The evolutionary history of the lethal amanitin biosynthesis genes in Amanita rubescens complex edible mushrooms and its potential health risk

Christian Quintero-Corrales[1], Melania Vega[1], Nallely Hernández-Rico[2], Pablo Octavio-Aguilar[2], and Roberto Garibay-Orijel[1]

[1]Universidad Nacional Autonoma de Mexico
[2]Universidad Autónoma del Estado de Hidalgo

October 7, 2022

Abstract

Horizontal gene transfer (HGT) is one of the most interesting evolutionary events in Eukaryotic life. It has been proven that fungi share genes or gene clusters with other organisms such as plants, animals, or other fungi. The amatoxins biosynthesis metabolic pathway produces lethal bicyclic peptides responsible for most of the deaths by mushroom poisoning. It is assumed that involved genes passed from Galerina and Lepiota to the Amanita secc. Phalloidae species by HGT. We used de novo genome assembling and gene mining for homologous amanitin genes among Agaricomycetes lineages. Phylogenetic and reconciliation trees were constructed to address the evolutionary history of POP gene family responsible for amanitin maturation. We also looked for the potential cytotoxicity of sequenced samples on plant bioassays. New and known edible species in Amanita rubescens complex have, partially or completely, the gene's package implied in the synthesis of amatoxins, making them potentially capable of synthesizing these lethal metabolites. The evolutionary history of these genes is more complex than previously reported involving at least ten HGT events, four duplication events, other fungal genera such as Piloderma and Russula, and several edible mushrooms. Furthermore, we showed that samples from a traditionally consumed species within Amanita rubescens complex had the same cytotoxicity capabilities as the lethal amanitas from the Phalloideae section. In conclusion, several old duplications and horizontal gene transfer events originated a cryptic diversity of the POP gene family in toxic and edible fungi raising questions on the safe consumption of species in the A. rubescens complex.

Title: The evolutionary history of the lethal amanitin biosynthesis genes in *Amanita rubescens* complex edible mushrooms and its potential health risk

CHRISTIAN QUINTERO-CORRALES[1,2], MELANIA VEGA[2,3] , GRISELDA NALLELY HERNÁNDEZ-RICO[4], PABLO OCTAVIO-AGUILAR[4], and ROBERTO GARIBAY-ORIJEL[1*]

1- Departamento de Botánica, Instituto de Biología, Universidad Nacional Autónoma de México. Circuito exterior s/n, Ciudad Universitaria, C.P. 04510, Coyoacán, Ciudad de México, México.

2- Posgrado en Ciencias Biológicas, Universidad Nacional Autónoma de México. Av. Ciudad Universitaria 3000, C.P. 04510 Coyoacán, Ciudad de México, México.

3- Jardín Botánico, Instituto de Biología, Universidad Nacional Autónoma de México. Circuito exterior, s/n, Ciudad Universitaria, C.P. 04510, Coyoacán, Ciudad de México, México.

4- Centro de Investigaciones Biológicas, Universidad Autónoma del Estado de Hidalgo. Ciudad del Conocimiento, Carretera Pachuca-Tulancingo Km 4.5, Col. Carboneras, C.P. 42184, Mineral de la Reforma, Hidalgo,

México.

*Corresponding author: Roberto Garibay-Orijel, email: rgaribay@ib.unam.mx

Summary

Horizontal gene transfer (HGT) is one of the most interesting evolutionary events in Eukaryotic life. It has been proven that fungi share genes or gene clusters with other organisms such as plants, animals, or other fungi. The amatoxins biosynthesis metabolic pathway produces lethal bicyclic peptides responsible for most of the deaths by mushroom poisoning. It is assumed that involved genes passed from *Galerina* and *Lepiota* to the *Amanita* secc.*Phalloidae* species by HGT. We used de novo genome assembling and gene mining for homologous amanitin genes among Agaricomycetes lineages. Phylogenetic and reconciliation trees were constructed to address the evolutionary history of POP gene family responsible for amanitin maturation. We also looked for the potential cytotoxicity of sequenced samples on plant bioassays. New and known edible species in*Amanita rubescens* complex have, partially or completely, the gene's package implied in the synthesis of amatoxins, making them potentially capable of synthesizing these lethal metabolites. The evolutionary history of these genes is more complex than previously reported involving at least ten HGT events, four duplication events, other fungal genera such as *Piloderma* and *Russula* , and several edible mushrooms. Furthermore, we showed that samples from a traditionally consumed species within *Amanita rubescens* complex had the same cytotoxicity capabilities as the lethal amanitas from the*Phalloideae* section. In conclusion, several old duplications and horizontal gene transfer events originated a cryptic diversity of the POP gene family in toxic and edible fungi raising questions on the safe consumption of species in the *A. rubescens* complex.

Keywords: Agaricomycetes, *Amanita rubescens* complex, amanitin, horizontal gene transfer, evolution.

Introduction

Lateral or horizontal gene transfer (HGT) is the genetic transmission between related or unrelated lineages by non-sexual events (Andersson, 2005a; Ayala-Ruano et al., 2019). The gain of single genes or gene clusters from other species is an effective evolutionary alternative to modify and increase the genetic pool of organisms, conferring potential adaptive advantages to themselves (as a selfish genes) or to the host (Husnik & McCutcheon, 2018; Kloesges et al., 2011; Schönknecht et al., 2014; Slot & Hibbett, 2007; Thomas & Nielsen, 2005). The transferred genes must "adapt" to the new genome (ameliorate) and then they can be vertically or horizontally inherited (Kloesges et al., 2011). Nonetheless, this inherence turns out in a highly unusual gene similarity among the involved organisms, due to the individual evolutionary history of the target gene and the host genome (Husnik & McCutcheon, 2018). Since the transfer involves the insertion of foreigner DNA into a specific lineage, new genes will be limited to the offspring of the recipient organism, producing a sparse phylogenetic distribution (Ochman et al., 2000) or phylogenetic incongruence (Rosewich et al., 2000).

It is well known that in prokaryotic organisms the ecological similarities (sympatric species or overlapping niches) or physical interactions (symbiotic relationships) instead of their phylogenetic or geographic distances explain the patterns of transferred genes (Andersson, 2005; Kloesges et al., 2011; Kunin et al., 2005). HGT has massive ecologically and evolutionary implications in the ecosystems as niche colonization (competition) or the emergence of new metabolic pathways as pathogenic capability (Ayala-Ruano et al., 2019; Huang, 2013; Van Etten & Bhattacharya, 2020). This phenomenon has been observed also in Eukaryotic organisms, but the transfer mechanisms are still unknown. It has been proposed that overlapping niches, direct physical interactions and sharing environmental selection pressures may promote genetic transfer (Reynolds et al., 2018). For instance, the interaction between the symbiotic *Amanita muscaria*and soil microbiota promoted the carbohydrate esterase genes transfer (necessary for organic matter decomposition) to the fungus (Chaib De Mares et al., 2015; Reynolds et al., 2018). Novo et al. (2009), showed that*Saccharomyces cerevisiae* EC118 wine strain acquired a 65 Kpb gene set from contamination yeasts during wine fermentation, increasing their fermentation potential. Another example is the acquisition of the psilocybin synthesis metabolic pathway, as a niche colonization tool, from lignin degradation fungi to dung decay fungi (Reynolds et al., 2018). In these three cases, the transferred gene clusters turned out in new metabolic pathway acquisitions, conferring

selective advantages to the new fungal hosts.

The secondary metabolites amatoxins are produced by an exclusive group of fungi that are associated with >90% of worldwide deaths by mushroom poisoning (Bresinsky, 1990). These metabolites are synthesized and matured by the MSDIN gene family (AMA and PHA) and the prolyl-oligopeptidase variant B (POPB) gene. The MSDIN gene family has a characteristic highly conserved five amino acids (MSDIN) that code for a 34-37 long peptide precursor (Hallen et al., 2007; He et al., 2020). These precursors are cleaved, trimmed and macrocycled to seven-ten amino acid length (known as the core peptides) by an exclusive proline specific enzyme, POPB. The outcome is the mature, active, and deathly metabolite. Recently, Luo et al. (2022) confirmed two new genes involved in amanitin biosynthesis, cytochrome P450s and flavin monooxygenase (FMO1) enzymes. These two enzymes metabolize compounds by adding molecular oxygen (hydroxylation and sulfoxidation) to their substrates.

It was thought that genes in the amanitin synthetic pathway were exclusive to *Amanita* section *Phalloideae* , where all deathly amanitas belong (Walton, 2018). However, Lüli et al. (2019) and Luo et al. (2018) found that MSDIN, POPB, FMO and P450 genes passed from an unknown fungus to*Lepiota* and *Galerina* to section *Phalloideae* by HGT. Nevertheless, a serious of deathly intoxications in the Mexican state of Hidalgo involving species in the *Amanita rubescens* complex built-up the hypothesis of the presence of these genes in these "edible" mushrooms consumed locally.

To understand the extent and nature of a possible HGT of the amanitin biosynthesis genetic pathway to edible amanitas outside the*Phalloideae* section we performed a comparative genomic analysis using Hernández-Rico et al. (2019) samples. We assembled the genomes and used a mining approach for the MSDIN, POPB, FMO and P450 homologous genes for phylogenetic inferences, gene neighborhood and evolutionary events detection. The comparison of the gene and species trees suggested that the evolution and diversity of the POP gene family are more complex than expected involving a diverse arrange of distantly related Agaricomycetes linages, including edible mushrooms. Even while we did not test the transcription of MSDIN genes, we tested the cytotoxicity of sequenced samples in broad bean meristematic root cells bioassays. The main goals of our research were to detect and test the likelihood of the acquisition of amanitin metabolic pathway due to HGT events in edible mushrooms; and to evaluate the potential gene source and transfer intermediaries of the amanitin biosynthesis genes among the Agaricomycetes.

Materials and Methods

2.1. Sample selection, DNA extraction and sequencing

All processed samples belong to the *Amanita rubescens* species complex, belonging to *Amanita* section *Validae* series*Rubescentes* . This clade is a cryptic species group with many undescribed taxa. Here we analyzed genomic data of the type species*Amanita rubescens s.s* ., a European species not distributed in America (even while the name has been used widely); and two undescribed species with the provisional names *Amanitacruentilemurum nom. prov.* and *Amanitaperezsilvae nom . prov.,* previously known as*A. rubescens s.l.* in Mexico.

Eleven samples from *A. rubescens* complex from Hernández-Rico et al. (2019) and one positive control (*A. suballiacea*) from*Amanita* section *Phalloideae* were chosen for whole genome sequencing. The DNA extraction was performed using 20 mg of dry stipe tissue, frozen at -70 °C for at least 24 hours before extraction, and grinded using TissueLyser LT. The DNeasy plant mini kit (QIAGEN) was used for DNA extraction. DNA purifying, quality and quantity were evaluated using Nanodrop, electrophoresis gels and Qubit4, respectively. Around 100 ng of high-quality DNA was used for sequencing at the UW-Madison Biotechnology Center, United States. The sequencing was performed with the Illumina Novaseq6000 technology paired end 150 pb (2x150) at 60X of depth.

2.2. Raw data and genome assembly

We used FASTQC V 0.11.9 (Andrews, 2010) to test raw reads quality. Trimmomatic V 0.40 (Bolger et al., 2014) was used for low quality (SLIDINGWINDOW:5:20), reads without pairs, adapter elimination

and reads length homogenization (MINLEN:80). Three different approaches were tested for genome assembly, SPAdes V 3.14-3.15 (Prjibelski et al., 2020), Velvet V 1.2.10 (Zerbino & Birney, 2008) and Abyss V 2.3.1 (Simpson et al., 2009), using corrected raw reads obtained by SPAdes (–careful). Different k-mers values (*i.e.* 21, 31, 41. . . .121) were used for ABySS and SPAdes. For Velvet, VelvetOptimiser V 2.2.6 (*http://bioinformatics.net.au/software.velvetoptimiser.shtml*) was used to choose the better k-mer value.

The assembled genomes quality was evaluated using BUSCO V 5.2.2 (Manni et al., 2021) and QUAST V 5.0.2 (Gurevich et al., 2013). Afterwards, scaffolds were removed under the following criteria: 1) >= 500 bp length; 2) corresponding to the mitochondrial genome; 3) with coverage < 1x. BLAST tools were used to locate POP genes. Only scaffolds containing the POP genes were used for gene prediction with AGUSTUS V 3.4.0 (Stanke et al., 2006) and gene annotation with blastp. The gene neighborhood was analyzed manually with CLIP STUDIO V 1.5 using the information of gene prediction and annotation.

2.3. Searching and mining of MSDIN and POP genes

The MSDIN and POP nucleotide and amino acid sequences were extracted from the assembled genomes using BLAST+ command line (Camacho et al., 2009). Known amatoxin biosynthesis gene sequences from *Amanita* section*Phalloideae* , *Galerina* and *Lepiota* were used as query sequences (Table S1, S2). The search was performed using blastn and blastp task tools with different word sizes.

Sequences alignments and intron/exon prediction for the POP genes were performed in MEGAX V 10.2.4 and UGENE V 43.0 (Kumar et al., 2018; Okonechnikov et al., 2012). Introns were manually extracted. ExPASy web service (Artimo et al., 2012) was used to corroborate the correct reading frame for each gene. The gene structure prediction was performed on the Splign website (*https://www.ncbi.nlm.nih.gov/sutils/splign/splign.cgi*).

2.4. Sequence alignments and phylogenetic analysis

Three data sets were used for phylogenetic inferences: 1) Coding sequence (CDS) from POP genes, 2) POP amino acid sequences, and 3) MSDIN amino acid sequences. For this, POP CDS and amino acid reference sequences of *Amanita* , *Galerina* , *Lepiota* and other Agarycomycetes were downloaded from NCBI and JGI databases (Table S2, Li et al., 2014; Lüli et al., 2019; Luo et al., 2018). Sequences were aligned using MUSCLE (Edgar, 2004) with default parameters and the alignments were manually edited in MEGAX. All posterior maximum likelihood (ML) analyses were run in CIPRES Science Gateway (Artimo et al., 2012; Miller et al., 2010), with the RAxML V 8.0 program (Stamatakis, 2014) using the GTR+CAT model with 1000 bootstraps. Bayesian inferences were performed in the CONABIO bioinformatic cluster using MrBayes V 3.2 (Ronquist et al., 2012), with 15 M iterations, 25% burning chains, sampling each 1000 and the GTR+I+G evolutionary model.

A concatenated matrix of ITS, LSU and rpb2 genes was used for the species tree. Each region was extracted from the assembled genomes using BLAST+ command line. Reference ITS, LSU and rpb2 sequences of*Amanita* , *Galerina* , *Lepiota* and Agaricomycetes were downloaded from NCBI and JGI database (Table S3). Each region was aligned, edited independently, and concatenated using MUSCLE and MEGAX. The best evolutionary model was chosen for each partition using PartitionFinder 2 (Lanfear et al., 2017). The ML and Bayesian analyses were performed using RAxML and MrBayes in the CIPRES Science Gateway and CONABIO's bioinformatic cluster, respectively. The ML analysis was run with 1000 bootstraps and the GTR+CAT evolutionary model. For MrBayes, 15 M iterations, 25% burning, sampling each 1000 and the GTR+G+I and GTR+I evolutionary models were used. The chain's convergence was tested with Tracer V 1.7.1 (Rambaut et al., 2018). All trees were visualized with FigTree V 1.4.4 (*http://tree.bio.ed.ac.uk/*).

2.4.1. Agaricomycetes POPB homologous genes

We explored the JGI Mycocosm database (Grigoriev et al., 2014) for*Amanita* section *Phalloideae* POPB homologous genes. The search was performed using the blastp and blastn tools from BLAST+. We used the *Amanita bisporigera* POPB and the *A. rubescens*complex POP (found here) as query sequences against the whole database (~2,100 fungal genomes). The first 400 match sequences with the higher homology values

(E-value and % identity) were downloaded. The sequences were aligned using MUSCLE in MEGAX and were used for a ML analysis. The analysis was performed with 1000 bootstraps (MLB) and the GTR+CAT evolutionary model.

2.5. Reconciliation trees: species vs genes

The species (ITS-LSU-RPB2 concatenated matrix) and gene (POP) trees were constructed by subsampling each original matrix. For this, species were chosen depending on POPB or POPD homologous genes presence. Each matrix had 25 species. Both trees were built with a maximum likelihood approach using RAxML, 1000 bootstraps and the GTR+CAT evolutionary model.

For the reconciliation analysis two different programs were used, Notung V 2.9 (Chen et al., 2000) and web service ecceTERA V 1.2.2 (Jacox et al., 2016, https://mbb.univ-montp2.fr/MBB/). Both programs infer evolutionary events based on a parsimonious approximation approach with two models: Duplication-Losses or Duplication-Losses-Transfers. Each program was run using their default values: for Notung was 1.5 for duplications, 3.0 for transfers and 1.0 for losses, and for ecceTERA was 2.0 for duplications, 3.0 for transfers and 1.0 for losses. The best evolutionary model was chosen by a minimum score (event score).

2.6. Citotoxicological bioassays in broad bean meristematic root cells

Amatoxins are potent inhibitors of the eukaryotic DNA-dependent RNA polymerase II or pol II. The inhibition of the pol II, responsible for all the messenger RNA (mRNA) transcription, results in cellular death. The latter has been proven on animal bioassays, mainly rodents, but it is known that they are lethal for humans and dogs. As pol II is universal in eukaryotes, plants bioassays also show transcription inhibition at low amatoxin concentration (Walton, 2018). Thus, to evaluate the potential cytotoxicity effects of *A. perezsilvaenom. prov.* , we performed a bioassay on the plant model *Vicia faba* roots cells. We used two species from the *Phalloideae* section as positive controls (*Amanita aff. virosa* and *Amanita aff. verna*), seven samples of *A. perezsilvae nom. prov.* as experimental samples and saline water solution as negative control. For each sample we used two different treatments (raw and boiled) with three replicates per sample. For all treatments, 0.56 gr of dried pileus per sample were macerated in sterile distilled water. The meristematic root´s cells were exposed to the macerated solutions at dark conditions for 12 hours. After that, apical meristematic root cells were fixed at 3:1 ethanol-acetic acid solution for 15 minutes and washed with saline water at 5%. Aceto-orcein staining was used for 15 minutes under dark conditions. Lastly, acetic acid at 45% was used for slide mounting using the "squash" technique.

To quantify cytotoxic capabilities of *A. perezsilvae* nom. prov. we used the mitotic index (MI). The index measures the cell proliferation (i.e. proportion of dividing cells in any mitotic phase) at controlled conditions. MI was calculated as follows: MI (%) = Mc*100/Tc, where Mc is the total number of cells at any mitotic stage, and Tc the total number of cells. Decreasing MI values reflect a cell's growth deficit that usually is related to cytotoxic agents in the given environment (Zendehboodi, 2018). Here, up to 500 cells were counted in each replicate using an optical microscopy at 40X. The obtained data were statistically tested using the nonparametric Kruskal-Wallis (H) and the post-hoc tests with Bonferroni correction.

3. Results

3.1 New MSDIN and Prolyl-oligopeptidase genes in *Amanita rubescens* complex

Twelve new draft genomes (ten *A. perezsilvae nom. prov* ., one *A. cruentilemurum nom. prov.* , and one *A. suballiacea*) were sequenced and assembled resulting in 40 Gb of raw data. For each sample, ˜10-15 M high quality reads (>35 quality value) and ˜4 Gb of clean data were used. SPAdes was the better assembly algorithm for our data, assembling genomes of ˜50 Mpb with a GC% of ˜48 (Table 1).

One MSDIN-like sequence gene was found in the ten *A. perezsilvae nom. prov.* and *A. cruentilemurum nom. prov* . genomes (supplementary material). The amino acid sequence has the same structure of known MSDIN genes divided into three sections: leader peptides, the first ten amino acids (the signature trait of the gene family); the hypervariable region flanked by Proline (P) residues (core peptides); and the follower region (Fig.

1a). The core peptide was 11 amino acids long (PTWHGHSMATHP) codifying for an unknown metabolite. The maximum likelihood and bayesian analyses showed that the new MSDIN gene found here is the most divergent sequence placing it as the outgroup for all known cycloamines: phalloidin, phallacidin, amanitin and cyclopeptides (100 MLB/1 pp, Fig. 1b). All *Amanita* section *Phalloideae*amanitin genes (AMA) are clustered together forming a sister clade with*Lepiota* and *Galerina* AMA (0.79 pp). For *A. suballiacea* , 21 different MSDIN gene sequences were found corresponding to known and unknown amatoxin metabolites (Table S4).

We found four different POP genes within *A. perezsilvae*nom. prov. and *A. cruentilemurum* nom. prov. genomes: one POPA, housekeeping gene in most eukaryotic organisms; and three different POPB homologous genes, called hereafter POPD. These genes had ˜50% of similarity and shared the same number and position of introns (18) and exons with known POPB genes (17, Fig. S1). Interestingly, two highly similarity sequences (>95%) from*Amanita rubescens* s.s. were found in NCBI database (KAF8346704 and KAF8331800). These sequences were labeled as "prolyl oligopeptidase" and were obtained by Miyauchi et al. (2020). The proposed names for the three POPD genes found are POPD1, POPD2 and POPD3 due to their highly gene similarity to POPDB, and because POPC corresponds to an exclusive *Lepiota venenata* gene not related to amanitin biosynthesis (Lüli *et al.,* 2019). The POPD1 gene was exclusive to*A. cruentilemurum nom. prov.* (GNHR-426), while POPD2 and POPD3 genes were from *A. perezsilvae nom. prov.* The POPD2 gene was slightly larger than POPD3 (˜3130 vs ˜3115 pb). The sequences were ˜2196 pb and ˜733 amino acid length after intron removal. All POPD genes had different scaffolds.

For *A. perezsilvae nom. prov.* samples, two gene copies of P450 and one FMO were found. Each P450 gene shared the genetic neighborhood with different POPD genes (POPD2 and 3) in different scaffolds (Fig. S2a). The FMO gene was flanked by POPD3 and one P450 gene. This pattern is conserved among all *A. rubescens* complex samples and *A. suballiacea* except for *A. perezsilvae nom. prov.* GNHR-435. For *A. cruentilemurum nom. prov.* only the FMO gene was found near to POPD3 gene together to an ABC transporter protein (Fig. S2b). Surprisingly, FMO and P450 genes were also found in*A. rubescens s.s* . with similar genetic neighborhoods as in*A. perezsilvae nom. prov.* samples (Fig. S2a).*Amanita cruentilemurum nom. prov.* and *A. perezsilvae nom. prov* . and *A. suballiacea* samples shared the same genetic neighborhood for the second P450 gene copy that was flanked by the POPD2 (POPB in *A. suballiacea*) and MATE (multidrug and toxic compound extrusion) transporter genes, changing the latter for a Major facilitator superfamily transporter (MFS) in *A. rubescenss.s.* (Fig. S2a).

3.2. Prolyl oligopeptidase B/D genes diversity within Agaricomycetes

The POPA phylogenetic distribution agrees as expected with the species tree (blue bar in Fig. 2) while the POPB genes formed their own clade, independently to their species position. All mayor clades are well supported, *Amanita* (100 MLB/ 1 PP) splits in its sections*Phalloideae* (100 MLB/ 1 PP), *Validae* (100 MLB/ 1 PP) and*Amanita* (100 MLB/ 1 PP), *Galerina* (87 MLB/ 1 PP) and*Lepiota* (80 MLB/ 1 PP). *Hebeloma* genes (100 MLB/ 1 PP) were grouped as sister clade of *Galerina* and *Cortinarius* . Both *Russula* (Order Russulales) genes and *Piloderma*(Subclass Agaricomycetidae) POP sequences were used as outgroups (96 MLB/ 1 PP, Fig. 2). *Tricholoma* and *Lepista* form a separate clade (89 MLB/ 1 PP).

The POPB sequences were clustered the same way as in Luo *et al.*(2018) and Lüli *et al* . (2019) forming a phylogenetic incongruence (100 MLB/ 1 PP). *Lepiota* is the most external branch (100 MLB/ 1 PP) followed by *Galerina* as the nearest branch to the*Phalloideae* section sequences (92 MLB/ 1 PP). However, we found many new POPB homologs genes in different fungal linages in*Piloderma* , *Russula* , *Hebeloma* and*Cortinarius* . Two *Piloderma byssinum* POPB sequences were clustered in the *Amanita* , *Lepiota* and *Galerina*clade (Fig. 2): one copy shares the same clade with *Lepiota* POPB and the second between *Galerina* and *Amanita* clade (96 MLB/ 1 PP). The remaining POPB homologous genes clustered together to*A. rubescens s.s.* sequences alongside the new POPD genes (95 MLB/ 1 PP). Within this group (red box in Fig. 2) four different clades were formed: 1) the exclusive GNHR-426's *Amanita cruentilemurum nom. prov.* POPD1 (100 MLB/ 1 PP); 2), POPD2 clade formed by *A. rubescens s.s.* and *Amanita perezsilvae nom. prov.* (GNHR-430) sequences (98 MLB/ 1 PP); 3) *Hebeloma brunneifolium* clade (94 MLB/ 1 PP); and 4) *A. rubescenss.s.* , *A. cruentilemurum*

nom. prov ., *A.perezsilvae nom. prov.* and *Cortinarius* sp. POPD3 clade (100 MLB/ 1 PP). Also, POPB/D homologous genes were found in*Russula dissimulans* and grouped as the most divergent genes (Fig. 2). To avoid tree polytomies due to many identical POPD2 and POPD3 copies only two samples (GNHR-426 and GNHR-430) with POPD sequences were chosen. The same approach was used for *P. byssinum* and *H.brunneifolium* as four identical copies of POPB and POPD were found, respectively.

3.3. Horizontal gene transfer as the main event of the POPB/D evolutionary pathway

Both Notung and eccTERA analyses showed that the duplication-transfer-loss (DTL) model is the best evolutionary scenario. The duplication-loss model (DL) explained 17 duplications and 65 losses with an event score value of 90.5, and 20 duplications and 65 losses with 117 event score value for Notung and ecceTERA. The DTL model in Notung scored with 47 with 4 duplications, 12 transfers and 5 losses, and 67 event score for ecceTERA with 4 duplications, 12 transfers and 6 losses.

Notung showed that four duplication events occurred exclusively within the POPB/POPD clade (Fig. 3). The first duplication event happened in the outgroup clade of *Russula* , resulting in two copies; the second occurred at the POPB crown group, one copy was inherited to the*Piloderma* and *Lepiota* clade, and the second to*Piloderma* , *Galerina* and *Amanita* ; the third occurred in the POPD2/3 clade, one copy turned out in the POPD2 clade and the other to the POPD3; and the fourth happened in *Hebeloma* clade. All transfer events except one were detected within the POPB/D clade. The oldest transfer event was from the hypothetical species "39" to the "n557". Followed by four transfer events in the POPD clade and five transfers in the POPB clade. In the POPD clade, one transfer was from *P. byssinum* to hypothetical species "n540"; the remaining transfers were within the POPD3 and *Hebeloma* clade after the duplication event where POPD2 and POPD3 diverged. The second transfer was from hypothetical species "n72" to *Hebeloma brunneifolium*followed by a duplication; the third transfer happened from the same hypothetical species "n72" to *Cortinarius* sp.; the fourth transfer occurred from *A. perezsilvae nom. prov.* (GNHR-430) to*A. cruentilemurum nom. prov.* (GNHR-426). For the POPB clade, all transfers occurred after the duplication event; the first copy was transferred to *Lepiota venenata* from *P. byssinum*and the second copy was transferred to the *Galerina* clade and then to *Amanita* section *Phalloideae* (Fig. 3).

3.4. Inhibition of cellular division

Both treatments (raw and boiled) showed cellular division changes compared to the negative controls (Fig. S3). Also, no differences were found between raw and boiled treatments in their effect on cell proliferation. The Kruskal-Wallis test showed significantly differences among species treatments (p = 4.909x10e-7). The pairwise post-hoc comparison showed differences between *A. perezsilvae nom. prov.* samples vs negative control ($p< 0.001$), differences between positive controls vs negative control ($p< 0.006$), and no differences between *A. perezsilvae nom. prov.* samples vs *Amanita aff. virosa* and *Amanita aff. verna* positive controls ($p= 1.0$).

4. Discussion

4.1. Amanitin biosynthesis pathway in *Amanita rubescens* complex

The new MSDIN-like genes found in three species of the *A. rubescens* complex (*A. rubescens s.s.* , *A. cruentilemurum nom. prov* . and *A. perezsilvae nom. prov.*) shared the same genetic structure with previously known MSDIN genes. If these genes are expressed, they should transcribe for a ribosomally synthesized and post-translationally modified peptide (RiPP), since they have the three characteristic domains: leader, core, and follower peptide (Hallen et al., 2007; He et al., 2020). The leader domain has the distinctive signature of the MSDIN gene family but has changes in the second and sixth positions in*A. perezsilvae nom. prov.* (M**Y** DIN**S** TRLP); and changes in the second, fourth and sixth positions in *A. cruentilemurum nom. prov.* (M**Y** D**V** N**S** TRLP). It has been proposed that the upstream amino acids residues (N[**A**/**S**]TRL) have a main role as recognition site for the POP enzyme (Sgambelluri et al., 2018; Walton, 2018). Thus, it must be conserved among the AMA and PHA genes (Fig. 1). However, the core (TWHGHSMATHP) and follower peptides (LDDDLIVNFVEKS) are too divergent compared to the known MSDIN genes or any homologous

gene found in the NCBI or JGI databases (Pulman et al., 2016). It is worth mentioning that we did not find MSDIN-like genes in*Russula* , *Piloderma* , *Cortinarius,* or *Hebeloma*genomes with POPB homologous genes (see next section).

The unusual hydroxylated amino acids (Leu, Trp and Pro), due to FMO and P450 enzymes are crucial for metabolite toxicity (Luo et al., 2022; Walton, 2018). The core domain of the MSDIN-like genes found here (...RL**P** TWHGHSMATH**P**) are flanked by the proline residues for the POP cleavage and it has Trp as the second amino acid but did not have a Cys for the triptationin cross-linked bridge. Thus, a chemical profile is needed to verify the double cyclic structure of the peptide. However, the presence of FMO and P450 genes near to POPD gene in the assembled genomes suggests that the metabolite oxygenation happens (Walton, 2018). Luo*et al.* (2022) proved that FMO and P450 genes are exclusive to*Amanita* , *Lepiota* and *Galerina* and that its knock down results in inactive peptides. Here, we found at least two P450 and one FMO gene in all *A. rubescens* complex studied species. Interestingly, we found that most of the amanitin biosynthetic homologous genes were together in different genetic clusters in *A. rubescens s.s.* , *A. cruentilemurum nom. prov.* and*A. perezsilvae nom. prov.* except for the MSDIN-like gene that is isolated. This genetic structure is like those of*Galerina* but contrasts with *Amanita* section*Phalloideae* and *Lepiota* where the genes are randomly distributed though their genomes (Fig. S2, Luo *et al.,* 2022). It should be considered that separation of MSDIN-like genes to the other amatoxin maturation genes might be an artifact due to the highly fragmented assembled genomes.

Most of the secondary metabolite pathways of fungal organisms are encoded by biosynthetic or metabolic gene clusters (BGC or MGC) (Rokas et al., 2020; Vignolle et al., 2020). We showed that in the POPD genes neighborhood three different superfamilies related to secondary metabolite transport were found: MATE (multidrug and toxic compound extrusion), MFS (major facilitator superfamily) and ATP-binding cassette proteins transporter (ABC). These proteins correspond to a group of major families of multidrug transporters in all kingdoms (Kusakizako et al., 2020; Quistgaard et al., 2016; Shoji, 2014). Most of the MATE transporters export metabolites out of the cell through the membrane, and are related to direct or indirect mechanisms of detoxification in plants (Santos et al., 2017). MFS transporters move a huge variety of small compounds across membranes between cells and between intracellular compartments such nutrients, extrusion of deleterious compounds, metabolites, signaling molecules and other substrates (Quistgaard et al., 2016). ABC transporters are responsible for drug resistance by pumping out a variety of drugs out cells at the expense of ATP hydrolysis (Choi, 2005). Using immunolocalization and confocal microscopy Luo *et al.* (2010) proved that amanitin has restricted distribution through the fungal fruitbody cells and is stored in vacuole-like structures. Thus, the transporter protein families mentioned above may move the metabolite to these stores.

Amanita rubescens complex species synthesize the hemolytic metabolite, rubescenslysin (Seeger et al., 1981). However, it is extremely thermolabile in contrast to amatoxins, which are highly stable to temperature and pH (Walton, 2018). The Kruskal-Wallis test found statistical differences between negative control and all experimental treatments and positive controls showing high cytotoxic activity, it also did not find differences among boiled and raw extracts. Additionally, it found no statistical differences between both positive controls and experimental samples. This means that*A. perezsilvae* nom. prov. raw and boiled samples are as cytotoxic as *A. aff. virosa* and *A. aff. verna* (both deathly species belonging to *A. Phalloideae* section), also that this cytotoxicity is not due to the action of rubescenslysin.

4.2 Evolution and diversity in POP genes

Given a vertical gene inheritance, it is expected that a gene tree shares the same topology with the species tree. The POPA genes distribution corresponded to those observed in the species tree, separating the different fungal genera and sections within*Amanita* . Our reconciled tree analysis suggests that POPB/POPD genes were originated from a modified ancient POPA that was horizontally transferred to an Agaricomycete species (hypothetical species "n557", Fig. 3). The POPB gene group phylogeny coincides with Lüli *et al.*(2019) and Luo *et al.* (2018) indicating a phylogenetic disagreement among *Amanita* , *Galerina* and *Lepiota*species due to HGT events (Fig. 3). These authors suggested that*Lepiota* was the original donor of POPB and MSDIN genes

who transferred them to *Galerina* and *Amanita* section*Phalloideae* , where the MSDIN genes diversified. Nevertheless, in the comparative genomic analyses performed by Luo *et al.* (2022), the author denies the previous hypothesis of direct transfer among these genera proposing an ancestral donor species. Our results support that the POPB genes were transferred to *Lepiota* , *Galerina* and*Amanita* from an ancestral species in Agaricomycetes (highly supported clades). Indeed, *Lepiota* POPB clustered together with one gene copy of *Piloderma* , while *Galerina* and*Amanita* POPB genes clustered with another *Piloderma* gene copy. Our analysis indicates that the hypothetical species "n517" in Agaricales acquired one POPB copy and then horizontally transferred it to known *Galerina* species. Another HGT event was from a species in Agaricales related to *Amanita* "n94". *Lepiota* got the other copy directly from *Piloderma* sp. Thus, *Lepiota* and*Galerina* /*Amanita* POPB are paralogous genes that maintain the same function, when most of the genes produced by duplication events tend to lose or change function (neofunctionalization) to flexible selective pressures in one of the copies (Comai, 2005). Nevertheless, this hypothesis could be partially true due to a sampling bias of the >400 fungal species available genomes. Even while we searched and mined all available Agaricomycetes genomes, we may not embrace the whole complex evolutionary history of POP genes. For instance, Lüli *et at.* (2019) proposed that the transference was from *Lepiota* to *Galerina* and then to *Amanita* (Fig. 3). Our wider sampling found that *Piloderma* was who transferred the POPB genes to all known gene hosts, and that *Lepiota* did not transferred POPB genes to *Galerina* (Fig. 3).

The POPD clade phylogenetic incongruence found here is new despite that*A. rubescens s.s.* POPD genes were stored in JGI Mycocosm and NCBI databases years ago. For POPD, our analysis suggested that an ancestral species in Agaricomycetes related to *Piloderma byssinum*transferred the POP gene to "n540" hypothetical Agaricomycete species. Unfortunately, we could not point out when or where exactly*Amanita* species acquired this gene. However, one hypothesis is that the "n540" species is related to the *Amanita* genus ancestor. This is supported due to the lack of any other evolutionary events but vertical inheritance within *Amanita* species. Nevertheless, Notung analysis suggested that POPD3 gene was transferred from "n72" *Amanita* related hypothetical species to*Hebeloma* and *Cortinarius* nodes.

For all POPD genes, *A. cruentilemurum nom. prov.*(GNHR-426) POPD1 is the oldest gene version and that POPD2 and POPD3 are paralogous genes that maintained the same structure. The fact that*A. rubescens s.s* . from Europe and, *A. cruentilemurum nom. prov.* and *A. perezsilvae nom. prov* . from North America share the POPD genes (D2 and 3) suggests that the horizontal transfer occurred before the species and continents separated ˜200 Million years ago. Our results show that the HGT of the amatoxin biosynthesis genes was not an isolated event to*Amanita* section *Phalloideae* , but a more extensive and complicated evolutionary history also involving *Amanita* series*Rubescentes* and many intermediaries.

4.3 Evolutionary and ecological implications of the amanitin lethal metabolic pathway horizontal transfer

Lüli *et al* . (2019) proposed that the POPB transfer was related to the ecological niche from saprophytic to ectomycorrhizal species. However, *Piloderma,* which is most likely the ancestral POPB/POPD gene donor, is ectomycorrhizal as *Russula brevipes* which is the outgroup of the POPB/POPD gene clade. Also, all species from the POPD gene clade are ectomycorrhizal, except for *Galerina* and*Lepiota* in the POPB clade. Hence, more than the ecological niche differences, it has been suggested that overlapping niches, direct physical interactions and sharing environmental selection pressures may promote the transferring events (Reynolds *et al.,* 2018). Here, we did an extensive gene sampling and mining in all available fungal genomes from different databases, obtaining a robust topology tree that showed a more complex evolutionary history than previously reported (Lüli *et al* ., 2019; Luo *et al* ., 2018; Walton, 2018). In the future it will be necessary to continue mining new fungal genomes to better explain the origin of the amatoxin synthesis genes and solve the ecological niche of its ancestor.

Transferred genes must provide selective advantages or fitness benefits to the new hosts. For instance, Reynolds *et al* ., (2018) suggested that the psilocybin gene clusters were transferred from *Psilocybe* sp. to*Podorospora* sp. for niche colonization from wood- to dug-decay, competitive and defense strategies. Theoretically, amanitin gene cluster must confer benefits to *Amanita* series *Rubescentes* as a defense mechanism. Walton (2018) suggested that the MSDIN gene diversification provided defense against all possible ant-

agonists by inhibiting the eukaryotic RNA polymerase II of most mammals, insects, worms, or parasites. However, the presence of this gene cluster in edible species of the *A. rubescens* complex contradict this. Even while the presence of all the genes necessary to produce amatoxins are present in the edible mushrooms *Amanita rubescenss.s.* , *Amanita cruentilemurum nom. prov.* and*Amanita perezsilvae nom . prov* . its transcription and metabolite production must be proven. Here we were able to prove that the same samples having the metabolic amatoxin pathway have the same levels of cytotoxicity in a vegetal model than lethal amanitas from section Phalloidae. As several mortal intoxications in Mexico have been related to the consumption these mushrooms it is imperative to communicate these results to the scientific community and decision makers. We recommend that the consumption of these species should be avoided until its chemistry and toxicology are fully solved.

Acknowledgements

The authors would like to thanks to the Posgrado en Ciencias Biologicas, the CONACyT postgraduate scholarship No. 696992, and UNAM "Programa de Apoyo a Proyectos de Investigación e Innovación Tecnológica" (UNAM PAPIIT IN212521) for founding. To Alicia Mastretta-Yanes and Luis David Peraza for their advice and commentaries. To CONABIO computational cluster.

References

Andersson, J. O. (2005). Lateral gene transfer in eukaryotes. *Cellular and Molecular Life Sciences: CMLS*, *62*(11), 1182–1197.

Artimo, P., Jonnalagedda, M., Arnold, K., Baratin, D., Csardi, G., de Castro, E., Duvaud, S., Flegel, V., Fortier, A., Gasteiger, E., Grosdidier, A., Hernandez, C., Ioannidis, V., Kuznetsov, D., Liechti, R., Moretti, S., Mostaguir, K., Redaschi, N., Rossier, G., ... Stockinger, H. (2012). ExPASy: SIB bioinformatics resource portal. *Nucleic Acids Research,40*(Web Server issue), W597–W603.

Ayala-Ruano, S., Santander-Gordón, D., Tejera, E., Perez-Castillo, Y., & Armijos-Jaramillo, V. (2019). A putative antimicrobial peptide from Hymenoptera in the megaplasmid pSCL4 of ATCC 27064 reveals a singular case of horizontal gene transfer with potential applications.*Ecology and Evolution*, *9*(5), 2602–2614.

Bolger, A. M., Lohse, M., & Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. In *Bioinformatics* (Vol. 30, Issue 15, pp. 2114–2120). https://doi.org/10.1093/bioinformatics/btu170

Bresinsky, A. (1990). *A Colour Atlas of Poisonous Fungi: A Handbook for Pharmacists, Doctors, and Biologists*. CRC Press.

Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K., & Madden, T. L. (2009). BLAST : architecture and applications. In *BMC Bioinformatics* (Vol. 10, Issue 1, p. 421). https://doi.org/10.1186/1471-2105-10-421

Chaib De Mares, M., Hess, J., Floudas, D., Lipzen, A., Choi, C., Kennedy, M., Grigoriev, I. V., & Pringle, A. (2015). Horizontal transfer of carbohydrate metabolism genes into ectomycorrhizal Amanita. *The New Phytologist*, *205*(4), 1552–1564.

Chen, K., Durand, D., & Farach-Colton, M. (2000). NOTUNG: A Program for Dating Gene Duplications and Optimizing Gene Family Trees. In *Journal of Computational Biology* (Vol. 7, Issues 3-4, pp. 429–447). https://doi.org/10.1089/106652700750050871

Choi, C.-H. (2005). ABC transporters as multidrug resistance mechanisms and the development of chemosensitizers for their reversal. *Cancer Cell International,5*, 30.

Comai, L. (2005). The advantages and disadvantages of being polyploid. *Nature Reviews. Genetics*, *6*(11), 836–846.

Edgar, R. C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput.*Nucleic Acids Research*, *32*(5), 1792–1797.

Grigoriev, I. V., Nikitin, R., Haridas, S., Kuo, A., Ohm, R., Otillar, R., Riley, R., Salamov, A., Zhao, X., Korzeniewski, F., Smirnova, T., Nordberg, H., Dubchak, I., & Shabalov, I. (2014). MycoCosm portal: gearing up for 1000 fungal genomes. *Nucleic Acids Research*, *42*(Database issue), D699–D704.

Gurevich, A., Saveliev, V., Vyahhi, N., & Tesler, G. (2013). QUAST: quality assessment tool for genome assemblies. *Bioinformatics* , *29*(8), 1072–1075.

Hallen, H. E., Luo, H., Scott-Craig, J. S., & Walton, J. D. (2007). Gene family encoding the major toxins of lethal Amanita mushrooms. *Proceedings of the National Academy of Sciences of the United States of America*,*104*(48), 19097–19101.

He, Z., Long, P., Fang, F., Li, S., Zhang, P., & Chen, Z. (2020). *Diversity of MSDIN family members in amanitin-producing mushrooms and the phylogeny of the MSDIN and prolyl oligopeptidase genes*. https://doi.org/10.21203/rs.2.22199/v2

Huang, J. (2013). Horizontal gene transfer in eukaryotes: the weak-link model. *BioEssays: News and Reviews in Molecular, Cellular and Developmental Biology*,*35*(10), 868–875.

Husnik, F., & McCutcheon, J. P. (2018). Functional horizontal gene transfer from bacteria to eukaryotes. In *Nature Reviews Microbiology* (Vol. 16, Issue 2, pp. 67–79). https://doi.org/10.1038/nrmicro.2017.137

Jacox, E., Chauve, C., Szöllősi, G. J., Ponty, Y., & Scornavacca, C. (2016). ecceTERA: comprehensive gene tree-species tree reconciliation using parsimony: Table 1. In *Bioinformatics* (Vol. 32, Issue 13, pp. 2056–2058). https://doi.org/10.1093/bioinformatics/btw105

Kloesges, T., Popa, O., Martin, W., & Dagan, T. (2011). Networks of gene sharing among 329 proteobacterial genomes reveal differences in lateral gene transfer frequency at different phylogenetic depths. *Molecular Biology and Evolution*, *28*(2), 1057–1074.

Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. In *Molecular Biology and Evolution* (Vol. 35, Issue 6, pp. 1547–1549). https://doi.org/10.1093/molbev/msy096

Kunin, V., Goldovsky, L., Darzentas, N., & Ouzounis, C. A. (2005). The net of life: reconstructing the microbial phylogenetic network. *Genome Research*, *15*(7), 954–959.

Kusakizako, T., Miyauchi, H., Ishitani, R., & Nureki, O. (2020). Structural biology of the multidrug and toxic compound extrusion superfamily transporters. *Biochimica et Biophysica Acta, Biomembranes*, *1862*(12), 183154.

Lanfear, R., Frandsen, P. B., Wright, A. M., Senfeld, T., & Calcott, B. (2017). PartitionFinder 2: New Methods for Selecting Partitioned Models of Evolution for Molecular and Morphological Phylogenetic Analyses. *Molecular Biology and Evolution*, *34*(3), 772–773.

Li, P., Deng, W., & Li, T. (2014). The molecular diversity of toxin gene families in lethal Amanita mushrooms. *Toxicon: Official Journal of the International Society on Toxinology*, *83*, 59–68.

Lüli, Y., Cai, Q., Chen, Z. H., Sun, H., Zhu, X.-T., Li, X., Yang, Z. L., & Luo, H. (2019). Genome of lethal Lepiota venenata and insights into the evolution of toxin-biosynthetic genes. *BMC Genomics*, *20*(1), 198.

Luo, H., Cai, Q., Lüli, Y., Li, X., Sinha, R., Hallen-Adams, H. E., & Yang, Z. L. (2018). The MSDIN family in amanitin-producing mushrooms and evolution of the prolyl oligopeptidase genes. *IMA Fungus*, *9*, 225–242.

Luo, H., Hallen-Adams, H. E., Lüli, Y., Sgambelluri, R. M., Li, X., Smith, M., Yang, Z. L., & Martin, F. M. (2022). Genes and evolutionary fates of the amanitin biosynthesis pathway in poisonous mushrooms. *Proceedings of the National Academy of Sciences of the United States of America*, *119*(20), e2201113119.

Manni, M., Berkeley, M. R., Seppey, M., Simão, F. A., & Zdobnov, E. M. (2021). BUSCO Update: Novel and Streamlined Workflows along with Broader and Deeper Phylogenetic Coverage for Scoring of Eukaryotic,

Prokaryotic, and Viral Genomes. In*Molecular Biology and Evolution* (Vol. 38, Issue 10, pp. 4647–4654). https://doi.org/10.1093/molbev/msab199

Miller, M. A., Pfeiffer, W., & Schwartz, T. (2010). Creating the CIPRES Science Gateway for inference of large phylogenetic trees. In *2010 Gateway Computing Environments Workshop (GCE)*. https://doi.org/10.1109/gce.2010.5676129

Miyauchi, S., Kiss, E., Kuo, A., Drula, E., Kohler, A., Sánchez-García, M., Morin, E., Andreopoulos, B., Barry, K. W., Bonito, G., Buée, M., Carver, A., Chen, C., Cichocki, N., Clum, A., Culley, D., Crous, P. W., Fauchery, L., Girlanda, M., … Martin, F. M. (2020). Large-scale genome sequencing of mycorrhizal fungi provides insights into the early evolution of symbiotic traits. *Nature Communications*, *11*(1), 5125.

Novo, M., Bigey, F., Beyne, E., Galeote, V., Gavory, F., Mallet, S., Cambon, B., Legras, J.-L., Wincker, P., Casaregola, S., & Dequin, S. (2009). Eukaryote-to-eukaryote gene transfer events revealed by the genome sequence of the wine yeast Saccharomyces cerevisiae EC1118.*Proceedings of the National Academy of Sciences of the United States of America*, *106*(38), 16333–16338.

Ochman, H., Lawrence, J. G., & Groisman, E. A. (2000). Lateral gene transfer and the nature of bacterial innovation. *Nature*, *405*(6784), 299–304.

Okonechnikov, K., Golosova, O., Fursov, M., & UGENE team. (2012). Unipro UGENE: a unified bioinformatics toolkit. *Bioinformatics* , *28*(8), 1166–1167.

Prjibelski, A., Antipov, D., Meleshko, D., Lapidus, A., & Korobeynikov, A. (2020). Using SPAdes De Novo Assembler. *Current Protocols in Bioinformatics / Editoral Board, Andreas D. Baxevanis … [et Al.]*, *70*(1), e102.

Pulman, J. A., Childs, K. L., Michael Sgambelluri, R., & Walton, J. D. (2016). Expansion and diversification of the MSDIN family of cyclic peptide genes in the poisonous agarics Amanita phalloides and A. bisporigera. In *BMC Genomics* (Vol. 17, Issue 1). https://doi.org/10.1186/s12864-016-3378-7

Quistgaard, E. M., Löw, C., Guettou, F., & Nordlund, P. (2016). Understanding transport by the major facilitator superfamily (MFS): structures pave the way.*Nature Reviews. Molecular Cell Biology*, *17*(2), 123–132.

Rambaut, A., Drummond, A. J., Xie, D., Baele, G., & Suchard, M. A. (2018). Posterior Summarization in Bayesian Phylogenetics Using Tracer 1.7. In *Systematic Biology*(Vol. 67, Issue 5, pp. 901–904). https://doi.org/10.1093/sysbio/syy032

Reynolds, H. T., Vijayakumar, V., Gluck-Thaler, E., Korotkin, H. B., Matheny, P. B., & Slot, J. C. (2018). Horizontal gene cluster transfer increased hallucinogenic mushroom diversity. *Evolution Letters*, *2*(2), 88–101.

Reynolds, H. T., Vijayakumar, V., Gluck-Thaler, E., Korotkin, H. B., Matheny, P. B., & Slot, J. C. (2018c). Horizontal gene cluster transfer increased hallucinogenic mushroom diversity. *Evolution Letters*, *2*(2), 88–101.

Rokas, A., Mead, M. E., Steenwyk, J. L., Raja, H. A., & Oberlies, N. H. (2020). Biosynthetic gene clusters and the evolution of fungal chemodiversity. In*Natural Product Reports* (Vol. 37, Issue 7, pp. 868–878). https://doi.org/10.1039/c9np00045c

Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D. L., Darling, A., Höhna, S., Larget, B., Liu, L., Suchard, M. A., & Huelsenbeck, J. P. (2012). MrBayes 3.2: Efficient Bayesian Phylogenetic Inference and Model Choice Across a Large Model Space. In *Systematic Biology* (Vol. 61, Issue 3, pp. 539–542). https://doi.org/10.1093/sysbio/sys029

Rosewich, U. L., Liane Rosewich, U., & Corby Kistler, H. (2000). Role of Horizontal Gene Transfer in the Evolution of Fungi. In *Annual Review of Phytopathology* (Vol. 38, Issue 1, pp. 325–363). https://doi.org/10.1146/annurev.phyto.38.1.325

Santos, A. L. D., Chaves-Silva, S., Yang, L., Maia, L. G. S., Chalfun-Júnior, A., Sinharoy, S., Zhao, J., & Benedito, V. A. (2017). Global analysis of the MATE gene family of metabolite transporters in tomato. *BMC Plant Biology*, *17*(1), 185.

Schönknecht, G., Weber, A. P. M., & Lercher, M. J. (2014). Horizontal gene acquisitions by eukaryotes as drivers of adaptive evolution. In *BioEssays* (Vol. 36, Issue 1, pp. 9–20). https://doi.org/10.1002/bies.201300095

Seeger, R., Odenthal, K. P., & Mengs, U. (1981). Toxic effects in mouse and rat of rubescenslysin from Amanita rubescens. *Toxicon: Official Journal of the International Society on Toxinology*, *19*(3), 409–417.

Sgambelluri, R. M., Smith, M. O., & Walton, J. D. (2018). Versatility of Prolyl Oligopeptidase B in Peptide Macrocyclization. *ACS Synthetic Biology*, *7*(1), 145–152.

Shoji, T. (2014). ATP-Binding Cassette and Multidrug and Toxic Compound Extrusion Transporters in Plants. In *International Review of Cell and Molecular Biology*(pp. 303–346). https://doi.org/10.1016/b978-0-12-800255-1.00006-5

Simpson, J. T., Wong, K., Jackman, S. D., Schein, J. E., Jones, S. J. M., & Birol, I. (2009). ABySS: a parallel assembler for short read sequence data. *Genome Research*, *19*(6), 1117–1123.

Slot, J. C., & Hibbett, D. S. (2007). Horizontal transfer of a nitrate assimilation gene cluster and ecological transitions in fungi: a phylogenetic study. *PloS One*,*2*(10), e1097.

Stamatakis, A. (2014). RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* , *30*(9), 1312–1313.

Stanke, M., Keller, O., Gunduz, I., Hayes, A., Waack, S., & Morgenstern, B. (2006). AUGUSTUS: ab initio prediction of alternative transcripts. *Nucleic Acids Research*, *34*(suppl_2), W435–W439.

Thomas, C. M., & Nielsen, K. M. (2005). Mechanisms of, and Barriers to, Horizontal Gene Transfer between Bacteria. In *Nature Reviews Microbiology* (Vol. 3, Issue 9, pp. 711–721). https://doi.org/10.1038/nrmicro1234

Van Etten, J., & Bhattacharya, D. (2020). Horizontal Gene Transfer in Eukaryotes: Not if, but How Much? In *Trends in Genetics* (Vol. 36, Issue 12, pp. 915–925). https://doi.org/10.1016/j.tig.2020.08.006

Vignolle, G. A., Mach, R. L., Mach-Aigner, A. R., & Derntl, C. (2020). Novel approach in whole genome mining and transcriptome analysis reveal conserved RiPPs in Trichoderma spp. *BMC Genomics*, *21*(1), 258.

Walton, J. (2018). *The Cyclic Peptide Toxins of Amanita and Other Poisonous Mushrooms*. https://doi.org/10.1007/978-3-319-76822-9

Zendehboodi, Z. (2018). Cytotoxicity and genotoxicity effects of water boiled in aluminum vessels on root tip cells. *Journal of Environmental Health Science & Engineering*, *16*(2), 337–341.

Zerbino, D. R., & Birney, E. (2008). Velvet: Algorithms for de novo short read assembly using de Bruijn graphs. In *Genome Research* (Vol. 18, Issue 5, pp. 821–829). https://doi.org/10.1101/gr.074492.107

Author contributions

CQ-C and RG-O designed the research and wrote the manuscript with contribution of all the authors. GH-R and PO-A collected the samples and performed the genotoxic analysis. CQ-C performed most of the analyses. MV performed genome assembly and gene neighborhood analysis.

Data Accessibility Statement

Unique haplotype data are deposited to NCBI Nucleotide Database:

ITS: MZ014386 to MZ014395

- rpb2: OP584270 to OP584280
- LSU: OP594911 to OP594921

- POP: MZ028175 to MZ028202

Tables

Table 1. Basic features of the assembled genomes

ID samples	GNHR426[1]	GNHR 430[2]	GNHR 431[2]	GNHR 432[2]	GNHR 433[2]	GNHR 434[2]	GNHR 435[2]
Genome size (Mb)	38.9	53	-	50.3	54.4	59.4	50.15
# Scaffolds	24663	10639	-	19599	12708	17108	21680
Depth	453	152	-	1672	265	365	918
N50	2923	34120	-	6843	27409	17264	4727
%GC	47.68	48.10	-	48.03	49.85	49.52	48.06
# Genes	1968	3534	-	3028	3510	3497	2871
Gene length	289	387	-	354	384	382	345

N50: length cutoff for the longest contigs that contain 50% of the total genome length

[1]: Correspond to *Amanita cruentilemurum* nom. pro.

[2]: Correspond to *Amanita perezsilvae*nom. pro.

[3]: Correspond to *Amanita suballiacea*

Figures

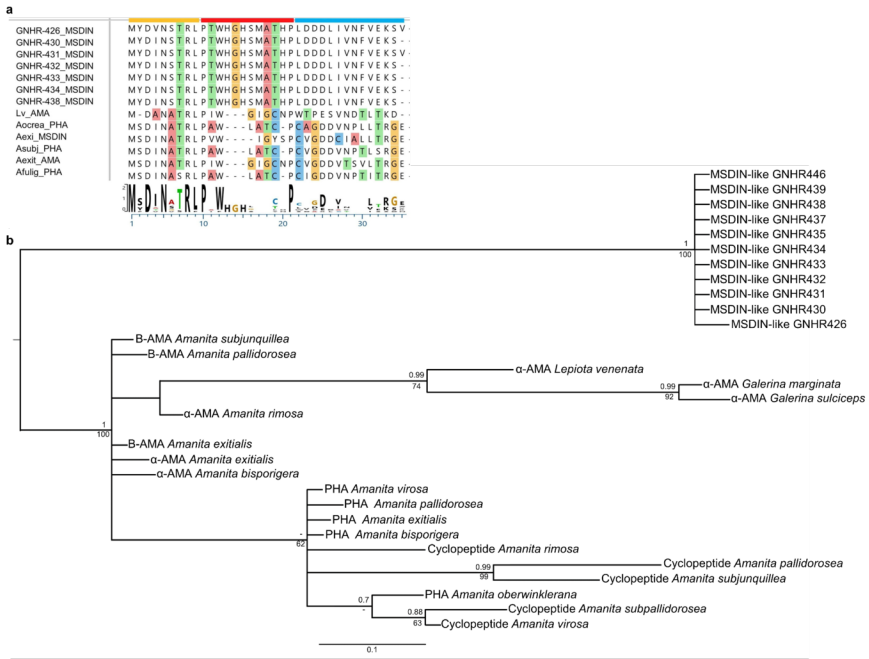

Figure 1. Alignment and phylogenetic inferences of MSDIN genes.**a)** Amino acids alignment of MSDIN sequences from *Amanita perezsilvae* nom. prov. (GNHR*), *Lepiota venenata* (Lv), *A. ocreata* (Aocrea), *A. exitialis* (Aexi), *A. subjunquillea*(Asubj), *A. fuliginea* (Afulg), amanitin (AMA) and phalloidin (PHA) genes. **b)** MSDIN phylogenetic tree. Posterior probability (pp) and bootstrap values greater than 0.64 and 60 are above and below each clade, respectively.

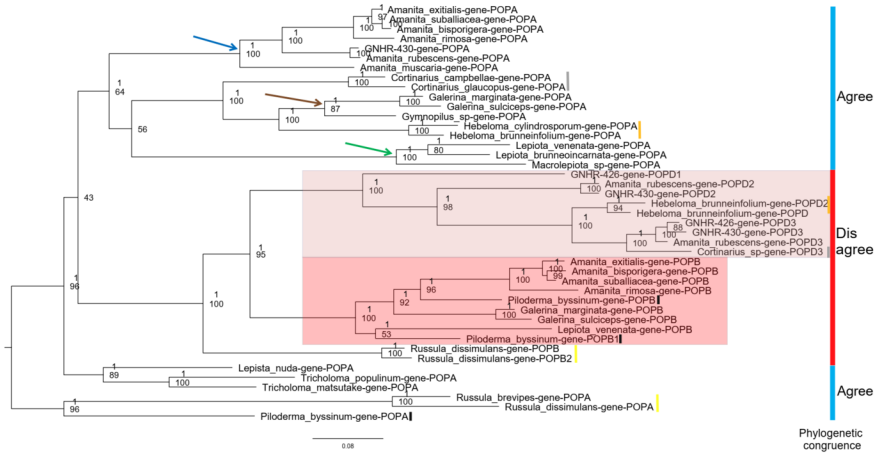

Figure 2. Maximum likelihood tree of prolyl oligopeptidase amino acid sequences in Agaricomycetes. The bootstrap values are in each clade. Each arrow points out the different POPB species, *Amanita* (blue arrow), *Galerina* (brown arrow) and *Lepiota* (green arrow). The red and pink boxes show the phylogenetic incongruences due to HGT events previously reported (Luo *et al* ., 2018; Lüli *et al.* , 2019) and the newly discovered here, respectively. The color bars at the end of some branches show the different genus that are related to the newly detected HGT event: *Russula* (yellow), *Piloderma*(black), *Hebeloma* (orange) and *Cortinarius* (gray). The red (disagree) and blue (agree) parallel bars mark out which clades follow the phylogenetic history.

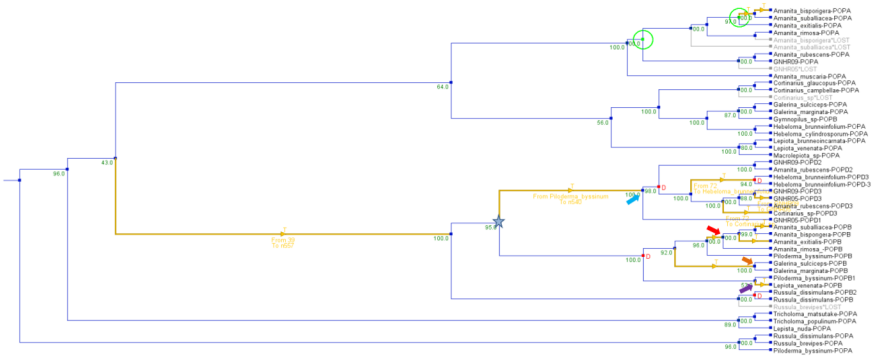

Figure 3. The DTL model reconciled tree constructed with Notung.**a**) The red D and yellow T letters in the clades point out the duplication and transferred events, respectively. The bootstraps values are below each clade in green. Each colored arrow indicates a transition event from *Piloderma byssinum* : to hypothetical species "n540" (blue), to species "94" (red), to species "n517" (orange), and to*Lepiota* sp. (purple). The blue star marks the *Piloderma byssinum* ancestor.