A. subjunquillea* for detection of known cyclic peptide toxins.

Results

Amanita genomes

Sequencing-quality fruiting bodies of *A. exitialis* (Ae) and *A. rimosa* (Ar) were collected in excellent condition and stored in liquid nitrogen (Fig. 1). For Ae, the N50 of the draft genome was 3 Mb with the genome size of 49 Mb (GC content = 46.6%), and the assembly contained 129 contigs. For Ar, the N50 was 690 Kb with the genome size at 45 Mb (GC content = 45.99%), and the assembly comprised 134 contigs. An independent strain of *A. subjunquillea* (As), AsYYH, was sequenced for comparison analysis and the genome had the N50 of 830 Kb. The draft size reached 77 Mb (GC content = 46.57%) with the assembly containing 119 contigs. These genomes were only meant for mining MSDIN genes, and therefore further details of the genomes will be discussed elsewhere.

Mining MSDIN sequences for candidate cyclic peptides

With a combination of multiple known MSDINs as queries, the genomes revealed the presence of MSDIN genes in all three sequenced *Amanita* species, i.e. As (strain AsYYH), Ae, and Ar, yielding 27, 23 and 39 MSDIN genes, respectively (Table 1–3). In total 89 MSDINs were discovered, and 37 were novel. All genomes possessed MSDIN genes coding for at least three major cyclic peptide toxins, namely α -amanitin, β -amanitin and phalloidin. Overall, the precursor genes share similar structures, designated as leader peptide, core peptide and recognition sequence [28]. Based on the newly discovered MSDINs, linear and cyclic peptides with and without further modifications, were predicted. A schematic diagram of the genome-guided approach was illustrated in Fig. 2.

Table 1
The MSDIN gene family from two independent strains of *Amanita subjunquillea*

Leader Peptide	Core Peptide	Recognition Sequence	Monoisotopic Mass	Expressed
MSDINATCLP	IWGIGCNP	CVGDEVAALLTRGEALC	918.3541(α -amanitin)	✓
MSDINATRLP	IWGIGCDP	CVGDEVTALLTRGEALC	919.3382 (β -amanitin)	✓
MSDINATRLP	IWGIGCDP	CIGDDVTALLTRGEALC	919.3382 (β -amanitin)	✓
MSDINATRLP	AWLATCP	CAGDDVNPTLTRGESLC	788.316 (phalloidin)	✓
MSDINATRLP	AWLVDCP	CVGDDINRRVVSAFA-C	846.3217 (phalloidin)	✓
MSDMNATRLP	LIQRPFP	CVSDDVDFALIRRCALVYAESSV	922.5461	✓
MSDINTARLP	HFAFIPP	CIGDDIEMVLKRGESLC	896.4617	✓
MSDINTARLP	TFLPPLFVPP ¹	CVSDDIEMVLTRGESLC	1108.6394	✓
MSDINATRLP	LNILPFMLPP	CVGDDVNPTLTRGEDLC	1135.6536	✓
MSDMNATRLP	LIQRPYAP	CVSDDVNSPLTRGESLC	938.5410	✓
MSDINTARLP	IGRPESIP	CVGDDIEMILERGQKLC	849.4781	✓
MSDINTARLP	LRLPPFMIPP	CVGDDIGMVLTRGENLC	1161.6805	✓
MSDVNATRLP	FNFFRFPYP	CIGDDSASVLGLGESLC	1215.5938	✓
MSDINATRLP	SSVLPRP	CVGDVDNIILTSREKLC	736.4304	✓
MSDMNVARLP	ISDPTAYP	CVGGDIHAVLRRGE	844.4039	×
MSDMNVARLP	ISDPTAYP	CVGGDIHAVLRRGE	844.4039	×
MSDINVTCLP	FIFWFFWPP	CVGDDAASIIK-GK	1267.6291	×
MSDINAARLP	FIFPPFFIPP	CVSDDIEMVLTRGE	1202.6601	×
MSDINTARLP ¹	AFFPPFFIPP ²	CVSDDIEMVLTRGESLC	1160.6131	✓
MSDINATRLP ¹	IPILPIPP	YCSDDANTTLTLGESLC	840.5545	✓
MSDINATRLP ¹	LFLAALGIP	-SDDADSTLTRGESLC	1008.6444	✓
MSDTNDARLP ¹	LFFWFWFLWP	SVSDDIDSVLNRGEDLC	1469.7397	✓
MSDINTVCLP	VQKPWSRP	CVGDDIEMILERGEDLC	978.5472	✓
MSDINTAALP	FFFPPFFIPP	CVSDDIEMVLTRGENLC	1236.6444	✓
MSDINITRLP	FFPIVFIPP	CIGDDAASIVKQGENLC	1057.6073	✓
MSDINTVCLP	LQKPWSRP	CVGDDIEMILERGE	992.5628	N/A
MFDINITRLP	IFWFIYFP	CVGDDVTALLTRGE	1113.5761	N/A
¹ Gray shade indicates MSDIN genes shared by the two strains. Black box for MSDINs present only in strain AsYYH; box of dashed line for MSDINs present only in the previously reported strain; ^ for newly found MSDINs compared to the previous report.				
² Red letters present differences in MSDINs from the two strains.				
³ Green letters indicate alternative splicing.				
⁴ <u> </u> and underlined letters indicate novel cyclic peptides detected with MS and MS/MS.				
⁵ The monoisotopic masses are for unmodified cyclic peptides based on MSDIN core peptides, except for major toxins.				

Table 2
 The MSDIN gene family in *Amanita rimosa*

Leader Peptide	Core Peptide	Recognition Sequence	Monoisotopic Mass
MSDINSTRLP	IWGIGCNP	SVGDEVTALLTRGEA	918.3541 (α -amanitin)
MSDINATRLP	IWGIGCNP	SVGDEVTALLASGEA	918.3541 (α -amanitin)
MSDINATRLP	IWGIGCDP	CVGDDVAALTTRGEA	919.3382 (β -amanitin)
MSDINATRVLP	AWLVDCP	CVGDDISRLLTRGEK	846.3217 (phalloidin)
MSDINATRLP	AWDSKHP	CVGDDVSRLLTRGE	821.3893
MSDINATRLP	AWDSKHP	CVGDDISRLLTRGE	821.3893
MSDINATRVLP	AWLAACP	CVGDDISHLLTRGE	770.3494
MSDINASRLP	FFIIVKP	CGNPYVSDDVNSTLTRGE	957.6124
MSDINTSRLP	FIPLGIITILP ¹	CVSDDVNTTITRGD	1177.7547
MSDINTACLP	FLFPVIPP	CLSEDANVVVLNSGE	910.5316
MSDINVTRLP	FFPIVFIPP	CI	1057.6073
MSDINIARLP	IFWFIYFP	CVGDDVDNTLSRGE	1113.576
MSDINVTRLP	IFLIMFIPP	CIGDDAASILKQGE	1071.6263
MSDINTSCLP	IFIAFPIPP	CVSDDIQTVLTRGE	995.5917
MSDTNTACLP	IFIAFPIPP	CVSDDIQTVLTRGE	995.5917
MSDINASRLP	ILKKPWAP	SVCDDVNSTLTRGE	933.5872
MSDINVARLP	ISDPTAYP ¹	CVGDDIQAVVKRGE	844.4039
MSDINATRLP	IIIVGLIIP	LCVSDIEMILTRGE	1044.7383
MSDINASRLP	IILAPIIP	CISDDVNTTLTCAE	830.5702
MSDINTTGLP	HFYNLMPP	CFSDDTGMVLVRGE	999.4709
MSDINATRLP	HPFPLGLQP	CAGDVDNFTLIKGE	986.541
MSDINASCLP	LILVANGMAYV	-SDDVSPTLTRGE	1144.6387
MSDINTARLP	SYIPFPPP	CLSEDNAVLMLGE	898.4661
MSDINTARLP	SYIPFPPP	CLSEDNAVLMLGE	898.4661
MSDINTSRFP	SYGYRAFP	CVGDDVEMVLMHGE	941.4468
MSDINVTRLP	VLVFIFFLP	CISDDAASIIKLGE	1075.6543
MSDIDTTRLP	LILFTLQP	SIGDDVNPTLTRGEK	925.5709
MSDIHAARLP	FPTRPVFP ¹	SAGDDMIEVVLGRGE	941.5196
MSDNNAARLP	FYFYLGP	SDDAHPILTRGERLA	1000.5058
MSDTNTARLP	ILFIQLEIP	CISDDVHPVLTTRGE	1066.6426
MSDVNTTRLP	FNFFRFPYP	CICDDSEKVELGE	1215.5865
MSEINTARFP	NHGHRTIP	CVGDDIEMVLMHGE	912.4678

¹ and underlined letters indicate novel cyclic peptides detected with MS and MS/MS.

²The monoisotopic masses are for unmodified cyclic peptides based on MSDIN core peptides, except for major toxins.

Leader Peptide	Core Peptide	Recognition Sequence	Monoisotopic Mass
MSEINTSRLP	LVFIPPYFAP	CVSDDIQMVLTLGE	1144.632
MFDMNNTTCLP	GFIIYAYV	–GDDVNHTLTRGE	926.4901
MLDINTARLP	FSLPTFPP	CVSDEIDVVLKRGE	886.4588
MLDINATRFPP	LGRPTHLP	CVGDDVNYIL	871.5027
MTDINDARLP	ILLLIFFWIP	CANDDDENILNRG	1255.7732
MTDINDTRLP	FVWILWLWLA	CVGDDTSILNRGE	1327.748
MPDINVTRLP	LLIIVLLTP	CISDDNNILNRGK	975.6732
¹ <u> </u> and underlined letters indicate novel cyclic peptides detected with MS and MS/MS.			
² The monoisotopic masses are for unmodified cyclic peptides based on MSDIN core peptides, except for major toxins.			

Table 3
 □ The MSDIN gene family in *Amanita exitialis*

Leader Peptide	Core Peptide	Recognition Sequence	Monoisotopic Mass
MSDINATRLP	IWGIGCNP	CVGDDVTSVLTRGEA	918.3541(α -amanitin)
MSDINATRLP	IWGIGCDP	CVGDDVTALLTRGEA	919.3382(β -amanitin)
MSDINATRLP	AWLVDCP	CVGDDVNRLLTRGE	846.3217(phalloidin)
MSDINATRLP	AWLTDCP	CVGDDVNRLLTRGE	786.3443
MSDINTTRLP	FVFVASPP ¹	CVGDDIAMVLTRGE	844.4556
MSDINTARLP	FIWVFGIP	G-DDIGTVLTRGEK	959.5342
MSDINLTRLP	GIIAIP	CVGDDDDVNSTLTRGQ	677.4549
MSDINATRLP	IILAPVIP	CISDDNDP-TLTRGQ	816.5546
MSDINTARLP	IPIPPFFFP	FVSDDIEIVLRRGEK	1055.5917
MSDINTARLP	IPIPPFFFP	FVSDDIEIVLRRGEK	1055.5917
MSDINATRLP	IGRPQLLP	CVGGDVNYILISGEK	874.5461
MSDINPTRLP	IFWFIYFP	CVSDVDST-LTRGE	1113.5761
MSDINTARLP	IYRPPFYALP	CVGDDIQAVLTRGE	1217.6670
MSDINTARLP	IWIIGNP	CVSDDVERILTRGE	906.5400
MSDINVIRAP	LLILSILP	CVGDDIEV-LRRGE	862.5964
MSDINATRLP	LFFPPDFRPP ¹	CVGDADNFTLTRGEK	1213.6357
MSDINATRLP	LFFPPDFRPP ¹	CVGDADNFTLTRGE	1213.6357
MSDINVIRLP	SMLTILPP	CVSDDASNTLTRGE	852.4852
MSDINTARLP	VFSLPVFFP ¹	-SDDIQAVLTRGE	1033.5709
MSDINVTRLP	VFIFFFIIPP	CVGDGTADIVRKGEK	1107.6230
MSDINATRLP	VWIGYSP	CVGDDCIALLTRGE	802.4086
MSDINATRLP	VWIGYSP	CVGDDCIALLTRGE	802.4086
MTDINDTRLP	FIWLLWIWLP	SVGDD-NNILNRGEE	1367.7867
¹ □ and underlined letters show novel and known cyclic peptides detected with MS and MS/MS.			
² The monoisotopic masses are for unmodified cyclic peptides based on MSDIN core peptides, except for major toxins.			

Transcriptome of *As*

As was chosen to be sequenced via Illumina RNA-Seq technique, as this was a readily available species. The specific sample was collected only a few meters apart from the strain *AsYYH*. In total 11.7 Gb clean reads were obtained. The assembled transcriptome yielded 46.1 Mb with 25,453 unigenes. BLAST search against the transcriptome with MSDINs from the two *As* genomes produced 24 MSDINs, four more than those in our previous result [14]. These four sequences were used as queries to re-search the genome, and the result confirmed their presence (Table 1). For the previously sequenced genome, 22 out of the 24 MSDIN genes were found to be expressed at the transcription level, i.e., 91.7% of the MSDINs were expressed (Table 1). For the new genome of the strain *AsYYH*, 23 out of 25 genes, i.e. 92.0%, were expressed (Table 1). Our result showed that most of MSDINs were expressed at the transcription level in *As*. Regarding the gene structure, exons and introns were conserved among most of the MSDIN genes, while alternative splicing was detected in two of the MSDIN transcripts marked in green in Table 1. The expressed MSDINs were the focus for potential cyclic peptide production in the following MS and MS/MS analyses.

With the Agilent LC-HRMS platform, correlations between measured masses and predicted peptides based on genomic data were carefully assessed. There were no linear versions of these peptides detected. Further, hydroxylation(s), sulfoxidation and cross-bridging, by themselves or by various combinations, were not detected on predicted linear or cyclic peptides. However, two matches were found between theoretical and measured masses of two predicted cyclic peptides. These two matches corresponded to the cyclized core peptide sequences of TFLPPLFVPP (named CylG1) and AFFPPFFIPP (named CylG2) without further modifications, respectively. The molecular formula for the candidate new cyclic peptide CylG1 is $C_{59}H_{84}N_{10}O_{11}$, and the theoretical molecular weight is 1109.6394 $[M + H]^+$. The measured molecular weight was 1109.6398 $[M + H]^+$, with mass discrepancy of 0.36 ppm (Fig. 3A). The molecular formula for CylG2 is $C_{65}H_{80}N_{10}O_{10}$ with the theoretical molecular weight at 1161.6131 $[M + H]^+$, and the measured molecular weight was 1161.6161 $[M + H]^+$, with mass discrepancy of 2.53 ppm (Fig. 3B). Two adduct ions of $[M + Na]^+$ and $[M + K]^+$ were shown in the figure as well. CylG1 and CylG2 were treated as candidate new cyclic peptides for further characterization.

In order to determine amino acid composition of CylG1 and CylG2, the candidate cyclic peptides were submitted to LC-MS/MS. The resultant spectra were first analyzed via Center for Computational Mass Spectrometry (CCMS) using the database of UniProtKB/Swiss-prot (Fig. 3C). The obtained peptide sequence for CylG1 was PPLVFTPPLE, only one amino acid different at the C-terminal (E vs. F, one letter codes used here and below for easy comparison), and this was due to that the CCMS only used linear peptide databases (Fig. 3C underlined). The molecular weight of F is 165.19 and losing H_2O will make the mass 147.18, close to that of E (147.13) and therefore accounting for the presence of E over F. For CylG2, similar processes were applied, and discrepancy was also found at the last predicted amino acid (Fig. 3D underlined). Some of our peptides did not return any results with CCMS, and they were then analyzed with other platforms (Mascot, pNovo and XCMS). In general, the results were largely in line with the above. Although the automated pipelines offered some evidence, we mostly relied on the following manual process to determine the peptide sequences.

Manual amino acid composition analysis was mostly based on y-type fragmentation of linear peptides. For CylG1, fragment ions were calculated using the core peptide, and the result of searching for these ions in the MS/MS spectrum was shown in Fig. 3E. Every fragment ion was manually checked and confirmed. Illustrated in Fig. 3E, y-type fragments (y2 to y9) were shown to be in strong agreement with the core peptide, and all the amino acids could be readily explained by the y-type ions. Immonium ions for P, L and F were also identified for CylG1. As a result, CylG1 was assigned as a novel cyclic peptide with the amino acid composition and combinations matching the circular MSDIN core peptide sequence Cyclo(TFLPPLFVPP) (Fig. 3C, E). CylG2 underwent the same analyses, in which case immonium ions for P, I and F were identified. Similarly, y-type fragments (y2 to y9) were shown to be in strong agreement with the core peptide, and all the amino acids complied with the y-type fragment ions (Fig. 3D, F). In conclusion, CylG2 was confirmed to be a novel cyclic peptide with the sequence Cyclo(AFFPPFFIPP). The peptide only differed from antamanide, Cyclo(FFVPPAFFPP), by one amino acid (I vs. V) when they were compared in circular structures.

Cloning MSDIN genes and LC-HRMS analysis in *As* AsBJ strain

Because the two cyclic peptides, CylG1 and CylG2, were not detected in the AsBJ strain, we were intrigued to see if the corresponding MSDINs were present. All attempted PCR reactions came back negative, while all the controls for α - and β -amanitins were positive. The result was consistent with that the lack of the two cyclic peptides in AsBJ was due to the absence of the two corresponding MSDIN genes.

Novel cyclic peptides in other *Amanita* species

CylG1 and CylG2 in *As* were our first effort towards finding novel cyclic peptides in the genus. Taking advantage of this genome-guided approach, we analyzed three other *Amanita* species, i.e., *A. pallidiorosea* (Apa), *Ae*, and *Ar*. In total, 10 additional new cyclic peptides were discovered (Table 4). Relevant data analyses of mass spectrometry were included in the supplementary information (Additional file 1: Figures S1–S6). Three new cyclic peptides were found in *Ar*, and their sequences, in the same order of their corresponding MSDIN core peptides, were Cyclo(ISDPTAYP), Cyclo(FIPLGIITILP) and Cyclo(FPTRPVFP) (Additional file 1: Figures S1 and S4), which were named CylH1, CylH2, and CylH3 respectively. Five new cyclic peptides were found in *Apa*, and their sequences were Cyclo(EFIVFGIFP), Cyclo(FVIIPPFIFP), Cyclo(YFNDHPP), Cyclo(TIHLFSAP) and Cyclo(MHILAPPP) (Additional file 1: Figures S2 and S5), which were named CylI1, CylI2, CylI3, CylI4, and CylI5, respectively. Further information on MSDIN genes in *Apa* were included in Additional file 1: Table S1. Two new cyclic peptides were found in *Ae*, and their peptide sequences were Cyclo(FVFVASPP) and Cyclo(LFFPPDFRPP) (Additional file 1: Figures S3 and S6), which were named CylJ1 and CylJ2 respectively. An previously known cyclic peptide called amanexitide with the sequence Cyclo(VFSLPVFF) [16] was also found in this study (Additional file 1: Figures S7). In conclusion, 12 novel and one known cyclic peptides were discovered in the four sequenced species (Table 4).

Table 4
Twelve novel and one known cyclic peptides discovered in *Amanita subjunquillea*, *A. rimosa*, *A. exitialis* and *A. pallidorozea*.

Species	Cyclopeptide sequence	Molecular formula	Theoretical(m/z)	Measured(m/z)	δ (ppm)
<i>A. subjunquillea</i>	TFLPPLFVPP (CylG1)	C ₅₉ H ₈₄ N ₁₀ O ₁₁	1109.6394	1109.6398	0.36
	AFFPPFFIPP (CylG2)	C ₆₅ H ₈₀ N ₁₀ O ₁₀	1161.6131	1161.6161	2.53
<i>A. rimosa</i>	ISDPTAYP* (CylH1)	C ₃₉ H ₅₆ N ₈ O ₁₃	845.4039	845.4040	0.36
	FIPLGIITILP (CylH2)	C ₆₁ H ₉₉ N ₁₁ O ₁₂	1178.7547	1178.7555	0.64
	FPTRPVFP (CylH3)	C ₄₈ H ₆₇ N ₁₁ O ₉	942.5196	942.5191	0.53
<i>A. pallidorozea</i>	EFIVFGIFP (CylI1)	C ₅₆ H ₇₅ N ₉ O ₁₁	1050.5658	1050.5694	3.35
	FVIIPPFIFP (CylI2)	C ₆₅ H ₉₀ N ₁₀ O ₁₀	1171.6914	1171.6941	2.30
	YFFNDHPP (CylI3)	C ₅₁ H ₅₉ N ₁₁ O ₁₂	1018.4417	1018.4421	0.35
	TIHLFSAP (CylI4)	C ₄₂ H ₆₂ N ₁₀ O ₁₀	867.4723	867.4733	1.14
	MHILAPPP (CylI5)	C ₄₁ H ₆₄ N ₁₀ O ₈ S	857.4702	857.4714	1.39
<i>A. exitialis</i>	FVVASPP (CylJ1)	C ₄₄ H ₆₀ N ₈ O ₉	845.4556	845.4582	3.07
	LFFPPDFRPP [#] (CylJ2)	C ₆₃ H ₈₃ N ₁₃ O ₁₂	1214.6357	1214.6357	0.00
	VFSLPVFFP [^]	C ₅₆ H ₇₅ N ₉ O ₁₀	1034.5709	1034.5734	1.97
¹ *Indicates that the sequence has been found in <i>A. subjunquillea</i> , <i>A. pallidorozea</i> and <i>A. rimosa</i> .					
² #The superscript number 2 indicates this sequence has two copies.					
³ ^Indicates amanexitide.					

LC-HRMS analysis on *As mycorrhizae* and gene cloning for major toxins

Successful tracing of *As* hyphae to plant roots were carried out in this study. Figure 4A illustrated a fresh fruiting body of *As* showing apparent association with a network of plant roots, and 4B displayed a cross section of a selected root tip with signs of mycorrhizal symbiosis. All four major toxins, i.e., α -amanitin, β -amanitin, phalloidin and phalloidin, were detected in LC-HRMS, with mass discrepancies of 2.12, 0.50, 0.76, and 2.93 ppm, respectively (Fig. 5). Cloning of the MSDINs for the major toxins was successful and the corresponding MSDINs of the toxins were shown above each section of the figure. We were not able to quantify the toxins because of limited amount of the material. However, α -amanitin was clearly the most significant as it showed as a robust peak in the HPLC chromatograms.

Discussion

To date, most cyclic peptides in *Amanita* are first discovered from *A. phalloides* with a few exceptions [6]. It has been suspected that lethal *Amanita* species could produce significant more cyclic peptides than what have been discovered [1, 6]. The genome-guided approach adopted in this study identified 12 novel cyclic peptides in four lethal *Amanita* species, providing strong support in this perspective. Around half of these peptides did not produce discernable peaks in HPLC chromatograms, indicating MS is highly sensitive in identifying these molecules. The discovered cyclic peptides lack further modifications besides cyclization and therefore can be readily synthesized for the purpose of screening for useful activities. Cyclic peptide pools in different species, even in different strains, have distinctions, although the core set (major toxins) is relatively stable. As a result, the collection of the peptides in dozens of known lethal species in the genus should constitute a large cyclic peptide reservoir, potentially valuable for useful biological activities. Cyclic peptides from *Amanita* including the toxins are highly valuable resources. α -Amanitin has been shown to have high potential in cancer research and treatment [29–31]. Phalloidin and phalloidin are valuable in cell research as generic molecular tools [32]. Antamanide functions as an antitoxin,

reversing the effort of phalloidin [33], and researchers now show that antamanide inhibits the mitochondrial permeability transition pore by targeting the regulator cyclophilin D [24]. In addition, cycloamanides and the related peptides possess potent activities useful in immunity-related medical situations [25, 26]. Logically, unknown cyclic peptides in *Amanita* species could be a valuable source for useful activities.

Cyclic peptide toxins in *Amanita* mushrooms have been considered to carry out a protective role against insects and other animals for the fruiting bodies, although the thought rarely materializes in publications. The finding of all four major toxins in *As mycorrhizae* was a rather unanticipated outcome. This was completely unknown to science mainly due to the fact that it is difficult to trace the mycelia from the mushrooms to the mycorrhizae, and these fungi are largely not culturable at the moment. As part of our other ongoing project, we investigated the toxin profile in the *As mycorrhizae*. The unexpected result is clearly against the previous hypothesis that the toxins were merely present in fruiting bodies [11]. This may be an indication that these toxins have role(s) in mycorrhizal symbiosis in lethal *Amanita* species. In *Galerina marginata*, the toxin production is detected in mycelia cultured in artificial media [34], serving as another example that the toxins could be produced outside the fruiting bodies. It would be close to a pure conjecture at this point to comment on the roles of the toxins in the mycorrhizal association. It could be that the fungi need to fend off animals or other fungi in soil, and it is also possible that the toxins play other unknown role(s). Given the molecular mechanism of α -amanitin, i.e. inhibition of RNA polymerase II, could it have regulatory function at the level of transcription? Does it have anything to do with the establishment of mycorrhizae in lethal *Amanita*? *Amanita phalloides* has rapidly spread from Europe to North America [35]; could the cyclic peptides aid in the process? It is largely blank in this area and perhaps all we can do at present is to keep an open mind. The pattern of the HPLC chromatograms on the mycorrhizae were very different from those of fruiting bodies. Although we were not able to quantify these toxins, α -amanitin was clearly the most significant shown as a major peak in the HPLC analysis. In contrast, the other three were present as small bumps in the chromatograms.

We believe that the genome-guided approach with mass spectrometry offered reliable results in this study. Regarding the data of LC-MS/MS, the measured molecular masses of the obtained cyclic peptides were all within 5 ppm mass discrepancies. MS/MS analyses showed that amino acid composition and combinations in these molecules matched nicely to their corresponding MSDIN core peptide sequences. When the *AsBJ* strain of *As* was analyzed, it was found through PCR that, the corresponding MSDINs for the two novel cyclic peptides were missing, coinciding with that the two novel peptides, CylG1 and CylG2, were absent from the strain. As another line of support, Cyclo(VFSLPVFFP) was found in *Ae* in this study, and this cyclic peptide was also discovered via biochemistry from the same species in 2011 [16]. These authors also showed that MS/MS analysis for this unmodified cyclic peptide was reliable. Last, manual analyses of MS and MS/MS data were the key for the pipeline. MS required good sample preparation and separation. Suitable MS/MS spectra oftentimes needed tweaking with collision energy and sample amounts. Fragment ions frequently could be recognized only by manual inspection.

Most of the MSDINs were expressed at the transcription level in *As*, as suggested by the RNA-Seq data. Previous studies detected as many as half of MSDINs expressed in *A. phalloides* and *A. bisporigera* [15], and additional expressed sequences were cloned from other *Amanita* species through RT-PCR [36, 37]. In our results with deep sequencing at 11.7 Gb data yield, around 92% MSDINs were shown to have corresponding transcripts in both strains. This indicates MSDINs are mostly real, active genes, which could potentially lead to production of many more true cyclic peptides than previously known in the genus. Our results also strongly suggest that, the amanitin biosynthetic pathway is highly versatile, capable of producing a wide range of cyclic peptides.

Strain differences were apparent in this study. It was observed that the *AsBJ* strain of *As* showed differential cyclic peptide production. In light of this, the strain *AsYYH* was sequenced, and similar results were obtained. Out of 27 MSDIN genes discovered in the two *As* genomes, 22 were shared, with two specific to the former sequenced strain [14], and three unique to *AsYYH*. The results showed that even at strain level, MSDIN gene sets from independent genomes can vary. The two independent strains sequenced were from two locations that were only a few hundred meters apart. This result suggests that MSDIN genes are highly dynamic, except for the core ones encoding major amatoxins and phallotoxins.

Regarding the structures of the novel cyclic peptides, CylG1 and CylG2 have a characteristic amino acid composition with double Pro residues, no Trp and Cys, and without any further posttranslational modifications. They and amanexitide share similarities to antamanide in that they are all decapeptides with a conserved sequence of XXXPPXXXPP; the two pairs of Pro residues are frequently found in MSDINs. Without Trp and Cys, sulfoxidation and tryptathionine are prohibited. According to the MS/MS data, hydroxylation on the 13 obtained peptides was not detected. Pro (100%), Phe (84.6%) and Ile (53.8%) were the most frequent residues in these cyclic peptides. CylG2 is very similar to antamanide with only one amino acid difference (Ile vs. Val). In *Ar*, the new peptide Cyclo(FIPLGIITILP), CylH2, has 11 amino acids, which is by far the longest in all *Amanita* cyclic peptides discovered. Despite of some structure similarities, the biological activities of these peptides require further study.

Posttranslational modifications other than cyclization, such as hydroxylation and sulfoxidation, were not detected in this study. The observation is probably due to that our current model pertaining to how these modifications occur and in which order, may not be entirely correct. And it is also possible that there may be other unknown modifications involved, which introduces more possibilities. Most of the cyclic peptides found in this study are present in low concentrations and therefore cannot be readily purified to a significant quantity. However, being without further modification does convey an advantage, as they can be readily synthesized at reasonable costs. Part of our on-going project is to produce and to screen these peptides against various targets.

Conclusion

This report provided a genome-guided approach for discovering cyclic peptides in lethal *Amanita* mushrooms. Three deadly *Amanita* species were sequenced, and in total 37 novel MSDIN genes discovered. Majority of the MSDIN genes were found to be expressed at the mRNA level in *A. subjunquillea*. Using mass spectrometry, 12 new cyclic peptides were identified, which are potentially valuable for potent activities. Our results show that the amanitin biosynthetic pathway is highly versatile, capable of producing a wide range of cyclic peptides. This is also the first report on toxins expressed in *Amanita* mycorrhizae, shedding light on their possible role(s) at the stage of mycorrhizal symbiosis.

Methods

Collection and preservation of samples

Fresh fruiting bodies of strains of *As* (strain *AsYYH* and the ones for RNA-Seq) were collected from Kunming, Yunnan Province of China, about 300 meters apart from the previously reported one [14]. An additional strain *AsBJ* was collected in Beijing, China for MS comparison. Fresh fruiting bodies of *Ae*, *Ar* and *Apa* were collected in the Guangzhou, Hunan and Shandong provinces of China, respectively. For mycorrhizal collections, the same site for *As* was chosen. Once a fresh fruiting body of *As* was located, soil was carefully removed to expose plant roots, and root tips with signs of mycorrhizal association were cut off and rinsed in ddH₂O several times to remove dirt and debris. All the above samples were immediately buried in dry ice on site, transferred to liquid nitrogen tanks the same day, freeze-dried, and then stored in -80 °C. All the mushrooms were taxonomically identified according to previous studies [2, 5]. Hand-cut sections were made from fresh *As* mycorrhizae, stained with Congo red, and viewed under a microscope (Leica DM1000 LED).

Genome and transcriptome sequencing

The sequencing platform for the genomes of *Ae*, *Ar* and the strain *AsYYH* of *As* was PacBio Sequel at NextOmics Biosciences, Wuhan, China. Sequencing and assembly were carried out using the company's standardized pipeline. High quality DNA was extracted as described before [14], and a 20-Kb library was constructed using a PacBio template prep kit and was analyzed by Agilent 2100 Bioanalyzer for quality control. After the completion of the library, the DNA template and enzyme complex were transferred to the Sequel sequencer for real-time single molecule sequencing. Illumina HiSeq X10 platform was used for nucleotide level correction, based on a 350 bp library and the company's a standard method. For the following analyses, two additional genomes of *As* and *Apa* were adopted from our previous studies [14].

One species, *As* was chosen for transcriptome sequencing largely due to its availability. The process was carried out via standardized pipeline at NextOmics Biosciences, Wuhan, China. The library was constructed after the samples passed quality inspection. The cDNA library was constructed by magnetic bead enrichment. Clean reads were obtained from Illumina RNA-Seq platform, which were then aligned to the two *As* genomes using HISAT2 software [38], and transcriptome assembly was performed using StringTie under the default parameter [39].

Cloning of MSDINs from *AsBJ* strain and mycorrhizae of *As*

Coding sequences of MSDINs from *As* (included α -amanitin, β -amanitin, CylG1 and CylG2) were obtained by RT-PCR using primers based on the genomic data. For α -amanitin encoding gene, the forward primer was 5'-CCAACCAAGGTAGGGAACG-3', and the reverse primer was 5'-TGGACATCTAGTATCATCGTTCG-3'. There were two MSDINs both encoding β -amanitin. Two sets of primers were designed accordingly. For the first MSDIN, MSDINATRLPIWGIGCDPCIGDDVTALLTRGEALC, the forward primer was 5'-CTTGACAGGATTGATTTGTAGGAT-3' and the reverse primer was 5'-CTGAATGTGCGAATGAGAATAGC-3'. For the second MSDIN, MSDINATRLP IWGIGCDPCVGDEVTALLTRGEALC, the forward primer was 5'-CCGCGACTTACAGGATTGAT-3', and the reverse primer was 5'-AAACAGTGCATTATTTCGGTCAT-3'. For CylG1 and CylG2, the forward primers were 5'-GGTTTGCTCTGCCTGAC-3' and 5'-GGTTTGCTCTGCCTGACT-3', and the reverse primers were 5'-GCTCTGTGCGACTTGTGG-3' and 5'-TCAAGCCACCGCAATAGAA-3', respectively. The PCR reactions were carried out under standard conditions.

With the *As mycorrhizae*, for α - and β -amanitins, the primers were as the above. For phalloidin, the forward primer was 5'-GAAACCTCTCAACCGCAATGT-3', and the reverse primer was 5'-AAAGGATTGAAAAAGCACACG-3'. For phallacidin, the forward primer was 5'-GACCCCAGCTCTAAATCACAATG-3', and the reverse primer was 5'-CGCAGATCATTTACGTGGGTTTT-3'.

Extraction of cyclic peptides

Roughly 0.05 g of freeze-dried material for all samples was ground to fine powder in a mortar and pestle with liquid nitrogen. Then 1 mL extraction buffer containing absolute methanol: water: 0.01 M hydrochloric acid (5: 4: 1) was added. The mixture was heated in a water bath at 75 °C for 15 min before centrifugation at 12,000 rpm for 10 min. The supernatant was transferred to a new 1.5 mL centrifuge tube, filtered with a 0.22 μ m PES syringe filter (BS-PES-22, Biosharp), and then stored in -80 °C before use.

HPLC analysis

The HPLC method analyses were performed using a reversed-phase C18 column on an Agilent 1260 Infinity II HPLC system equipped with a UV detector (Agilent Technologies Inc). Separations were carried out on an InfinityLab Poroshell 120 EC-C18 reverse phase HPLC column (4.6 $\times$ 100 mm I.D., particle size 2.7 μ m, Agilent Technologies) at 28 °C. The mobile phases were (A) 0.02 M aqueous ammonium acetate-acetonitrile (90:10, v/v) and (B) 100% acetonitrile. The pH of solvents A was adjusted to 5.0 with glacial acetic acid. All solutions were prepared with HPLC grade reagents, and were degassed before use. For HPLC separation, the flow rate was 0.5 mL/min with a step of constant 100% solution A for 2 min, followed by a gradient from 100% solution A to 100% solution B in 8 min. The absorbance of the elution was monitored at 280 and 295 nm.

Mining MSDIN data

All nucleotide sequences of MSDINs from their genomes and the *As* transcriptome were obtained through standalone BLAST searches (NCBI BLAST⁺ 2.6.0) with query MSDIN sequences from *A. bisporigera* and *G. marginata*, which are well characterized by our molecular and biochemical approaches [40, 41]. In addition, MSDINs with high divergence at the core region from our previous reports were added as queries for more thorough excavation of potential MSDIN genes [14]. In the case of *As*, the resulted MSDIN sets were used as queries to further cross-search the two genomes and the *As* transcriptome for the purpose of finding MSDINs with high divergence.

Predicted molecular masses of candidate peptide sequences

To obtain molecular masses of predicted peptides based on MSDIN sequences, calculation websites (<https://www.ncbi.nlm.nih.gov/CBBresearch/Yu/webresources.html>, and <https://www.expasy.org/>) were adopted for calculating monoisotopic masses. This report mainly used the NCBI online calculation under the following conditions: Coarse-grained isotopic distribution mass accuracy (Da) = 1, Fine-grained isotopic distribution mass accuracy (Da) = 0.01, and the algorithm based on polynomial. Linear peptides adopted the amino acid sequences directly for core regions of MSDIN genes. Cyclized versions equaled to the linear versions minus a water molecule. Besides cyclization, this study attempted to predict other possible posttranslational modifications, including hydroxylation, sulfoxidation and bridge formation. One hydroxylation adds one O atom, which can multiply by up to 4 according to known amatoxins. Sulfoxidation adds an O atom, which only happens to the peptides containing Cys and Trp. The bridge formation between Trp and Cys loses two H atoms, resulting in a structure called tryptathionine [42]. How many and in which order these modifications would occur to a given MSDIN-related peptide are unknown. Our strategy was to try different combinations and look for matches in LC-HRMS.

LC-MS and LC-MS/MS analyses

HPLC separation described as above was coupled with Agilent 6540 UHD precision mass Q-TOF LC/MS (Agilent Technology) equipped with an electrospray source. Eluant was monitored in positive ESI mode with the capillary voltage at 3.5 kV. The drying gas (N_2) temperature was 350 °C and the flow rate was 8 L/min. The mass scan range was 500–1700 m/z. The electrospray electrical (ESI) was performed in a positive mode. For collision energy in MS/MS analysis, a range of 10 to 70 eV was applied.

After LC-MS/MS data were obtained, amino acid composition and combinations of candidate cyclic peptide was investigated first through the platform at Center for Computational Mass Spectrometry (CCMS) based on UniProtKB/swiss-port databased (<https://proteomics.ucsd.edu/ProteoSAFe/>). The workflow used MS-GF plus (V1.3.0) on the website by extracting masses of fragments using Agilent Mass Hunter v8.0.0 (Agilent Technology). In detail, the fragmentation diagrams were saved as MGF files and uploaded to CCMS [43]. When no results were obtained with CCMS, other platforms were included in the MS/MS analysis, including Mascot (with NCBIprot database), pNovo v3.1 [44], and XCMS online [45]. MS Convert (<http://proteowizard.sourceforge.net/tools.shtml>) was used for obtaining suitable file formats for each application.

All results from the above online tools were further confirmed or corrected via a thorough manual pipeline. First, high quality MS/MS spectra were obtained by optimizing the energy in the Q-TOF process. Best spectra were selected by choosing the ones with the most y-

type ions characteristic to certain amino acids. In some cases, increased amounts of samples were applied for optimal MS/MS spectra. Second, common fragmentation patterns for linear peptides were adopted for analyzing unmodified cyclic peptides because our preliminary results suggested that they were useful. Third, y-type ions for each MS/MS spectrum were determined based on core peptides of MSDINs using Molecular Weight Calculator v6.50 (<http://omics.pnl.gov>). Last, immonium ions were searched based on amino acid compositions. Only in situations that most or all amino acids (by themselves or in the right combinations) in a given candidate cyclic peptide matched a MSDIN sequence, did we call the cyclic peptide real with the sequence designated by the core region of the MSDIN gene.

Declarations

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Authors' contributions

HL and SWZ designed and carried out the genome sequencing, LC-MS/MS and the analyses on mycorrhizae; SWZ did the online database analysis. XCL and XL performed gene cloning and helped write the manuscript. ZHC and GHL collected the samples. HL assembled and analyzed the transcriptome. ZLY helped design the study and helped improve the final manuscript. HL and SWZ prepared the manuscript. All authors read and approved the final manuscript.

Availability of data and materials

The complete datasets of MS/MS analysis for all 13 cyclic peptides in this study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

This article does not contain any studies with human participants or animals performed by any of the authors.

Consent for publication

All authors have given consent for publication.

Competing interests

The authors declare that they have no competing interests.

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Supplementary Files Legend

Supplementary information accompanies this paper at <https://doi.org/10.1038/s41598-020-70000-0>:

Additional file 1: Figure S1. The monoisotopic masses of candidate new cyclic peptides in *Amanita rimosa*. Figure S2. The monoisotopic masses of candidate new cyclic peptides in *Amanita pallidorozea*. Figure S3. The monoisotopic masses of candidate new cyclic peptide in *Amanita exitialis*. Figure S4. Tandem mass spectrometry of novel cyclic peptides in *Amanita rimosa*. Figure S5. Tandem mass spectrometry of novel cyclic peptides in *Amanita pallidorozea*. Figure S6. Tandem mass spectrometry of novel cyclic peptides in *Amanita exitialis*. Figure S7. Mass spectrometry of amanexitide from *Amanita exitialis*. Table S1. MSDIN family and novel cyclic peptides in *Amanita pallidorozea*.

Figures

Figure 1

Deadly *Amanita* species for genome sequencing and peptide analyses. (A) *A. rimosa*; (B) *A. exitialis*.

Figure 2

A flowchart for characterizing cyclic peptides in *Amanita* species.

Figure 4

Mycorrhiza of *Amanita subjunquillea*. (A) A fruiting body of *A. subjunquillea* and the connected plant roots. (B) A cross section of a root tip associated with *A. subjunquillea* (Hartig net distinguishable).

Figure 5

Cyclic peptide toxins discovered in the mycorrhiza of *Amanita subjunquillea*. Three adduct ions of α -amanitin (A), β -amanitin (B), phalloidin (C) and phallacidin (D). The corresponding MSDIN genes cloned were listed (shaded) above each section.

Supplementary Files

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