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Gene family encoding the major toxins of lethal Amanita mushrooms

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Gene family encoding the major toxins of lethal *Amanita* mushrooms

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Amatoxins, the lethal constituents of poisonous mushrooms in the genus *Amanita*, are bicyclic octapeptides. Two genes in *A. bisporigera*, *AMA1* and *PHA1*, directly encode α-amanitin, an amatoxin, and the related bicyclic heptapeptide phallacidin, a phallotoxin, indicating that these compounds are synthesized on ribosomes and not by nonribosomal peptide synthetases. α-Amanitin and phallacidin are synthesized as proproteins of 35 and 34 amino acids, respectively, from which they are predicted to be cleaved by a prolyl oligopeptidase. *AMA1* and *PHA1* are present in other toxic species of *Amanita* section *Phalloidae* but are absent from nontoxic species in other sections. The genomes of *A. bisporigera* and *A. phalloides* contain multiple sequences related to *AMA1* and *PHA1*. The predicted protein products of this family of genes are characterized by a hypervariable "toxin" region capable of encoding a wide variety of peptides of 7–10 amino acids flanked by conserved sequences. Our results suggest that these fungi have a broad capacity to synthesize cyclic peptides on ribosomes.

amanitin | cyclic peptide | phalloidin | phallotoxin | amatoxin

Mushrooms in the genus *Amanita* section *Phalloideae* account for >90% of all fatal mushroom poisonings (1). The human LD_{50} for α-amanitin (Fig. 1*A*) is ≈0.1 mg/kg, and one mature destroying angel (*A. bisporigera*, *A. virosa*, *A. suballiacea*, and allied species) (Fig. 2*A*) or death cap (*A. phalloides*) (Fig. 2*B*) can contain a fatal dose of 10–12 mg (2). Only the carpophores (fruiting bodies) contain high concentrations of the toxins. Like other ectomycorrhizal basidiomycetes, species of *Amanita* grow slowly and do not form carpophores in culture (3). There are ≈900–1,000 species of *Amanita*, but most do not produce amatoxins or phallotoxins, and some are edible (Fig. 2*C*) (4, 5).

The mammalian toxicity of amatoxins is because of active cellular uptake followed by inhibition of RNA polymerase II (6–9). The typical symptoms of amatoxin poisoning are gastrointestinal distress beginning 6–12 h after ingestion, a remission phase lasting 12–24 h, and progressive loss of liver function culminating in death within 3–5 days. One of the few effective treatments is liver transplantation (10).

In addition to amatoxins, several members of *Amanita* section *Phalloideae* produce bicyclic heptapeptides called phallotoxins (Fig. 1*B*). Although structurally related to amatoxins, phallotoxins have a different mode of action, which is the stabilization of F-actin (11). Phallotoxins are poisonous when administered parenterally, but not orally because of poor absorption.

The biosynthetic origin of the *Amanita* toxins has been unknown. Because of the difficulty of working with *Amanita* fungi in culture, we took a genomic approach to identify genes involved in the biosynthesis of the amatoxins and phallotoxins.

Results and Discussion

The genome of *A. bisporigera*, an amatoxin- and phallotoxin-producing species native to North America (Fig. 2*A*), was shotgun-sequenced to approximately two times the coverage of the genome (≈70 MB total based on the known size of other homobasidiomycetes) (12) by a combination of automated Sanger sequencing and pyrosequencing (13). Because all known fungal cyclic peptides are biosynthesized by nonribosomal peptide synthetases (NRPSs) (14, 15), the genome survey sequences were first queried with known bacterial and fungal NRPSs. No evidence for any NRPS was found in *A. bisporigera*; the most closely related sequences were orthologs of aminoadipate reductase and acyl-CoA synthase, which are other members of the aminoacyl-adenylating superfamily (15).

We then searched the *A. bisporigera* genome for DNA encoding amanitins' amino acid sequences. Simplifed to the unmodified 20 proteogenic amino acids (i.e., ignoring the hydroxyla-

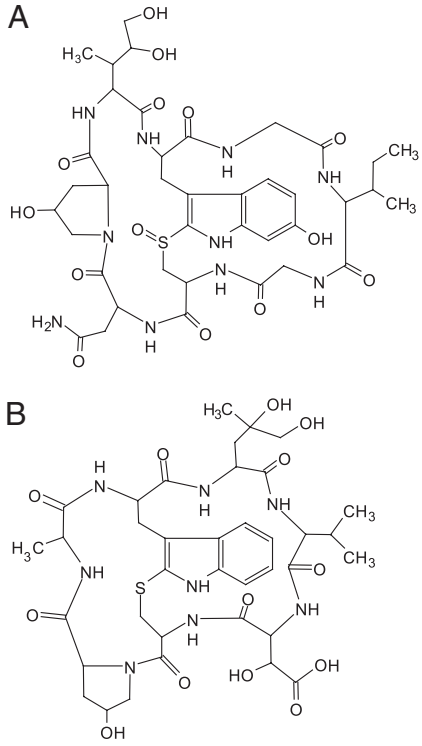

Fig. 1. Structures of α-amanitin (*A*) and phallacidin (*B*). All of the amino acids have the L configuration except hydroxyAsp in phallacidin (Thr in phalloidin).

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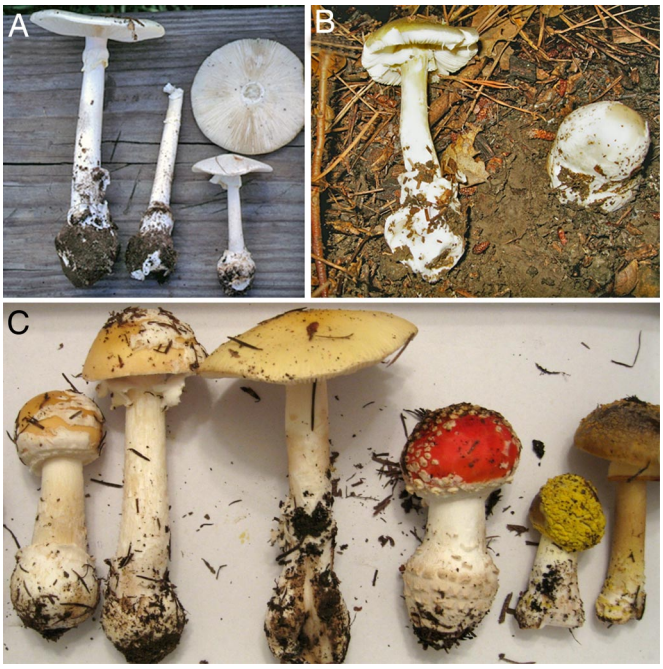

Fig. 2. Fungi of the genus *Amanita*. (*A*) *A. bisporigera* (Oakland County, MI). (*B*) *A. phalloides* (Alameda County, CA). (*C*) Nondeadly species of *Amanita*. Shown from left to right are three specimens of *A. gemmata*, one specimen of *A. muscaria*, and two specimens of *A. franchetii* (Mendocino County, CA).

tions and Trp-Cys cross-bridge) (Fig. 1), the sequence of the amatoxins is a cyclic permutation of either IWGIGCNP (α- and γ-amanitins) or IWGIGCDP (β- and ε-amanitins). Nucleotide sequences that could encode the amino acid sequence of α-amanitin were found in the genome of *A. bisporigera*. This sequence is not present in any protein or gene in the GenBank database, therefore it is not likely to be present in *A. bisporigera* by chance. Inverse PCR by using the restriction enzyme PvuI resulted in the isolation of a 2.5-kb fragment of flanking genomic DNA. An RNA blot probed with this DNA indicated that this region of the genome is transcribed into an mRNA of <400 nt (data not shown). PCR primers based on the genomic sequence were used to amplify a cDNA of ≈380 bp by 3′ and 5′ rapid amplification of cDNA ends (RACE). Comparison of the cloned, polyadenylated cDNA to the genomic sequence indicated that the gene, *AMA1*, has three introns with conventional GT/AG intron borders. Two of the introns (53 and 59 nt in length) are in the 3′ untranslated region, and one intron (58 nt) interrupts the fourth from the last codon (Fig. 3*A*). The presence of these features indicates that *AMA1* constitutes a true transcribed and processed gene. Assuming that translation starts at the first ATG downstream of the transcriptional start site, *AMA1* encodes a proprotein of 35 amino acids (Fig. 3*A*).

A genomic survey sequence of *A. bisporigera* also predicted the peptide AWLVDCP, which matches phallacidin, one of the major phallotoxins (Fig. 1*B*). Inverse PCR using PvuI and SacI was used to isolate genomic fragments of 1.6 and 1.9 kb, respectively, covering the *PHA1* gene. Two different classes of sequences were found, which were identical in the region of phallacidin but diverged ≈135 nt upstream. This finding indicates that *A. bisporigera* has at least two copies of the *PHA1* gene, both of which could encode phallacidin. A cDNA for *PHA1* was isolated by using 3′ and 5′ RACE. Like *AMA1*, the cDNA for *PHA1* also has three introns (57, 70, and 51 nt in length) in approximately the same positions as the introns in *AMA1*. The proprotein of *PHA1* is 34 amino acids (Fig. 3*B*).

AMA1 and *PHA1* and their translation products are similar in overall size and sequence (Fig. 4). The translated regions upstream of the toxin sequences have 28 of 30 nt in common (93%), the regions downstream have 40 of 48 nt in common (83%), but the toxin regions have only 11 of 24 nt in common (46%). Thus, the proproteins of α-amanitin and phallacidin are composed of two domains, a variable toxin region flanked by conserved regions (Fig. 4).

Many secondary metabolites are limited in their taxonomic distribution, and most species of *Amanita* do not make amatoxins or phallotoxins. To test whether the lack of toxin production among other species of *Amanita* were because of absence of the encoding genes, a blot of genomic DNA from 12 species of *Amanita* was hybridized with *AMA1* and *PHA1*. The species include four from section *Phalloideae* (this section contains all of

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A                                                                   M   S
 a ccc aac tcc cat tcg aac cta act cca aga cct cta aac ctc aca atc cca atg tct

 D   I   N   A   T   R   L   P   I   W   G   I   G   C   N   P   C   I   G   D
gac atc aat gct acc cgt ctt ccc atc tgg ggt atc ggt tgc aac ccg tgc atc ggt gac

 D   V   T   T   L   L   T   R   G   E   A   L   C   *
gac gtc act aca ctc ctc act cgt ggc gag gc^c ctt tgt taa attccccatccatttgtccg
ctgctatgacacgaag^tagtgggcgatacaagttgtggacgttatcaggcttgggccgttgagcctgcatcggaa^ac
aacttatgttccttctttttctgttttcatttgttaaaatacagaacccatgtcgatgatctgtgttgtagtcaatat
aaagttgtactgtgtttcttgtcaaaaaaaaaaaaaaaaaaaaaa

B                                   M   S   D   I   N   A   T   R   L   P   A   W
ga cct ctg ctc taa atc aca atg tct gac atc aat gcc acc cgt ctt ccc gct tgg

 L   V   D   C   P   C   V   G   D   D   V   N   R   L   L   T   R   G   E
ctt gta gat tgc cca tgc gtc ggt gac gat gtc aac cgt ctc ctc act cgt ggc gag

 S   L   C   *
ag^c ctt tgg taa atgtctcatccactagtcaag^gcaagttgttgacaatgtcaggcttgcgggtttg
agcctgcatcggaa^acgactcacgttctttctcattctttctgattctcatttgtaaacatataaaacccacgt
aaatgatccgttgtgctatggaatgcaatatacttgtgaaaaaaaaaaaaaaaaaaaaaaaaa
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Fig. 3. Nucleotide sequences of cDNAs for *AMA1* and *PHA1*. (*A*) *AMA1*. The sequence of α-amanitin is underlined. Carets indicate the positions of the three introns. (*B*) *PHA1*. The sequence of phallacidin is underlined. Carets indicate the positions of the three introns.

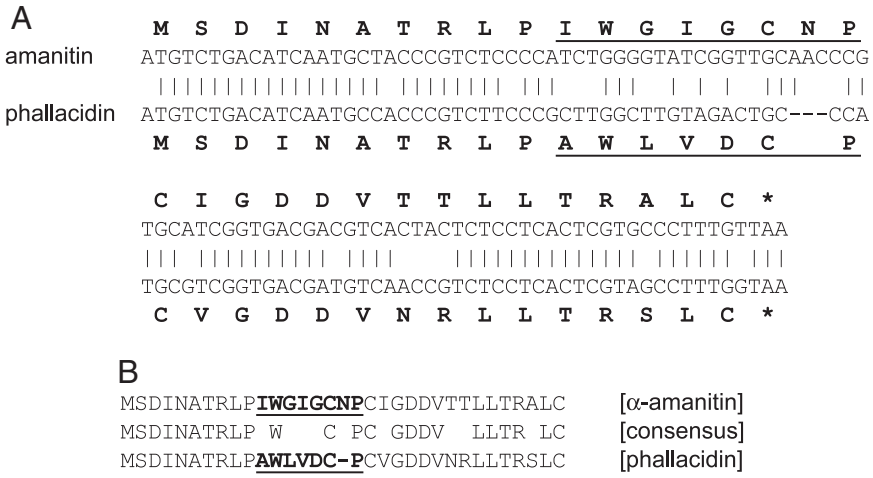

Fig. 4. Alignment of the cDNA nucleotide (*A*) and predicted amino acid sequences (*B*) of the coding regions of *AMA1* and *PHA1*. The mature toxin sequences are underlined.

the species that make amatoxins and phallotoxins), three from section *Validae* (the sister group to section *Phalloideae*), two from section *Amanita*, one from section *Caesarea*, and two from section *Vaginatae* (4, 5). All mushrooms were tested and confirmed by HPLC for the expected presence or absence of amatoxins and phallotoxins. All of the tested species that synthesize amatoxins and phallotoxins, but none of the nonproducers, hybridize to *AMA1* and *PHA1* (Fig. 5). This finding is consistent with *AMA1* and *PHA1* being responsible for amanitin and phallacidin biosynthesis and provides a molecular explanation for why *Amanita* species outside of section *Phalloideae* are not deadly poisonous. (Some of the *Amanita* species that do not make amatoxins or phallotoxins are edible, but others make different toxic compounds.)

The complex hybridization patterns shown in Fig. 5 indicate that *AMA1* and *PHA1* are members of gene families. Therefore, the conserved upstream and downstream amino acid sequences of *AMA1* and *PHA1* were used as queries to search for additional related sequences in the *A. bisporigera* genome. We thereby found at least 13 new, related complete or almost complete sequences (Fig. 6*A*) and another 10–15 sequences missing one end or the other (data not shown). All of these new sequences have an upstream conserved consensus sequence MSDINTARLP (MSDIN, R, and P are invariant) and a downstream conserved consensus sequence CVGDDV (the first D is invariant). The putative toxin regions, which start immediately downstream of the invariant Pro residue and end after an invariant Pro residue, are hypervariable compared with the upstream and downstream sequences. The hypervariable regions contain 7–10 amino acids, and all 20 proteogenic amino acids are represented at least once.

To detect related genes in *A. phalloides*, which worldwide accounts for the majority of fatal mushroom poisonings, degenerate PCR primers were designed against the conserved upstream and downstream sequences of *AMA1* and *PHA1*. The predicted translations of four amplicons from *A. phalloides* and one from *A. ocreata* are shown in Fig. 6*B*. One of them (IWGIGCDP) matches the amino acid sequence of β-amanitin,

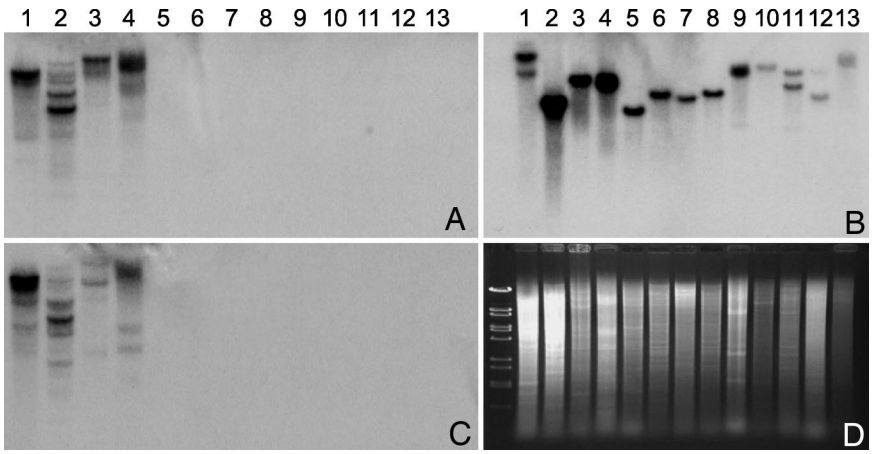

Fig. 5. DNA blots of different species of *Amanita*. (*A*) Probed with *AMA1* cDNA. (*B*) Probed with *PHA1* cDNA. (*C*) Probed with a fragment of the β-tubulin gene isolated from *A. bisporigera* (see *SI Text*). (*D*) Ethidium-stained gel showing relative lane loading. Markers are λ phage DNA cut with BstEII. Species and provenances are as follows: lane 1, *A.* aff. *subalbiacea* (Ingham County, MI); lane 2, *A. bisporigera* (Ingham County); lane 3, *A. phalloides* (Alameda County, CA); lane 4, *A. ocreata* (Sonoma County, CA); lane 5, *A. novinupta* (Sonoma County); lane 6, *A. franchetii* (Mendocino County, CA); lane 7, *A. porphyria* (Sonoma County); lane 8, a second isolate of *A. franchetii* (Sonoma County); lane 9, *A. muscaria* (Monterey County, CA); lane 10, *A. gemmata* (Mendocino County); lane 11, *A. hemibapha* (Mendocino County); lane 12, *A. velosa* (Napa County, CA); and lane 13, *Amanital* section *Vaginatae* (Mendocino County). Mushrooms represent sections *Phalloideae* (1–4), *Validae* (5–8), *Amanita* (9 and 10), *Caesareae* (11), and *Vaginatae* (12 and 13). Four separate gels were run; the lanes are in the same order on each gel, and approximately the same amount of DNA was loaded per lane. *A* and *B* are to the same scale, and *C* and *D* are to the same scale.

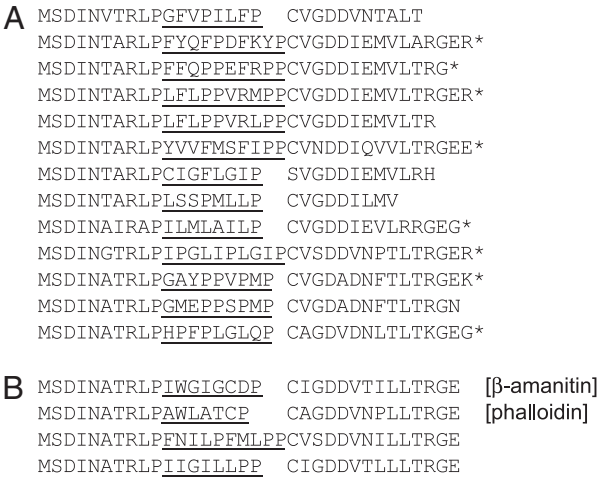

Fig. 6. Sequences related to *AMA1* and *PHA1*. (*A*) Related, predicted amino acid sequences identified in the *A. bisporigera* genome. (*B*) PCR products amplified from *A. phalloides* and *A. ocreata* (phalloidin) with degenerate primers based on the conserved sequences of *AMA1* and *PHA1*. Spaces have been inserted after some of the toxin regions (underlined) to emphasize the conservation of the downstream sequences. Asterisks indicate stop codons.

one matches phalloidin (AWLATCP), and the other two predict novel peptides.

The results in Fig. 6 suggest that species of *Amanita* section *Phalloideae* have the capacity to synthesize small, cyclic peptides in addition to amatoxins and phallotoxins. In fact, *A. phalloides* is known to produce other cyclic peptides, including CyA-A, CyA-B, CyA-C, CyA-D, and antamanide, which have the structures cyclo(GVAFFP), cyclo(SFFFPIP), cyclo(MLGFLVLP), cyclo(MLGFLPLP), and cyclo(FFVPPAFFPP), respectively (2, 16–18). None of these amino acid sequences were found in the genome survey sequences of *A. bisporigera*, but FFQPPEFRPP (Fig. 6*A*) is 70% identical to antamanide (18).

Small, modified, and biologically active peptides were previously identified from bacteria and several animals, including arachnids, snakes, cone snails, and amphibian skin (19–21). Like the *Amanita* toxins, the animal peptides are synthesized as precursor proteins and often undergo posttranslational modifications, including, like the *Amanita* toxins, hydroxylation and epimerization (22–24). Both the conotoxin and the *Amanita* toxin genes are characterized by the presence of conserved and hypervariable regions, resulting in the capacity to synthesize a large number of peptides on the same fundamental biosynthetic scaffold (Fig. 6) (25).

The *Amanita* toxins differ from these other small peptides in several key aspects. First, the animal peptides are not cyclized by peptide bonds, but acquire their essential rigidity by extensive disulfide bonds. Second, although ribosomally synthesized cyclic peptides have been described for bacteria, plants, and animals (e.g., the cyclotides and microcin J25) (26, 27), to the best of our knowledge, all previously known fungal cyclic peptides are synthesized by nonribosomal peptide synthetases (14, 15). Third, the *Amanita* toxins are not secreted (3), and, consistent with this fact, they lack predicted signal peptides (Figs. 3–5). Fourth, whereas the animal peptides are processed from their respective proproteins by proteases that recognize basic amino acid residues (Arg or Lys) (19, 24), we predict that the toxins of *Amanita* are cleaved from their proproteins by a protease that hydrolyzes peptide bonds specifically at Pro. All of the known *Amanita* cyclic peptides contain Pro, the last amino acid in the upstream conserved region is always Pro, and the predicted toxin sequences all have Pro as the last amino acid (Figs. 4 and 6).

Based on the properties of the known proline-specific peptidases (28, 29), the prolyl oligopeptidase family (POP) (EC 3.4.21.26) is the most promising to be involved in the processing of the proproteins of the *Amanita* toxins. We identified sequences related to human POP (GenBank accession no. NP_002717) in the genome survey sequences of *A. bisporigera* [see supporting information (SI) *Text*]. Orthologs of human POP also were found in every other basidiomycete for which whole genome sequences are available (*Laccaria bicolor*, *Coprinus cinereus*, *Phanerochaete chrysosporium*, *Ustilago maydis*, *Sporobolomyces roseus*, *Puccinia graminis*, and *Cryptococcus neoformans*) (see *SI Text*). A POP has been characterized from the mushroom *Lyophyllum cinerascens* (30). In contrast, orthologs of human POP are rare or nonexistent in fungi outside of the basidiomycetes. BLASTP (default parameters) identified no orthologs of human POP with a score >53 and E value $<10^{-6}$ in any fungus outside of the basidiomycetes, except perhaps in the ascomycete *Setosphaeria nodorum* (SNOG11288; score = 166; E value = 3×10^{-40}). Thus, it appears that at least one component of the biochemical machinery necessary for the biosynthesis of the *Amanita* toxins is both widespread in, and restricted to, the basidiomycetes.

The results presented here indicate that species of *Amanita* section *Phalloidae* synthesize their notoriously toxic cyclic peptides on ribosomes. Furthermore, these fungi have evolved a unique mechanism of combinatorial biosynthesis that endows them with the ability to biosynthesize a multitude of cyclic peptides. Further elucidation of the biosynthetic pathway of *Amanita* toxin biosynthesis could take advantage of the tractability of some basidiomycete fungi such as *C. cinereus* (31).

Materials and Methods

Mushrooms were harvested from the wild in 2002, 2006, and 2007; frozen at −80°C; and lyophilized. DNA was extracted from lyophilized fruiting bodies or cultures by using cetyltrimethylammonium bromide, phenol, and chloroform (32). RNA was extracted by using TRIzol (Invitrogen) (33).

PCR products were purified by using Wizard SV Gel and PCR Clean-Up System (Promega) and were cloned into TOPO pCR 4 (Invitrogen) for sequencing. For 3′ RACE, initial and nested primers from GeneRacer (Invitrogen) were used, and gene-specific primers were derived from the genomic sequence. Primer sequences may be found in *SI Text*.

Probe labeling, DNA blotting, and filter hybridization followed standard protocols (34, 35). DNA for blotting was cut with PstI and electrophoresed in 0.7% agarose. Hybridizations were performed overnight at 65°C in 4× SET, 0.1% sodium pyrophosphate, 0.2% SDS, 10% dextran sulfate, and 625 μg/ml heparin. SET (20×) is 3 M NaCl, 0.6 M Tris, and 0.04 M EDTA (pH 7.4). A 551-bp fragment of the *A. bisporigera* β-tubulin gene used as a control probe on DNA blots was amplified by PCR.

Variability in toxin content is known within species of *Amanita* (36, 37). All fungi analyzed for the presence of *AMA1* and *PHA1* (Fig. 5) were analyzed for amatoxins and phallotoxins by established HPLC methods (32, 38). Standards of α-amanitin, β-amanitin, phalloidin, and phallacidin were purchased from Sigma–Aldrich.

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SI Text

Primers for inverse PCR for *AMA1* were CCATCTGGGGTATCGGTTGC and TTGGGATTGTGAGGTTTAGAGGTC, and for *PHA1* CGTCAACCGTCTCCTC and ACGCATGGGCAGTCTAC.

For 3' RACE, initial and nested primers from GeneRacer (Invitrogen) were used. For the other 3' *AMA1* RACE primers, the initial primer was CCCATTCGAACCTAACTCCAAGAC and the nested primer was CCTCTAAACCTCACAATCCCAATG . For 5' RACE of the *AMA1* cDNA, the initial primer was GCCCAAGCCTGATAACGTCCACAACT and the nested primer was TATCGCCCACTACTTCGTGTCATA. For *PHA1*, the 3' initial primer was GACCTCTGCTCTAAATCACAATG and the 3' nested primer was ATCAATGCCACCCGTCTTCCTG. The 5' initial *PHA1* primer was CGGATCATTTACGTGGGTTTTA and the 5' nested primer was AACTTGCCTTGACTAGTGGATGAGAC.

Degenerate primers for amplification of toxin genes from *A. phalloides* were ATGTCNGAYATYAAYGCNACNCG (forward) and AAGGSYCTCGCCACGAGTGAGGAGWSKRKTGAC (reverse), in which W indicates A or T, S indicates C or G, K indicates G or T, R indicates A or G, and Y indicates T or C.

Primers to amplify a portion of the *A. bisporigera* tubulin gene were ACCTCCATCTCGTCCATACCTTCC and TGTTTGCCACGCTGCATACTA.

Sequences orthologous to human prolyl oligopeptidase (POP) in *A. bisporigera* and other fungi. The following genome survey sequences were identified in the *A. bisporigera* genome (subject) by TBLASTN using human POP (GenBank NP_002717) as query:

```
>ECGK9LO02JKSHR R length=112
          Length = 112

 Score = 47.8 bits (112), Expect = 4e-06
 Identities = 20/33 (60%), Positives = 26/33 (78%)
 Frame = +3

Query: 436 QTVQIFYPSKDGTKIPMFIVHKKSIKLDGSHPA 468
           ++ Q++Y SKDGTK+PMFIV  KS K DG+ PA
Sbjct: 3   ESTQVWYESKDGTKVPMFIVRHKSTKFDGTAPA 101

>contig26093 length=206   numReads=6
          Length = 206

 Score = 41.2 bits (95), Expect = 3e-04
 Identities = 18/32 (56%), Positives = 23/32 (71%)
 Frame = +1

Query: 440 IFYPSKDGTKIPMFIVHKKSIKLDGSHPAFLY 471
           ++Y S DGTKIPMFIV  K+ K +G+ PA  Y
Sbjct: 109 VWYDSYDGTKIPMFIVRHKNTKFNGTAPAIQY 204

>ECIMO1V02I2IO5 S length=107
          Length = 107

 Score = 35.8 bits (81), Expect = 0.014
 Identities = 18/27 (66%), Positives = 21/27 (77%), Gaps = 1/27 (3%)
 Frame = +2

Query: 546 KRLTINGGSNGGLLVAAC-ANQRPDLF 571
           ++L I+GGSNGGLLV A    QRPDLF
Sbjct: 26  EKLAISGGSNGGLLVGASRLTQRPDLF 106


>ECIMO1V01CKHE5 R length=94
          Length = 94

 Score = 35.4 bits (80), Expect = 0.019
 Identities = 16/27 (59%), Positives = 19/27 (70%)
 Frame = +2

Query: 120 SDDGTVALRGYAFSEDGEYFAYGLSAS 146
           S DGT +L  Y FS  G+YFAYG+S S
Sbjct: 2   SSDGTASLSMYDFSHCGKYFAYGISLS 82

>EEISCGG02IHTSV R length=106
          Length = 106

 Score = 33.1 bits (74), Expect = 0.093
 Identities = 14/20 (70%), Positives = 15/20 (75%)
 Frame = -2

Query: 669 PLLIHVDTKAGHGAGKPTAK 688
           PLL+ VD KAGHG GK T K
Sbjct: 105 PLLLRVDKKAGHGGGKSTEK 46
```

```
>ECIMO1V02H2WNR S length=78
          Length = 78

 Score = 28.9 bits (63), Expect = 1.7
 Identities = 12/16 (75%), Positives = 13/16 (81%)
 Frame = +2

Query: 446 DGTKIPMFIVHKKSIK 461
           DGTK+PMFIV  KS K
Sbjct: 2   DGTKVPMFIVRHKSTK 49
```

Orthologs of human POP in other Basidiomycetes are: *Coprinus cinereus* (GenBank CC1G_09936), *Ustilago maydis* (GenBank UM05288), *Cryptococcus neoformans* (GenBank XP_567311 and XP_567292), *Laccaria bicolor* (Lacbi1|303722), *Phanerochaete chrysosporium* (Phchr1|1293), *Puccinia graminis* (PGTG_14822.2), and *Sporobolomyces roseus* (Sporo1|33368).

The genome sequences of *L. bicolor*, *P. chrysosporium*, and *S. roseus* are available at http://genome.jgi-psf.org/. The genome sequence of *P. graminis* is available at http://www.broad.mit.edu/annotation/genome/puccinia_graminis.