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ARTICLE

Evolution and horizontal transfer of the psilocybin biosynthetic gene cluster drive the diversification of magic mushrooms

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Abstract

Psilocybin, the psychoactive compound responsible for the hallucinogenic effects of "magic mushrooms," is synthesized by a biosynthetic gene cluster (BGC) traditionally associated with *Psilocybe* species. However, psilocybin production has also been identified in multiple genera across the Strophariaceae, Hymenogastraceae, and Galeropsidaceae families. Here, we sequenced the genomes of 30 putative psilocybin-producing mushroom species from ten genera using PacBio long-read technology. Comparative analysis across 20,608 bacterial, plant, and fungal genomes indicates that the psilocybin BGC most likely originated from endogenous fungal homologs through gene duplication and rearrangement, rather than horizontal gene transfer (HGT) from non-fungal sources. We identified four independent HGT events and three distinct BGC configurations, and propose an evolutionary framework integrating vertical inheritance, HGT, and strong purifying selection. Transcriptomic profiling revealed high expression of *PsiK* during the mycelial stage, while *PsiH* and *PsiM* remained inactive—correlating with the absence of psilocybin in mycelial tissue confirmed by UHPLC-MS/MS, and suggesting stage-specific regulation. Divergence time estimation, coupled with the coprophilous habit of most psilocybin-producing species, supports a post-Cretaceous-Tertiary radiation coinciding with the rise of mammals and novel ecological niches such as grasslands and dung substrates. Pangenomic analysis further reveals that horizontal BGC transfer contributes substantially to genetic innovation, facilitating species diversification and ecological adaptation. These findings highlight the pivotal role of secondary metabolites in fungal evolution and provide a genomic foundation for future research on psilocybin biosynthesis and its therapeutic potential.

Keywords – biosynthetic gene cluster – fungal diversity – horizontal gene transfer – magic mushrooms – psilocybin

INTRODUCTION

Secondary metabolite (SM) biosynthetic gene clusters (BGCs) are common features in fungal genomes, where genes involved in the synthesis of secondary metabolites tend to cluster together. These metabolites play crucial roles in fungal ecology, including defense mechanisms, interspecies

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interactions, and adaptation to diverse ecological niches. However, understanding the genetic basis and evolution of these clusters is challenging due to their high divergence and species-specific presence (Rokas et al. 2018). Horizontal gene transfer (HGT), initially observed in prokaryotes, also plays a significant role in fungal genome evolution (Fitzpatrick 2012, Liu et al. 2024). In fungi, HGT has played a significant role in the evolution of BGCs responsible for producing a wide range of secondary metabolites with roles in defence, virulence, and communication (Lind et al. 2017, Rokas et al. 2020). For example, a complete operon involved in siderophore biosynthesis was horizontally transferred from bacteria to a group of budding yeasts (Kominek et al. 2019).

Psilocybin, a serotonergic hallucinogen found in psychedelic mushrooms, profoundly alters perception and mood, inducing hallucinations upon ingestion (Nichols 2016). Current research, both in humans and animals, suggests that psilocybin is not associated with addiction risk (van Amsterdam et al. 2011). Psilocybin has the potential to offer short- and long-term benefits for individuals suffering from various conditions, such as resistant depression, mood disorders, and chronic pain (Reiff et al. 2020, Goodwin et al. 2022). The enzymatic pathway for synthesizing the psychoactive compound psilocybin was first elucidated in *Psilocybe cubensis* (Fricke et al. 2017). Four core genes encoding enzymes (tryptophan decarboxylase *PsiD*, kinase *PsiK*, methyltransferase *PsiM*, and P450 *PsiH*) that convert the amino acid tryptophan into psilocybin occur in a cluster and one gene that encodes a putative transporter (*PsiT*) that is co-inherited with the cluster (Fricke et al. 2017, Reynolds et al. 2018). The psilocybin gene cluster responsible for psilocybin synthesis is horizontally transferred across various mushroom-forming fungal lineages (Reynolds et al. 2018). Phylogenetic analysis suggests that this cluster was horizontally acquired by psilocybin-producing mushrooms in other genera that share common ecological niches (Reynolds et al. 2018, Awan et al. 2018), although the origin of psilocybin biosynthetic genes and the direction of these transfers remain unclear.

Psilocybin-producing mushrooms, spanning genera such as *Pholiota* (Fr.) P. Kumm., *Gymnopilus* P. Karst., *Galerina* Earle, *Psilocybe* (Fr.) P. Kumm., *Inocybe* (Fr.) Fr., *Pluteus* Fr., *Hypholoma* (Fr.) P. Kumm., and *Panaeolus* (Fr.) Quél. (Walters 1965, He et al. 2022), are globally distributed, with *Psilocybe* being the major contributor (Meyer & Slot 2023). The majority of these mushrooms trace their origins to the families Strophariaceae, Hymenogastraceae and Galeropsidaceae based on the outline of Agaricales, Agaricomycetes, and Basidiomycota (He 2019). Specifically, six genera from Hymenogastraceae viz. *Flammula* (Fr.) P. Kumm, *Galerina*, *Gymnopilus*, *Hebeloma*, *Phaeocollybia* R. Heim and *Psilocybe* and four from Strophariaceae viz. *Hypholoma*, *Leratiomyces* Bresinsky & Manfr. Binder ex Bridge, Spooner, Beever & D.C. Park, *Pholiota* and *Protostropharia* Redhead, Moncalvo & Vilgalys include the reported psilocybin-producing species. Conversely, the intertwined binary branched phylogenetic distribution of toxic and edible species within these genera reveals unclear systematic evolution patterns (Moreno et al. 2017, Sato et al. 2020, Tian & Matheny 2021). Understanding these dynamics is essential, especially in the context of biodiversity loss and conservation, which are pressing global concerns (Antonelli et al. 2020, Gonçalves et al. 2021).

Genetic diversity, a fundamental dimension of biodiversity, plays a crucial role in species survival and the functioning of ecosystems (Toro & Caballero 2005). It enhances evolutionary potential and increases the ability of species to respond to environmental changes (Pemberton 2004). Numerous studies have indicated that genetic factors significantly influence species endangerment and extinction (Murray et al. 2017). Moreover, genetic diversity is positively correlated with species diversity across various taxa, including terrestrial mammals, marine and freshwater fishes, and wildlife in China (Manel et al. 2020, Theodoridis et al. 2020, Hu et al. 2021). This relationship underscores the importance of maintaining genetic diversity, particularly in ecologically significant groups like psilocybin-producing mushrooms, which could have important implications for their conservation and ecological roles.

Despite the widespread distribution of psilocybin-producing species, our understanding of the genomic structures underlying the psilocybin biosynthetic pathway and the evolutionary processes driving the diversification of these mushrooms remains limited (Van Court et al. 2022). To date,

only a few species from *Psilocybe, Panaeolus,* and *Gymnopilus* have been investigated at a genomic scale (Van Court et al. 2022, Dörner et al. 2022, Bradshaw et al. 2024), which does not fully represent the diversity of those species, thus limiting comprehensive evolutionary inferences. To address this gap, we conducted a comprehensive genomic analysis of psilocybin BGCs by sequencing 30 genomes of potentially psilocybin-producing mushrooms. This study provides new insights into the evolutionary history of the psilocybin BGC and emphasizes the importance of the evolution of complex secondary metabolic pathways in driving fungal diversity.

MATERIALS AND METHODS

Strain culture and genome sequencing

Basidiospores were inoculated on potato dextrose agar (PDA) and incubated at 25°C for 10 days until germination. Single spore isolates without clamp connections were selected and sub-cultured individually on PDA plates at 25°C. High-quality genomic DNA was extracted using the QIAGEN® Genomic kit (Qiagen, Dusseldorf, Germany) following the manufacturer's instructions. DNA quality was assessed via agarose gel electrophoresis and quantified by Qubit® 2.0 Fluorometer (Thermo Scientific). Libraries for single-molecule real-time (SMRT) sequencing were constructed with an insert size of 20 kb using the SMRTbell™ Template Prep Kit v1.0. The process involved DNA fragmentation, concentration, damage repair, end repair, blunt ligation reaction preparation, SMRTbell template purification with 0.45X AMPure PB Beads, size-selection using the BluePippin System, and post size-selection damage repair. The library quality was confirmed using the Qubit® 2.0 Fluorometer and Agilent 2100 (Agilent Technologies). Whole-genome sequencing was performed using the PacBio Sequel platform at Beijing Novogene Bioinformatics Technology Co., Ltd.

Genome assembly and completeness assessment

Genome assembly was performed with Flye version 2.9-b1768 (Kolmogorov et al. 2020), a long-read metagenomic assembler that constructs a repeat graph to assemble and polish contigs. Genome completeness was assessed using BUSCO v5.2.0 (Manni et al. 2021) with the agaricales_odb10 dataset, which contains 3,870 conserved single-copy orthologs. BUSCO genes were classified as complete (single-copy or duplicated), fragmented, or missing.

Genome annotation

Gene prediction was analyzed by GeneMark-ES (Ter-Hovhannisyan et al. 2008), which has a special option for fungal genomes to account for fungal-specific intron organization. For the prediction of Carbohydrate-Active Enzymes (CAZymes), we conducted an HMM search using dbCAN2 (Zhang et al. 2018) with dbCAN-HMM profiles (https://bio.tools/dbcan) as our query templates. Putative polyketide synthases (PKS), non-ribosomal peptide synthases (NRPS) genes, PKS-NRPS hybrids, and their modules of different domains were identified using antiSMASH 6.1.1 with default settings (Blin et al. 2021). To further assess the ability of fungi to produce new chemical scaffolds, we grouped BGCs into families using the pairwise distance between BGCs and a clustering algorithm to yield GCFs. BGCs from antiSMASH were converted to arrays of protein domains and then compared based on the fraction of shared domains and backbone protein domain sequence identity. BGCs within a family are expected to encode identical or similar metabolites depending on the similarity threshold. Identifying BGCs that have known metabolite products is an important component of genome mining, enabling researchers to prioritize known versus unknown biosynthetic pathways for discovery. The development of the Minimum Information about a Biosynthetic Gene Cluster (MIBiG) repository (Medema et al. 2015), housing 438 fungal BGCs, has greatly supported genomic dereplication efforts. These BGCs consist of 415 from Ascomycota and 23 from Basidiomycota, all well-documented. By anchoring unknown BGCs to these known references, the Genomic Cluster Family (GCF) approach allows for large-scale annotation, identifying clusters likely to produce known or derivative metabolites. GO terms were annotated

using InterProScan v5.0 with default options (Jones et al. 2014). Enriched genes in mushrooms with the psilocybin BGC were statistically assessed using Fisher's exact test, with p-values adjusted for multiple comparisons (Bonferroni method, p-value < 0.01). Functional enrichment analysis was performed using Gene Set Enrichment Analysis algorithm in the clusterProfiler (Wu et al. 2021) package.

Phylogenetic analysis

A phylogenomic data matrix was constructed using 3,870 single-copy orthologous genes from representatives of Basidiomycota. Each gene was aligned with MAFFT (Katoh & Standley 2013) v7.490 with default options. Ambiguously aligned regions were removed using trimAl version 1.4 with the gappyout option. The AA alignments of these BUSCO genes, each of which has more than 50% taxon occupancy, were then concatenated into the full data matrix. Phylogenetic analyses were performed using IQ-TREE (Minh et al. 2020) version 2.0.3, suited for maximum likelihood (ML) frameworks in phylogenomic data analysis. Molecular dating was estimated using r8s (Sanderson 2003) with calibration points set as 106 mya to represent the divergence time between Panaelous and Gymnopilus (He et al. 2024). Node ages inferred from this analysis were used as divergence time (t) in the calculation of corrected nucleotide diversity.

Identification of psilocybin biosynthetic genes

Sequence and domain alignment were combined to confidently identify psilocybin-related proteins. First, the protein sequences of the psilocybin gene cluster (PsiD, ASU62239.1, PPQ83218.1; *PsiM*, ASU62241.1, PPQ83230.1; *PsiH*, PPQ83216.1, PPQ83215.1; *PsiK*, PPQ83229.1; *PsiT*, PPQ83222.1) were used as bait to search psilocybin genes in genomes by BLASTP. The results were filtered by parameters of e-value less than 1e-30. Then, each protein sequence of each genome was searched against the Pfam database using a profile-sequence alignment tool PfamScan with default parameters to detect the occurrence of any protein domain families in the psilocybin gene cluster (*PsiT*, PF07690; *PsiM*, PF05971; PsiD, PF12588 and PF02666; *PsiK*, PF01636; PsiH, PF00067). Next, we scanned all genomes within a window of 20 kb using in-house scripts to select candidate regions where *PsiD*, *PsiK*, *PsiM*, and *PsiH* genes coexisted. This approach identified complete psilocybin biosynthetic genes in numerous fungal species across the fungal kingdom. Some species may potentially produce psilocybin, warranting further investigation. Only the closest homologous copies of psilocybin genes across the Agaricales order were kept as native homologs. All publicly available fungal genomes used in this study are obtained from the National Center for Biotechnology Information GenBank database (Table S4). One hundred representative complete fungal genomes and 2,515 representative fungal genomes were downloaded at the NCBI as of June 2023. All the plant genomes were from the *Phytozome* (Goodstein et al. 2012) database.

UHPLC-MS/MS

Dried fruiting bodies underwent homogenization using a ceramic mortar and pestle, resulting in a fine powder. For sample extraction, 200 mg of fungal powder was combined with 0.5 mL of 0.5% acetic acid (v/v) in methanol. The mixtures were vortexed at 25℃ for 30 minutes. Subsequently, the testing tubes underwent centrifugation for 10 minutes at 10,000 rpm at 4℃. The suspensions were then filtered using Eppendorf tubes equipped with a 0.2 μm PTFE microfilter (Ciro, Hudson, WI, USA) in a centrifuge (10,000 rpm, 10 minutes, 4℃). Re-extraction was performed with 0.4 mL methanol under the same conditions. The final supernatant was used for UHPLC-MS/MS analysis. Separation and quantification of tryptamines were conducted using the UHPLC 1290 Infinity assembly (Agilent Technologies) with a Zorbax Eclipse Plus C18 column. The system was coupled with a triple quadrupole 6460 spectrometer. Gradient elution was employed with mobile phases A (10 mM ammonium formate with 0.1% formic acid) and B (10 mM ammonium formate with 0.1% formic acid in methanol). The flow rate was 5.0 mL/min, with a sample injection volume of 0.40 μL. Electrospray ionization in positive mode (ESI+) was applied.

Optimal MS conditions included a drying gas temperature (nitrogen) of 340℃, drying gas flow of 10 L/min, nebulizer (nitrogen) of 25 psig, sheath gas temperature (nitrogen) of 400℃, sheath gas flow of 12 L/min, and a capillary voltage of 2800 V. Dynamic multiple reaction monitoring (dMRM) acquisition mode was used, with a detection limit (LOD) of 0.1 ng/mL and a quantification limit (LOQ) of 0.5 ng/mL.

The origin and evolution of the psilocybin BGC

To analyze the origin and evolution of psilocybin BGC genes, including *PsiD*, *PsiK*, *PsiM*, and *PsiH* genes, homologs were identified by BLAST searching against all fungal proteins in our database and NCBI's NR database. Each class retained only one gene with the highest consistency based on multiple sequence alignment. The remaining homologs were then used for phylogenetic analyses after removing overly short homologs. The phylogenetic analyses were performed using the MAFFT+TrimAl+IQ-TREE as above.

To identify potential transfer (T) events, we used RANGER-DTL v2.0, reconciling rooted gene trees with the rooted species tree. This approach allowed differentiate HGT events from gene duplication (D) and loss (L) events, using the tool's default costs (duplication = 2, transfer = 3, loss = 1) (Bansal et al. 2018). All inferred transfer events were visualized on the species tree using the iTOL online service. The selection pressure indicator ω, the ratio between non-synonymous substitution rate (dN) and synonymous substitution rate (dS), was used to measure selection pressure, with $\omega = 1$ indicating neutral mutations, $\omega < 1$ indicating purifying selection, and $\omega > 1$ indicating positive selection. The dN and dS values were calculated using KaKs_Calculator 3.0 with the NG model for all gene pairs within each group (psilocybin biosynthetic genes or native homologs) (Zhang 2022). Amino acid alignments for gene pairs were performed using MAFFT, followed by the conversion of the results into coding sequence alignments using PAL2NAL (Suyama et al. 2006). The alignments were then processed through KaKs_Calculator to derive Ka/Ks values.

Transcriptome sequencing and data analysis

Gymnopilus luteofolius, *Gymnopilus* sp. ZRL20210270E, *Panaeolus* sp. ZRL20220850, and *Psilocybe keralensis* were selected for transcriptome sequencing. For each species, three independent biological replicates of mycelial samples were collected for RNA sequencing. Samples were immediately frozen in liquid nitrogen and stored at −80°C prior to RNA extraction. Total RNA was extracted using the TIANGEN RNAprep Pure Plant Kit. For each sample, 2 μg of RNA was used as input material for RNA library preparation. Sequencing libraries were generated using the NEBNext Ultra RNA Library Prep Kit for Illumina, and the libraries were sequenced on the Illumina HiSeq 4000 platform, generating 150-bp paired-end reads. Clean reads were mapped to the assembled genomes using HISAT2 with default parameters (Kim et al. 2019), and FPKM values were calculated using Cufflinks (Trapnell et al. 2012).

Genetic diversity analysis

To test the hypothesis that the horizontal transfer of the psilocybin gene cluster promotes the diversification of psychedelic mushrooms, we performed a genetic diversity analysis. Whole-genome sequence alignment files, previously constructed for the genomic phylogenetic tree (Figure 1), were used for this analysis. The genomic data were divided into two groups: one consisting of 13 psilocybin-producing species with the psilocybin gene cluster, and the other comprising 30 related species without the cluster. For each group, the genetic diversity Pi (π) value, representing the average pairwise difference between all possible pairs of individuals, was calculated using an in-house Python script, providing a quantitative measure of genetic variation within each group. Higher Pi values indicate greater genetic diversity, which could suggest an evolutionary advantage for the group. To account for divergence time between species, observed Pi values were corrected using the formula Corrected Pi=Observed Pi/t, where t represents the divergence time between species. This adjustment ensures that Pi values reflect the appropriate evolutionary context.

Pairwise comparisons were conducted within each group to analyze genetic diversity across individual samples and observe variations in corrected Pi values. Finally, the significance of differences in corrected Pi values between the two groups was assessed using Wilcoxon rank-sum tests on the plot averages, performed with the stat_compare_means function from the R package ggpubr (v.0.2.1). This analysis provided statistical support for the observed patterns of genetic diversity.

RESULTS

Assessment of newly sequenced genomes of potentially psilocybin-producing mushrooms

Although the primary contributors to psilocybin production are from the genus *Psilocybe*, psilocybin-producing mushrooms are also found in other genera, particularly within the sister families Strophariaceae and Hymenogastraceae. In this study, we sequenced the whole genomes of 30 potentially psilocybin-producing mushrooms from these two families using PacBio single-molecule long-read sequencing. This included species from *Pholiota* (7), *Gymnopilus* (6), *Panaeolus* (5), *Agrocybe* (2), *Hypholoma* (2), *Stropharia* (2), *Deconica* (2), *Psathyrella* (2), *Psilocybe* (1), and *Protostropharia* (1). We compared these 30 genomes to 13 previously sequenced representative species of psilocybin-producing mushrooms, including species from *Gymnopilus* (Reynolds et al. 2018, Ruiz-Dueñas et al. 2021), *Panaeolus* (Reynolds et al. 2018), and *Psilocybe* (McKernan et al. 2021, McTaggart et al. 2023a) (Figure 1A). Additionally, these 30 genomes were compared to 90 previously sequenced representative species within Agaricales to further elucidate their phylogenetic context (Figure S1).

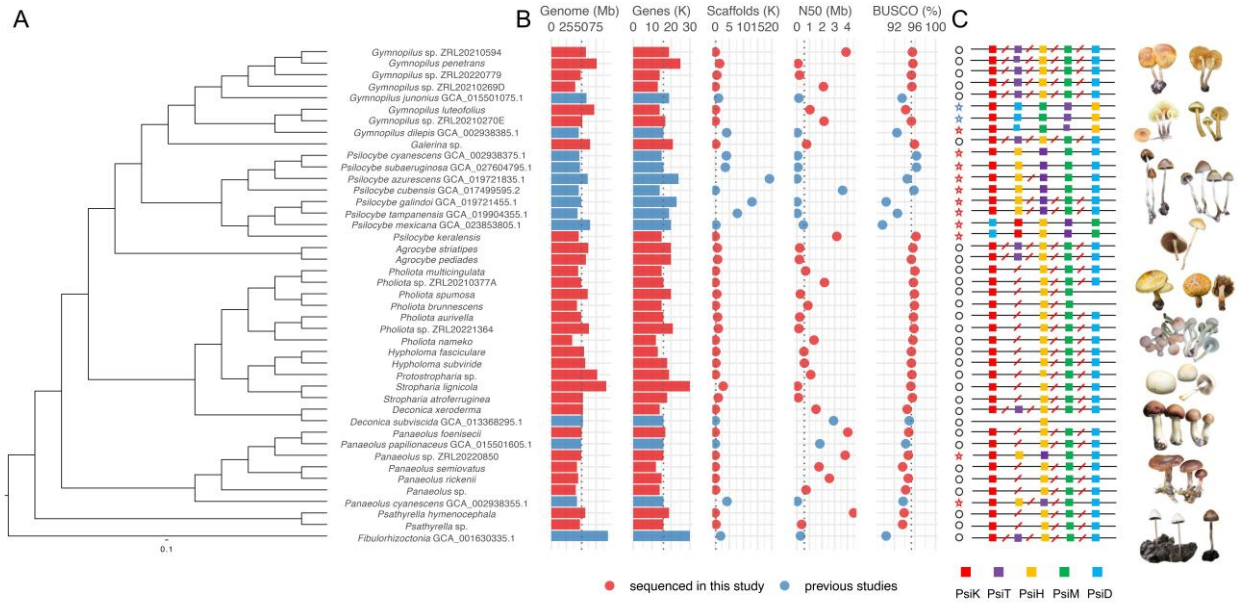

Figure 1 – Phylogeny, genome statistics, and distribution of psilocybin biosynthetic genes in potentially psilocybin-producing mushrooms. (A). Phylogenetic tree constructed using IQ-TREE based on 3,870 single-copy orthologous genes. (B). Bubble plots illustrating key genome quality parameters. Genomes sequenced in this and previous studies are color-coded as shown in the bottom panel. The dotted lines indicate median values. Genome (Mb): genome size in Mb, Genes (K): number of genes, Scaffolds (K): number of scaffolds, N50 (Mb): N50 length, BUSCO (%): genome completeness in % based on BUSCO survey. (C). Distribution of psilocybin biosynthetic genes. Each rectangle represents a gene element. Genes flanking the black dashed slash are clustered. The red star indicates a psilocybin-producing mushroom with the psilocybin BGC. The blue star indicates a mushroom that harbors the psilocybin BGC but lacks detectable psilocybin under mycelial growth conditions. The black circle indicates the non-psilocybin-producing

mushroom without the psilocybin BGC. Gene arrangements are schematic representations and are not drawn to scale.

The quality of the genome assemblies was compared based on genome size, gene number, number of scaffolds, N50 length, and genome completeness (Figure 1B and Table S1). Our results showed high-quality draft genome assemblies, with 21 out of the 30 genomes assembled into fewer than 300 scaffolds. The Benchmarking Universal Single-Copy Orthologs (BUSCO) evaluation indicated that, on average, 95% of the 3,870 single-copy genes found in the Agaricales species were fully assembled in the analyzed genomes. Therefore, the genome annotation is of sufficient quality for analyzing the psilocybin gene cluster and comparing gene content.

Identification of psilocybin BGCs

Psilocybin BGCs typically do not include core biosynthesis genes like polyketide synthases, terpene synthases, and non-ribosomal peptide synthases, making them difficult to identify using common BGC detection tools like antiSMASH (Rokas et al. 2020, Blin et al. 2021). Here, we employed a sophisticated methodology to identify homologs of psilocybin biosynthetic genes in genomes of potentially psilocybin-producing mushrooms (Materials and Methods). In total, the psilocybin gene cluster was identified in thirteen of the forty-three genomes analyzed (30 newly sequenced and 13 previously published): eight from *Psilocybe*, three from *Gymnopilus,* and two from *Panaeolus*. Four of these were newly detected psilocybin gene clusters from *Psilocybe keralensis*, *Panaeolus* sp. ZRL20220850, *Gymnopilus luteofolius* and *Gymnopilus* sp. ZRL20210270E (Figure 1C). We identified three distinct patterns of the psilocybin BGC based on gene orders: patterns 1 and 2 in *Psilocybe*, pattern 3 in *Gymnopilus* and pattern 1 in *Panaeolus* (Figure 6). To illustrate our approach of linking metabolites to their corresponding gene clusters, we identified three closely associated compounds—psilocybin, psilocin, and baeocystin—in the 30 sequenced samples using liquid chromatography–mass spectrometry (Table S2). Importantly, all species lacking the psilocybin biosynthetic gene cluster showed no detectable levels of psilocybin or related metabolites, supporting a robust genotype–phenotype correspondence.

Origin of psilocybin biosynthetic genes

To investigate the origin of the psilocybin gene cluster, we conducted extensive homology searches across 17,708 bacterial genomes from NCBI, spanning the entire bacterial diversity available as of January 2022. Despite obtaining homologs of several individual genes (*PsiD*: 5,383, *PsiH*: 2,877, *PsiM*: 4,506, *PsiK*: 41), we did not find any instances of the complete psilocybin gene cluster or all of its biosynthetic genes in any bacterial genome. This suggests that the psilocybin gene cluster likely did not originate from bacteria. We also performed homology searches on 385 plant genomes from the Phytozome database (release v13) and found no evidence of the psilocybin gene cluster or its complete biosynthetic genes in any of the plant genomes examined. However, within 100 representative fungal genomes downloaded from NCBI, we discovered homologs of the complete psilocybin biosynthetic genes that are widely distributed among these fungal genomes. This finding provides further evidence that the psilocybin BGC likely originated within the fungal lineage.

Expanding our search to 2,515 fungal genomes, we classified the genes into four types: *PsiD* genes (Pfam ID PF02666/PF12588), *PsiK* genes (PF01636), *PsiM* (PF05971), and *PsiH* (PF00067). A total of 2,350 *PsiD* homologs were found and subsequently used to analyze their phylogenetic relationship (Figure S2). Our analysis shows that the *PsiD* clade is nested within the Phosphatidylserine decarboxylase clade, and *PsiD* and its closest homologs have the same pfam domains (PF12588 and PF02666). Furthermore, the sequence of the *PsiD* gene shows a high amino acid sequence similarity to the closest Phosphatidylserine decarboxylase gene, and there are *PsiD* lineage-specific substitutions (Figure S2). Altogether, these observations suggest that the *PsiD* gene might have originated from the duplication of Phosphatidylserine decarboxylase. Similarly, we obtained 2,476 *PsiH*, 1,113 *PsiK*, and 2,265 *PsiM* homologs in fungi for phylogenetic analyses and

combined annotations from the NR database. We concluded that *PsiH*, *PsiK*, and *PsiM* might have originated from cytochrome P450, kinase-like domain-containing protein, and S-adenosyl-L-methionine dependent methyltransferase, respectively. Additionally, we obtained all *PsiH*, *PsiK*, and *PsiM* homologs within Agaricales for phylogenetic analyses (Figures S3-S5) and found that *Fibularhizoctonia* G.C. Adams & Kropp (a synonym for the genus *Athelia* Pers.) is closely related to mushrooms with the psilocybin BGC.

HGT and evolution of the psilocybin gene cluster

We used previously described approaches to assess the potential contribution of HGT to the discontinuous distribution of psilocybin gene clusters among fungi. The approaches were: 1) reconciliation analysis with the program RANGER-DTL, which infers HGT, duplication, or loss of genes to reconcile branch conflicts between gene trees and species trees; 2) estimates of the selection pressure value.

Each of the four psilocybin biosynthetic genes was individually aligned and then concatenated into one data. The gene tree constructed with the concatenated data was reconciled against the species tree to infer the HGT scenario using the Ranger-DTL program, which includes four interspecies HGT events (Figure 2). In addition, to investigate the splitting of these potentially psilocybin-producing mushrooms, we performed molecular dating using r8s. Firstly, the psilocybin BGC was transferred from an ancestor of *Psilocybe cyanescens* and *Psilocybe cubensis* to *Panaeolus* sp. ZRL20220850 and *Panaeolus cyanescens* (GCA_002938355.1), respectively, between 30 and 8 mya. Secondly, an ancestor of *Psilocybe keralensis* and *Psilocybe mexicana* transferred the psilocybin BGC to the most recent common ancestor of *Gymnopilus dilepis* (GCA_002938385.1), *Gymnopilus luteofolius* and *Gymnopilus* sp. ZRL20210270E between 32 and 22 mya; and thirdly, an interspecies HGT event within the genus *Psilocybe* occurred; an ancestor of *Psilocybe cyanescens* and *Psilocybe cubensis* transferred the psilocybin BGC to an ancestor of *Psilocybe keralensis* and *Psilocybe mexicana* between 50 and 22 mya.

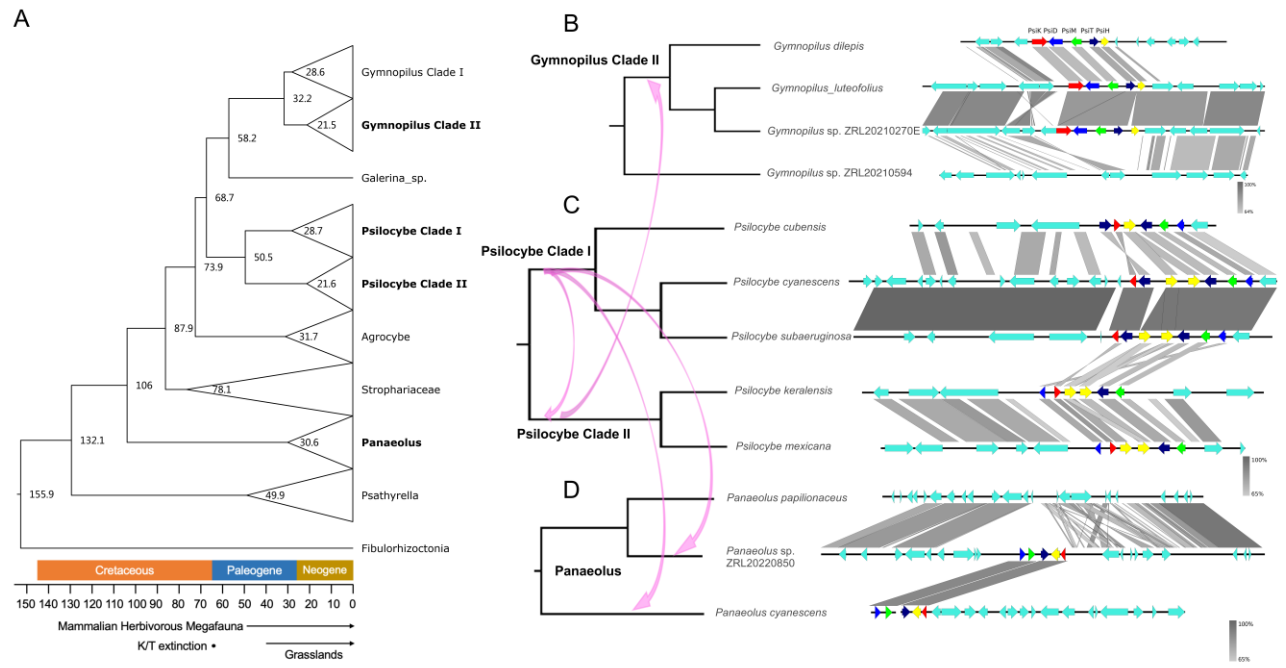

Figure 2 – Horizontal gene transfer (HGT) events and comparative analysis of psilocybin biosynthetic genes in potentially psilocybin-producing mushrooms. (A) Time-calibrated phylogeny of representative psilocybin-producing lineages, illustrating inferred horizontal transfer events of the psilocybin BGC in the context of major geological transitions. Divergence times (million years ago, mya) were estimated using r8s. (B–D) Comparative organization of psilocybin biosynthetic genes in *Panaeolus* (B), *Gymnopilus* (C), and *Psilocybe* (D). Pink lines indicate all inferred HGT

events of the psilocybin BGC mapped onto the species phylogeny, with arrows denoting the inferred directions of transfer. Gene arrangements are schematic representations and are not drawn to scale.

Furthermore, a more comprehensive evolutionary analysis of psilocybin biosynthetic genes was carried out using easyfig (Sullivan et al. 2011), with an emphasis on *Gymnopilus, Psilocybe,* and *Panaeolus.* The number, order, and orientation of genes within psilocybin clusters in the three *Gymnopilus* species are identical (Figure 2B), and the sequences flanking the clusters were similar, indicating a single origin and vertical evolutionary transmission of the psilocybin cluster within *Gymnopilus*. There were two types of psilocybin clusters between the two main clades within the genus *Psilocybe* and the sequences flanking the two types of clusters were divergent, indicating the psilocybin cluster is not a single origin (Figure 2C). In genus *Panaeolus*, we identified a complete psilocybin cluster in *Panaeolus* sp. ZRL20220850, which has the same order and orientation within the psilocybin cluster as in *Psilocybe cubensis* (Figure 2D), supporting the above HGT event that an ancestor of *Psilocybe* transferred the psilocybin BGC to *Panaeolus* sp. ZRL20220850.

The selection pressure value, quantified as ω (Ka/Ks), captures the ratio of non-synonymous site substitutions to synonymous site substitutions within each lineage of the individual psilocybin genes. Across the board, both the psilocybin genes and their respective native homologs are subject to purifying selection (conserved protein evolution), with an average ω = 0.22 (Figure 3A). Moreover, our analysis reveals that the psilocybin genes (ω = 0.14) are subject to a significantly more rigorous purifying selection compared to their native homologs (ω = 0.24), with a statistically significant difference at $P < 0.0001$.

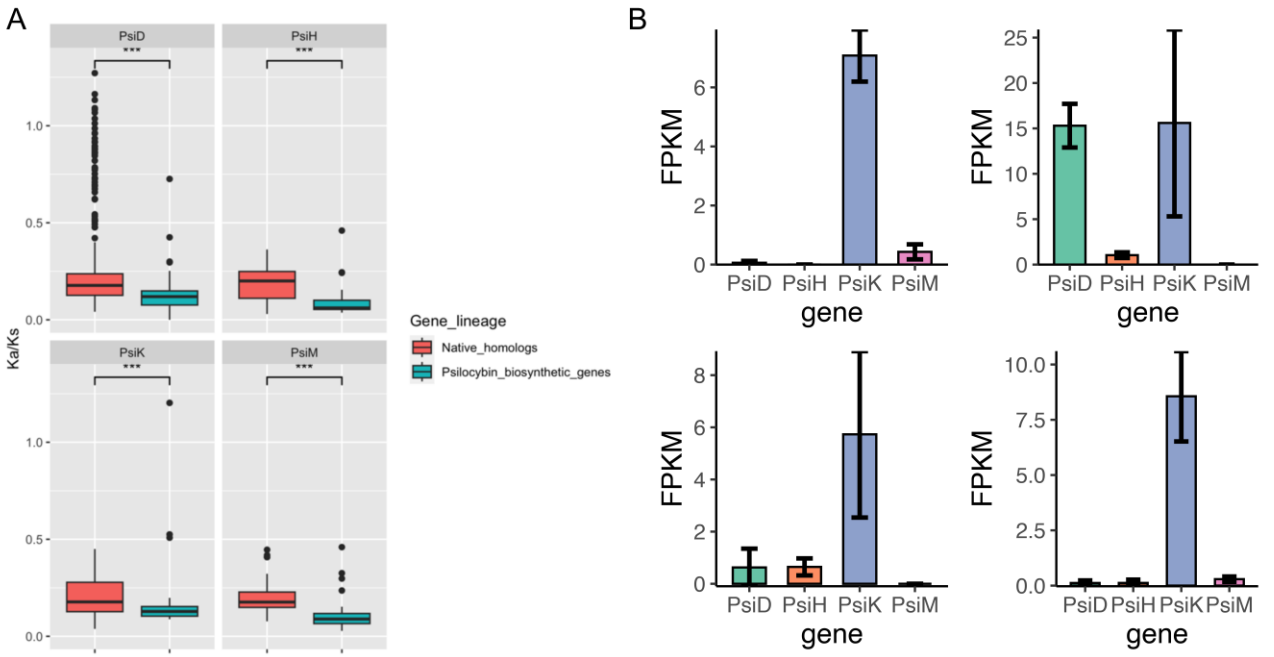

Figure 3 – Selection pressure and developmental regulation on psilocybin biosynthetic genes. (A) Selection pressure estimated by ω (Ka/Ks) of psilocybin biosynthetic genes (*PsiD*, PsiK, *PsiM*, and *PsiH*) and their homologs. The psilocybin genes are subject to a significantly more rigorous purifying selection compared to their native homologs. ***$p < 0.0001$, Wilcoxon rank-sum test. (B) Expression levels (FPKM) of the four psilocybin biosynthetic genes (*PsiD*, *PsiK*, *PsiM*, and *PsiH*) across four species (From top-left to bottom-right: *Gymnopilus luteofolius, Gymnopilus sp.* ZRL20210270E*, Panaeolus sp.* ZRL20220850*, and Psilocybe keralensis*) during the mycelial stage. FPKM is the number of fragments per kilobase per million mapped reads. *PsiK* shows consistently high expression in all sampled species, indicating its crucial role in maintaining basal pathway activity, while the expression of *PsiM* and *PsiH* varies across species.

Transcriptomic analysis of the four newly identified psilocybin gene clusters, encompassing species *Gymnopilus luteofolius*, *Gymnopilus* sp. ZRL20210270E, *Panaeolus* sp. ZRL20220850, and *Psilocybe keralensis*, revealed that *PsiK* exhibited high expression levels across all sampled species during the mycelial stage, consistent with its potential role in maintaining basal pathway activity (Figure 3B and Table S3). However, key biosynthetic genes *PsiM* and *PsiH* were not expressed in at least one species, correlating with the absence of detectable psilocybin in mycelial samples. This indicates that the full activation of the psilocybin biosynthetic pathway, and thus psilocybin production, is likely restricted to later developmental stages, such as the fruiting body. These findings provide new insights into the developmental regulation of the psilocybin gene cluster, highlighting its intricate transcriptional control and its potential ecological roles in mature fruiting bodies. Together, our approach combining genomic, transcriptomic, and metabolomic data enables a comprehensive understanding of the psilocybin biosynthetic pathway and its evolutionary dynamics.

Comparative and pangenomic analysis revealed genomic evolution of psilocybin-producing species

Our comparative and pangenomic analysis of psilocybin-producing mushrooms focused on four genera viz. *Psilocybe, Pholiota*, *Gymnopilus,* and *Panaeolus* each comprising 7–8 species-level genomes. Among these, *Gymnopilus* exhibited the largest genome size, averaging around 58 Mb (Figure 4A), with *G. penetrans* having the largest genome size of 76 Mb. Although there was no significant difference in genome assembly size and gene number (Figure 4B) among the four genera, the genus-level average nucleotide identity (ANI) values varied significantly (Figure 4C).

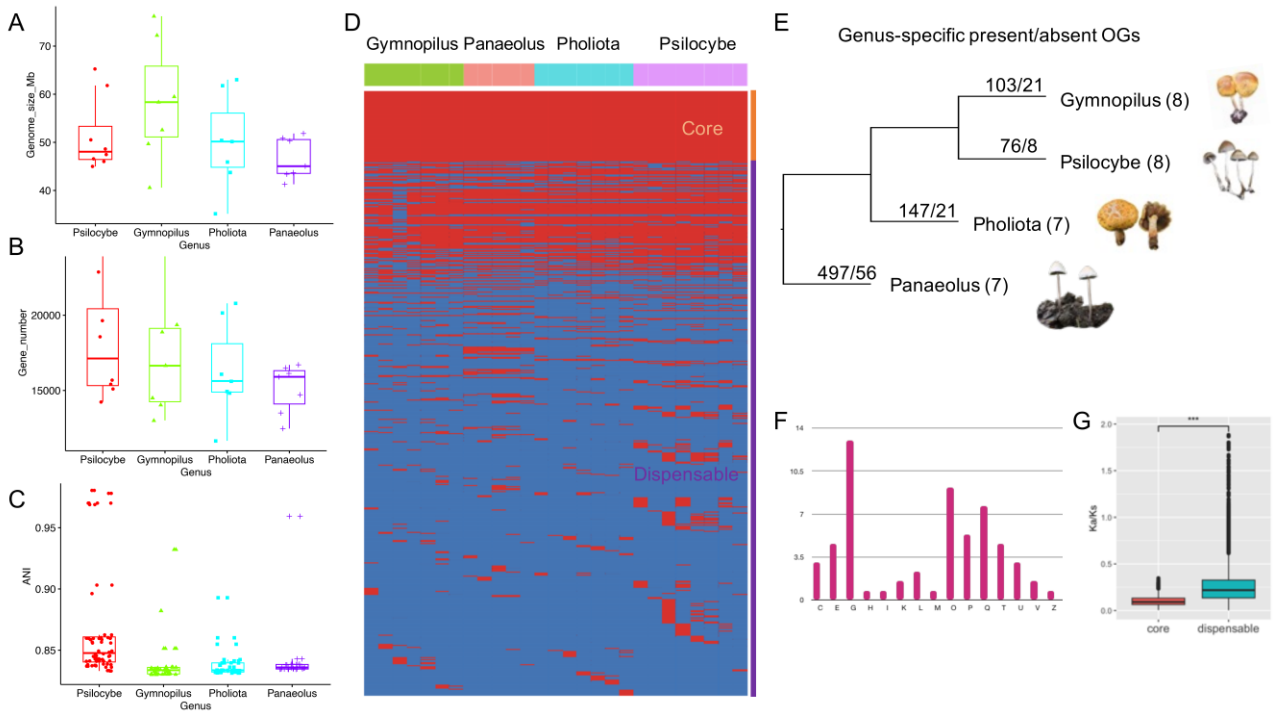

Figure 4 – Genome comparison among four genera. (A). Comparison of genome assembly size among four genera. (B). Comparison of the number of genes among four genera. (C). Comparison of ANI among four genera. The genus-level ANI can describe the genetic relationships between species within the same genus. The genus-level ANI value of *Psilocybe* is higher (87.0%) than those of *Panaeolus*, *Gymnopilus*, and *Pholiota*, which were 84.5%, 84.1%, and 84.0%, respectively. Additionally, the ANI values between the *Psilocybe* species varied widely from 83.3% to 99.0%, indicating the high genus-level genomic diversity. (D). Presence/absence matrix of orthologous gene clusters identified from 30 genomes. The pan-genome is subdivided into core (orthogroups (OGs) present in all genomes), and dispensable (absent in at least a single genome)

genes. (E). Genus-specific present and absent orthogroups. Representative hosts are illustrated above each genus. Numbers in parentheses indicate the number of species studied in each genus. (F). The proportion of genus-specific present OGs assigned to each major COG category (C, Energy production and conversion; E, Amino acid metabolism and transport; G, Carbohydrate metabolism and transport; H, Coenzyme metabolism; I, Lipid metabolism; K, Transcription; L, Replication and repair; M, Cell wall/membrane/ envelope biogenesis; O, Post-translational modification, protein turnover, and chaperone functions; P, Inorganic ion transport and metabolism; Q, Secondary metabolites biosynthesis, transport and catabolism; T, Signal transduction; U, Intracellular trafficking and secretion; V, Defense mechanisms; Z, Coenzyme metabolism). (G). Pairwise nonsynonymous/synonymous substitution ratios (Ka/Ks) within core and dispensable genes. The upper and lower edges of the boxes represent the 75% and 25% quartiles, and the central line denotes the median. ***$p < 0.0001$, Wilcoxon rank-sum test.

Orthofinder assigned 503,189 proteins from 30 species to 28,233 orthogroups (Figure 4D), defined as set of genes that descend from a single gene in the last common ancestor of all the species being considered (Emms & Kelly 2019). In summary, the pangenome provides a cross-genus census of the core and dispensable gene content for potentially psilocybin-producing mushrooms (Figure 4D). We identified a core gene content of 3,269 genes across the four genera, while the core gene content within *Gymnopilus, Psilocybe, Pholiota*, and *Panaeolus* is 4,435, 6,394, 6453, and 6,586, respectively. To elucidate genus-specific genes, we identified orthogroups exclusive to each genus, categorizing them as either 'present' or 'absent'. We identified 76–497 genus-specific 'present' and 8–56 genus-specific 'absent' genes, respectively (Figure 4E). Annotation of these genus-specific genes using eggNOG-mapper (Cantalapiedra et al. 2021) revealed associations with carbohydrate transport and metabolism (G), posttranslational modification, protein turnover, chaperones (O), and secondary metabolite biosynthesis, transport, and catabolism (Q) (Figure 4F). The non-synonymous/synonymous substitution ratio (Ka/Ks or dN/dS) analysis indicated that core genes had lower pairwise Ka/Ks values than variable genes (Figure 4G). These results suggest that the functions of core genes in different genera are relatively conserved, while variable genes evolve more rapidly to acquire new functions or adapt to new environments.

Using the CAZy database, we predicted a total of 23,970 carbohydrate-active enzymes (CAZymes) for the 43 genomes, averaging 557 per species (Figure S6A). Genomes with high numbers of CAZymes (>700) included *Protostropharia sp., Fibulorhizoctonia*, and *Psilocybe mexicana*. In contrast, *Hypholoma fasciculare, Gymnopilus sp.* ZRL20220779*, Gymnopilus sp.* ZRL20210269D, and *Pholiota nameko* contained only 367, 434, 436, and 437 CAZymes per genome, respectively, placing them at the low end of the comparison (Figure S6A). Glycoside hydrolases (GHs) and auxiliary activities (AAs) were the most abundant classes of CAZymes (Figure S6B). The abundance of each class was also different for each genus. For example, the highest average number of GHs per genome was detected in *Panaeolus* (308) and *Agrocybe* (283), while the genomes of *Gymnopilus*, *Pholiota*, and *Hypholoma* encoded on average only 217, 213, and 176 GHs, respectively. CBMs were most abundant in *Agrocybe*, *Gymnopilus*, and *Panaeolus* at 40, 36, and 34 per genome, respectively. To investigate what type of SM gene clusters potentially psilocybin-producing mushrooms have in common, the SM gene clusters were predicted for each genome, using an implementation of antiSMASH, yielding an output of 1,349 BGCs (average 31/genome). *Gymnopilus strains* exhibited the highest count (40/genome) compared to *Pholiota* (34), *Psilocybe* (26), and *Panaeolus* (23). This elevated number of BGCs is associated with the larger genome size of *Gymnopilus*. An overview of the predicted clusters in each genome is presented in Figure S6C, with terpene BGCs being the most abundant. The prediction of SM gene clusters also revealed considerable differences between closely related genera. For example, *Agrocybe* has 56 predicted SM clusters per species, whereas *Psilocybe* has only 16 per species (Figure S6C). Gene Cluster Families (GCFs) analysis grouped 1,349 BGCs into 769 families, with 580 being species-specific, indicating highly specialized compounds at the species level. Upon

excluding the species-specific GCFs, our analysis revealed that the remaining GCFs are specific to the genus level, as illustrated in Figure S6D. Notably, no GCFs contained known BGCs from MIBiG, suggesting numerous undiscovered metabolites.

We observed no significant differences in genome assembly size and gene number between mushrooms with and without the psilocybin BGC (Figures 5A and 5B). However, we identified 1,294 genes enriched in mushrooms with the psilocybin BGC (Figure 5C, $P < 0.01$, Fisher's exact test Benjamini Hochberg adjusted P values). To unveil functional signatures in psilocybin-producing mushrooms, we conducted functional annotation of enriched genes, revealing that these genes were mainly associated with replication and repair (L), carbohydrate transport and metabolism (G), and secondary metabolite biosynthesis, transport, and catabolism (Q) (Figure 5D). GO enrichment analysis highlighted that specific genes present in psilocybin-producing mushrooms were significantly enriched for protein transport (GO:0015031) (Figure 5E, $P < 0.01$). These genes are involved in the directed movement of proteins into, out of, or within a cell, or between cells, facilitated by a transporter. Ka/Ks analysis indicated that core genes within mushrooms possessing the psilocybin BGC exhibited higher pairwise Ka/Ks values compared to mushrooms without the psilocybin BGC (Figure 5G). Additionally, several classes of CAZymes were significantly overrepresented in psilocybin-producing mushrooms (Figure 5F), including GT31, AA1, AA13, CBM20, CE1, GH94, GH24, and CH16 ($P < 0.01$). Furthermore, we identified three BGCs - two terpenes and one nonribosomal peptide synthetase (NRPS) - that were significantly overrepresented in psilocybin-producing mushrooms ($P < 0.01$).

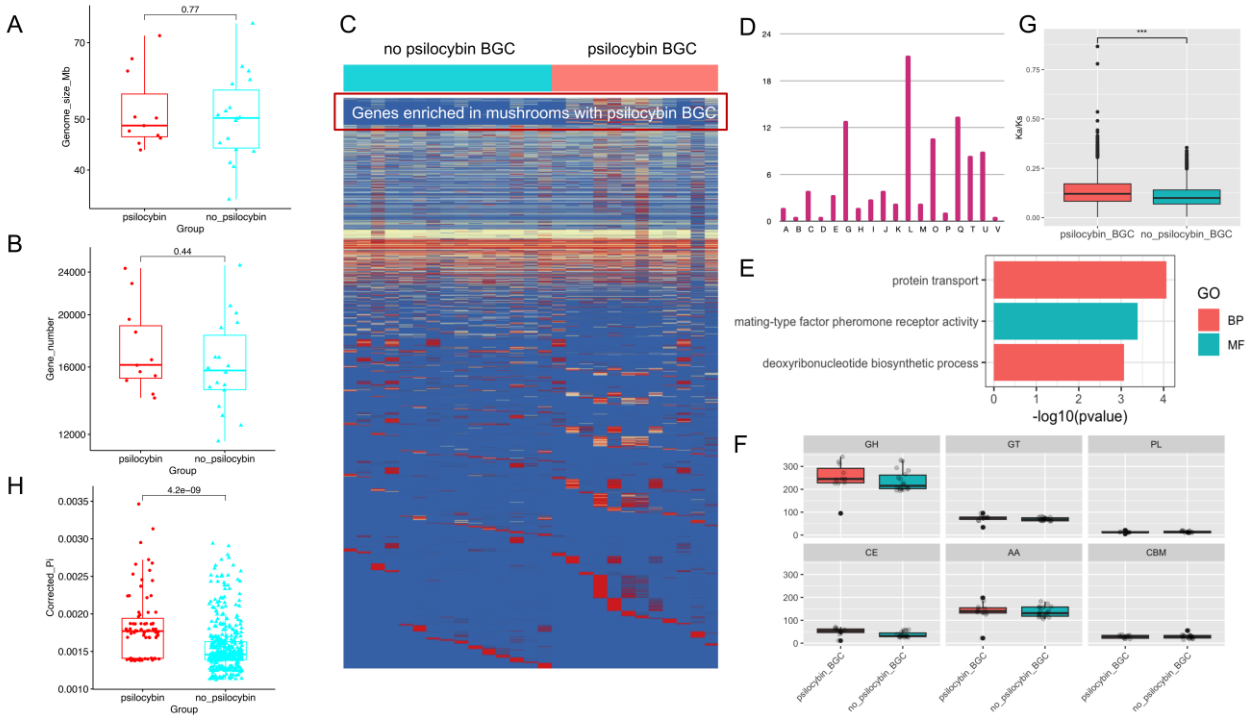

Figure 5 – Genome comparison between mushrooms with and without the psilocybin biosynthetic gene cluster (BGC). (A). Comparison of genome assembly size between mushrooms with and without the psilocybin BGC. (B). Comparison of gene number. (C). Presence/absence matrix of orthologous gene clusters. (D). The proportion of OGs enriched in mushrooms with the psilocybin BGC assigned to each major COG category (A, RNA processing and modification; B, chromatin structure and dynamics; C, Energy production and conversion; D, cell cycle control and mitosis; E, Amino acid metabolism and transport; G, Carbohydrate metabolism and transport; H, Coenzyme metabolism; I, Lipid metabolism; K, Transcription; L, Replication and repair; M, Cell wall/membrane/ envelope biogenesis; O, Post-translational modification, protein turnover, and chaperone functions; P, Inorganic ion transport and metabolism; Q, Secondary metabolites biosynthesis, transport and catabolism; T, Signal transduction; U, Intracellular trafficking and

secretion; V, Defense mechanisms). (E). Gene Ontology (GO) enrichment analysis of functional categories in mushrooms with the psilocybin BGC. (F). Boxplot representing the diversity of CAZyme family content and abundance between mushrooms with and without the psilocybin BGC. In the boxplot, the midline represents the median, the upper and lower limit of the box represents the third and first quartile, and the whiskers extend up to 1.5 times the interquartile. (G). Pairwise Ka/Ks of core genes between mushrooms with and without the psilocybin BGC. The upper and lower edges of the boxes represent the 75% and 25% quartiles, and the central line denotes the median. ***$p < 0.0001$, Wilcoxon rank-sum test. (H) Comparison of genetic diversity between fungi with and without the psilocybin BGC. Pairwise comparisons were conducted within each group to analyze genetic diversity among individual samples and to observe variations in corrected Pi values. Wilcoxon rank-sum tests were employed to evaluate the significance of differences in corrected Pi values between the two groups, providing robust statistical support for the observed patterns of genetic diversity.

The comparative and pangenomic analyses of psilocybin-producing mushrooms offer profound insights into their genomic evolution. The study indicated the conservation of core gene functions across genera and the rapid evolution of variable genes to adapt to new environments. The specific carbohydrate-active enzymes and secondary metabolite gene clusters enriched in psilocybin-producing species, suggesting unique metabolic adaptations. Additionally, the presence of enriched genes associated with protein transport and higher Ka/Ks values in core genes indicates ongoing evolutionary pressures in these mushrooms.

Genetic diversity and the role of the psilocybin gene cluster in promoting diversification of psychedelic mushrooms

To assess the relationship between the psilocybin gene cluster and genetic diversity in psychedelic mushrooms, we compared nucleotide diversity (π) between species with and without the psilocybin BGC. Genetic diversity was calculated using π values and further corrected for divergence time to account for lineage age effects. Our results indicated that species harboring the psilocybin BGC exhibited significantly higher corrected π values than closely related species lacking the cluster (Figure 5H). This supports the notion that the horizontal transfer of the psilocybin BGC has contributed to the diversification of psilocybin-producing species, as higher genetic diversity is generally associated with greater evolutionary potential and the ability to occupy diverse ecological niches (Murray et al. 2017, Hu et al. 2021). This finding adds a new layer to our understanding of how HGT enhances adaptive evolution and ecological niche expansion in psilocybin-producing fungi.

DISCUSSION

To explore the origin and evolutionary mechanisms of the psilocybin biosynthetic gene cluster (BGC), we constructed high-quality genome assemblies for 30 presumed psilocybin-producing mushroom species from ten genera. Our findings suggest that the psilocybin BGC originated from native fungal homologs through gene duplication, neofunctionalization, and genomic relocation, rather than through horizontal gene transfer (HGT) from bacteria or plants. A comprehensive evolutionary model that includes vertical inheritance, four HGT events, and strong purifying selection was proposed, elucidating the origin and evolution of the psilocybin BGC. Comparative and pangenomic analyses provided significant insights into the genomic evolution of psilocybin-producing mushrooms, particularly in relation to their ecological adaptation and diversity. Through our comprehensive genomic analysis, we found that the horizontal transfer of the psilocybin gene cluster contributes significantly to the genetic diversity of these fungi, promoting their adaptation to diverse ecological niches. This diversification, driven by increased genetic variation and facilitated by HGT, underscores the evolutionary importance of the psilocybin BGC in shaping the ecological success and distribution of psychedelic mushrooms.

Origin of the psilocybin BGC and its ecological significance

Psilocybin-producing fungi represent a remarkably diverse group, spanning various geographic regions and phylogenetic lineages. Although there are reports of psilocybin in ectomycorrhizal fungi (Awan et al. 2018), an insect pathogen (Boyce et al. 2019), and an unconfirmed account of psilocybin in a lichen, most known psilocybin producers are mushroom-forming fungi that inhabit dung and late-wood decay niches (Schmull et al. 2014, Reynolds et al. 2018) and mainly from the families Strophariaceae, Hymenogastraceae, and Galeropsidaceae. The recent discovery of psilocybin BGCs has significantly advanced our understanding of psilocybin biosynthesis (Fricke et al. 2017, Wu et al. 2022). Our investigation of 20,608 representative genomes across bacteria, plants, and fungi suggests that the ancestral psilocybin gene cluster in fungi likely arose through a process involving gene duplication of native homologs, followed by neofunctionalization, and subsequent genomic relocation (Figure 6), which consisted with the primary origins of BGCs in most fungi (Wong & Wolfe 2005, Rokas et al. 2018). An analysis of phylogenies encompassing all psilocybin biosynthetic genes reveals a close evolutionary affinity between *Fibularhizoctonia* and psilocybin-producing mushrooms, as shown in Figures S2–S5. Although an intact psilocybin BGC was not detected in *Fibularhizoctonia*, dispersed homologs of individual *Psi* genes were identified across its genome, consistent with a stepwise assembly of the gene cluster within fungal lineages. These observations support the hypothesis that the psilocybin BGC may have originated from dispersed ancestral homologs in a *Fibularhizoctonia*-like lineage, prior to subsequent clustering and horizontal transfer into mushroom-forming fungi, where complete *Psi* gene sets are present but not always organized in a conserved cluster (Reynolds et al. 2018). We note that inference of deep evolutionary origins based on gene phylogenies is inherently subject to uncertainty, including potential effects of long-branch attraction, incomplete lineage sorting, and taxon sampling. These limitations should be considered when interpreting the origin and early evolution of the psilocybin BGC. Together, these results provide new insights into how secondary metabolite gene clusters can emerge through gradual assembly followed by horizontal dissemination during fungal evolution.

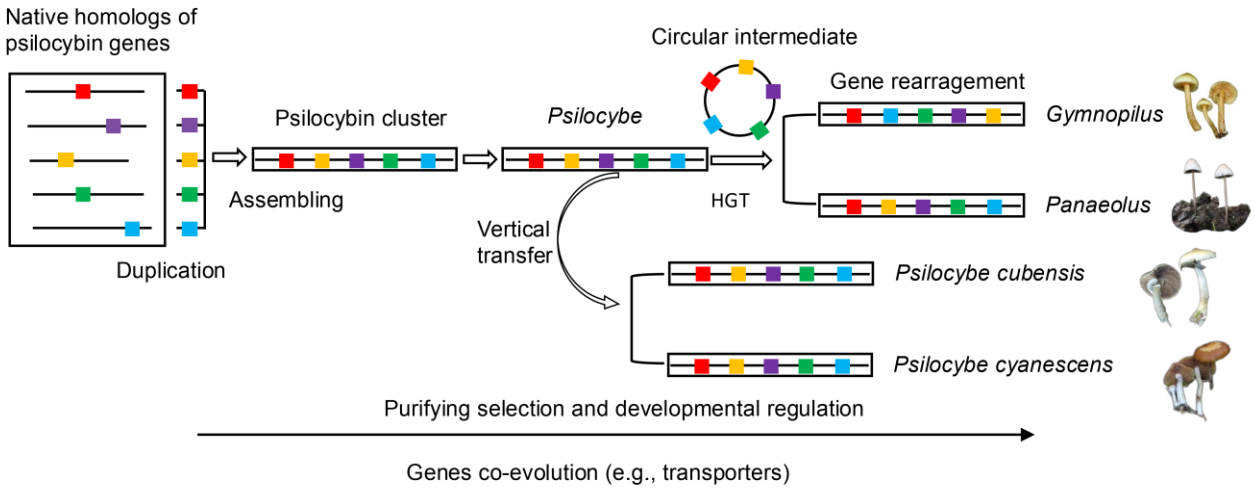

Figure 6 – A proposed model for the origin and evolution of the psilocybin cluster in fungi. The psilocybin BGC likely originated *de novo* in fungi through gene duplication events involving native homologs of psilocybin-related genes. It evolved through a complex process, including vertical inheritance and HGT. HGT events, coupled with subsequent strong purifying selection and developmental regulation, have shaped the distribution of the psilocybin BGC. Three distinct patterns of the psilocybin BGC, including two in *Psilocybe*, one in *Gymnopilus*, and one in *Panaeolus*, were identified. Psilocybin-producing fungi exhibited an enrichment of enzymes, including transporters, shedding light on their unique metabolic adaptations. All features are schematic representations and are not drawn to scale.

Secondary metabolites (SMs) can confer a competitive edge in resource competition, but their necessity diminishes in stable environments (Rokas et al. 2018). Variations in SM potential across species in our study likely reflect environmental differences and competitive pressures. This observation aligns with earlier studies in *Aspergillus* species (Vesth et al. 2018), suggesting that HGT plays a significant role in shaping the evolutionary dynamics of fungal secondary metabolism. Our results indicated that the psilocybin BGC was first acquired by *Psilocybe* between 50-30 mya and by *Gymnopilus* and *Panaeolus* between 32–8 mya (Figure 2). This suggests that psilocybin-producing mushrooms likely thrive in highly competitive environments, necessitating a diverse repertoire of SMs. The proliferation of psilocybin-producing mushrooms predominantly occurred after the Cretaceous-Tertiary (K/T) extinction event, coinciding with the onset of mammals (Springer et al. 2019). This event created new ecological niches, including grass and dung decay. Psilocybin production likely emerged and dispersed horizontally, coinciding with the expansion of grasslands and the emergence of dung-producing mammalian herbivorous megafauna 50-40 million years ago (MacFadden 2000, Retallack 2001, Meyer & Slot 2023) (Figure 2). In these niches, diverse animals inhabit, with invertebrates exerting stronger selective pressures on mushrooms due to their higher numbers compared to most vertebrates. Arthropods and gastropods, in particular, represent plausible ecological agents influencing the evolution of psilocybin production. Terrestrial gastropods diversified rapidly beginning around 110 million years ago and continued to radiate through the estimated emergence of psilocybin-producing fungi (~40 million years ago), potentially shaping fungal chemical defenses during this period (Ayyagari & Sreerama 2020, Meyer & Slot 2023). In addition to psilocybin biosynthesis, psilocybin-producing mushrooms exhibit enrichment of transporter genes. Although transporters are not directly involved in secondary metabolite synthesis, they are often associated with biosynthetic gene clusters and may facilitate metabolite export or self-protection against toxic compounds (Amnuaykanjanasin & Daub 2009, Wang et al. 2014). Taken together, potential ecological roles of psilocybin, such as insect deterrence or chemical signaling, should be regarded as testable hypotheses rather than definitive functions. Experimental validation through ecological interaction studies and behavioral assays will be required to determine their adaptive significance.

The evolutionary trajectory and horizontal transfer of the psilocybin BGC

Numerous studies have documented the transfer of BGCs across fungi (Reynolds et al. 2017, Li et al. 2020), however, the evolutionary trajectory of the psilocybin BGC remains largely unexplored beyond the genus *Psilocybe* (McTaggart et al. 2023b, Bradshaw et al. 2024). In this study we identified four HGT events and three different patterns of psilocybin BGC among *Psilocybe, Gymnopilus*, and *Panaeolus*. Combined with taxonomic dating, our findings indicate that *Psilocybe* might be the oldest lineage to acquire the psilocybin BGC (Figure 5). The ubiquity of the psilocybin BGC in *Psilocybe* strongly suggests that psilocybin might be a shared derived trait among species within this genus. We identified two distinct patterns of the psilocybin gene cluster in *Psilocybe*, consistent with prior research (Bradshaw et al. 2024). Our results indicated that an ancestor of *Psilocybe* Clade I (pattern 1) transferred the psilocybin BGC to an ancestor of *Psilocybe* Clade II (pattern 2). While the precise origins of these patterns remain uncertain, their early divergence within the two clades in *Psilocybe* suggests the possibility of additional variations occurring during the initial stages of psilocybin biosynthesis (Figure 6). These variations could have developed in response to selective pressures influencing the function of the BGC. In *Gymnopilus*, the number, order, and orientation of genes within psilocybin clusters in the three *Gymnopilus* species are identical, and the sequences flanking the clusters are similar, indicating a single origin of the psilocybin cluster and suggesting vertical inheritance within this genus. This finding aligns with our inferred HGT event, where an ancestor of *Psilocybe* transferred the psilocybin BGC to an ancestor of *Gymnopilus* (pattern 3). In *Panaeolus*, we identified a complete psilocybin cluster in *Panaeolus* sp. ZRL20220850 (pattern 1), which has the same order and orientation within the psilocybin cluster as in *Psilocybe cubensis* (pattern 1), supporting the proposed HGT event from a *Psilocybe* ancestor to *Panaeolus* sp. ZRL20220850. An intriguing

hypothesis is the potential occurrence of HGT of the core cluster via a circular intermediate (Figure 6), which could undergo linearization at different integration points within a new genome (Awan et al. 2018, Bucknell & McDonald 2023). However, no starship-like mobile genetic elements (Gluck-Thaler et al. 2022, Liu et al. 2025) were identified in the genomic regions flanking the psilocybin BGC in this study. Therefore, the precise molecular mechanisms underlying the horizontal transfer of the psilocybin BGC remain to be fully elucidated. In addition to differences in transfer mechanisms, this observation raises the intriguing possibility that post-transfer evolutionary processes, such as divergence in transcriptional regulation, may vary among taxa (Yin & Keller 2011), thereby contributing to the observed variation in psilocybin production across fungi.

Our study suggests the horizontal transfer of the psilocybin BGC among mushrooms with shared ecological niches. Wood decomposition is considered the ancestral ecological niche of *Psilocybe*, and *Gymnopilus* is indeed a wood decomposer as well (Bradshaw et al. 2024). Specific species like *Psilocybe cubensis* and *Panaeolus cyanescens* have adapted to a coprophilous lifestyle (Bradshaw et al. 2024). Interestingly, we identified an HGT event between the two main clades within the genus *Psilocybe*, coinciding with the gene arrangement of the psilocybin BGC. This finding suggests a potential motive and mechanism for the spread of psilocybin BGCs is related to their ecological niches, and the phenomenon of HGT significantly contributes to the expansion of psilocybin BGCs in fungi.

Psilocybin BGC experiences purifying selection and developmental regulation

The evolutionary drivers governing the origination and persistence of BGCs remain a central debate. While the role of natural selection is widely acknowledged, comprehensive analyses of BGC selection are hindered by limited data. Previous studies on galactose BGCs in Saccharomycetes and thalianol BGCs in plants have exemplified the role of purifying selection in maintaining these clusters (Wong & Wolfe 2005, Liu et al. 2020). In contrast, balancing selection, as observed in the aflatoxin gene cluster, promotes genetic diversity (Carbone et al. 2007). Our study employs the psilocybin BGC and its native homologs across diverse fungi to dissect psilocybin BGC selection. The psilocybin BGC experiences strong purifying selection, which is notably stronger than that experienced by its native homologs (Figure 3). This underscores the crucial role of selective forces in shaping the evolution and maintenance of BGCs. Additionally, core genes had lower pairwise Ka/Ks values than variable genes in the pangenome (Figure 4G), indicating stronger purifying selection on core genes. Interestingly, core genes in mushrooms with the psilocybin BGC have higher pairwise Ka/Ks values compared to those in mushrooms without the psilocybin BGC (Figure 4F), suggesting that the selective pressure on core genes may have decreased following the acquisition of the psilocybin biosynthetic capability.

Our transcriptomic data offer critical additional insights into the dynamics of selection pressure on the psilocybin BGC. The expression analysis reveals that *PsiK*, a core gene of the psilocybin biosynthetic pathway, is highly expressed in the mycelial stage across all sampled species. This suggests that *PsiK* plays a fundamental role in maintaining basal pathway activity during early fungal development. In contrast, genes such as *PsiM* and *PsiH*, which are essential for the later stages of psilocybin biosynthesis, show no expression in at least one species during the mycelial stage, aligning with the absence of detectable psilocybin in mycelial samples. This highlights the role of developmental regulation in the activation of the psilocybin biosynthetic pathway, with full activation likely occurring only during fruiting body development.

Horizontal transfer of the psilocybin BGC and fungal diversification

Our pangenomic analyses reveal a strong association between the horizontal transfer of the psilocybin BGC and increased genetic diversity in psilocybin-producing mushrooms. Species harboring the psilocybin BGC exhibit higher levels of genetic diversity compared with closely related taxa lacking the cluster, suggesting that BGC acquisition may be linked to enhanced evolutionary potential. This finding is consistent with previous studies indicating that HGT can lead

to increased genetic diversity and the potential for novel traits to arise, allowing organisms to occupy new ecological niches (Soucy et al. 2015).

Similar associations between horizontal transfer of biosynthetic gene clusters and ecological diversification have been reported in other fungal systems, where acquired secondary metabolic pathways are thought to facilitate niche expansion and ecological adaptation (Khaldi et al. 2008, Slot & Rokas 2011, Gluck-Thaler & Slot 2015, Tran et al. 2019). These observations are consistent with the idea that access to novel secondary metabolites can provide selective advantages under conditions of intense ecological competition.

Importantly, however, we emphasize that the relationship observed here is correlative rather than strictly causal. It remains possible that already diverse lineages are more permissive to the acquisition and retention of biosynthetic gene clusters, or that shared ecological factors, such as niche breadth or exposure to heterogeneous environments, simultaneously promote both lineage diversification and the frequency of horizontal gene transfer.

In summary, our study offers a comprehensive exploration of the psilocybin BGC, uncovering its intricate evolutionary history shaped by vertical inheritance, HGT, and strong purifying selection. By sequencing genomes from a wide array of psilocybin-producing mushrooms, we have generated valuable genomic resources that pave the way for the discovery of novel neurotrophic natural products. Our findings highlight the critical role of secondary metabolic pathways in fostering fungal diversity and facilitating adaptive evolution. This work lays a robust foundation for future studies aimed at unraveling the complex biology of psilocybin biosynthesis and exploring its potential applications in medicine, ecology, and biotechnology.

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Conflict of interest

The authors declare that they have no conflict of interest.

Contributions

RLZ and RC designed and supervised this study. FL, JXL, WQY, BC, and MQH performed experiments. FL and QW analyzed data. FL wrote the manuscript with input from all authors. All the authors read and approved the manuscript.

Data availability – All genomes used in the study are available from GenBank (ftp://ftp.ncbi.nlm.nih.gov/genbank). All sequencing raw data and genome assemblies generated in this study have been deposited in the NCBI Sequence Read Archive and are publicly accessible under BioProject accession numbers PRJNA1046270 and PRJNA1223616.

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SUPPLEMENTAL MATERIAL

All supplementary materials are available at DOI: https://doi.org/10.5281/zenodo.18046710

Table S1 – The quality of the genome assemblies.

Table S2 – Liquid chromatography-mass spectrometry analysis.

Table S3 – Transcriptome sequencing and mapping statistics.

Table S4 – Bacterial and fungal genomes used in the study.

Figure S1 – Phylogenetic tree constructed using IQ-TREE based on 3,870 core genes within Agaricales.
Figure S2 – The phylogeny of *PsiD* in fungi.
Figure S3 – Maximum-likelihood tree of *PsiK* and its homologs across the order Agaricales.
Figure S4 – Maximum-likelihood tree of *PsiM* and its homologs across the order Agaricales.
Figure S5 – Maximum-likelihood tree of *PsiH* and its homologs across the order Agaricales.
Figure S6 – Analysis of CAZymes and BGCs.