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Wood-loving magic mushrooms from Australia are saprotrophic invaders in the Northern Hemisphere

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Abstract: Magic mushrooms are fungi that produce psilocybin, an entheogen with long-term cultural use and a breakthrough compound for treatment of mental health disorders. Fungal populations separated by geography are candidates for allopatric speciation, yet species connectivity typically persists because there is minimal divergence at functional parts of mating compatibility genes. We studied whether connectivity is maintained across populations of a widespread species complex of magic mushrooms that has infiltrated the Northern Hemisphere from a hypothesised centre of origin in Australasia. We analysed 89 genomes of magic mushrooms to examine erosion of species connectivity in disparate populations with support from gene flow, kinship, structure, allelic diversity, and mating compatibility. We used comparative genomics and synteny to test whether the genes that produce psilocybin are under selection in natural populations of magic mushrooms. Despite phenotypic plasticity and intercontinental distribution, sexual compatibility is maintained across geographically isolated populations of magic mushrooms. Psilocybin loci have high allelic diversity and evidence of balancing selection. Australasia is the centre of origin of wood-degrading magic mushrooms and geographically separated populations are fully sexually compatible, despite minimal gene flow since differentiation from a shared ancestor. Movement of woodchips, mulch, or plants has most likely facilitated invasion of these mushrooms in the Northern Hemisphere.

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INTRODUCTION

Mushrooms (*Agaricomycetes*, *Basidiomycota*) disperse large quantities of wind-borne spores (Dam 2013), yet populations of many different species are structured by geography (Amend *et al.* 2010, Branco *et al.* 2017, Dabao Sun *et al.* 2023). Speciation of geographically isolated populations mostly occurs when mating compatibility ceases. Mushrooms are typically obligately sexual (heterothallic), and mating compatibility is either

tetrapolar, in which alleles at two independent loci must differ for gametes to fuse, or bipolar, controlled at one locus (Coelho *et al.* 2017). Mating-compatibility loci have high allele diversity and long allele residence times (Skrede *et al.* 2013, van Diepen *et al.* 2013), and this diversity is maintained across populations by negative frequency selection that favours rare mating types, with minimal divergence of functional parts of mating genes that control compatibility (Peris *et al.* 2022). The relationship between speciation and erosion of mate compatibility in



Fig. 1. Phenotypic diversity of *Psilocybe subaeruginosa* from populations in Australia, including pilei that are conical, papillate, or sinuate, and fruiting from diverse substrates, including grass, leaf litter, moss, and wood. **A.** Ellendale, Tasmania. **B.** Dover, Tasmania. **C.** Tasmania (image courtesy of K. Keats). **D.** Ellendale, Tasmania. **E.** Victoria (image courtesy of T. Coolhaas). **F.** Tasmania (image courtesy of K. Keats). **G.** Kunanyi, Tasmania. **H.** Ellendale, Tasmania. **I.** Western Australia (image courtesy of O. Keats). **J.** Victoria (image courtesy of T. Coolhaas). **K.** Victoria. **L.** Bunya Mountains, Queensland.

reproductive isolation needs better understanding to delineate populations from species of *Basidiomycota* (Peterson & Hughes 1999).

Mushrooms that produce psilocybin, magic mushrooms, occupy diverse environmental niches as saprotrophs that degrade leaves, wood, and dung (Stamets 1996). A key diagnostic character of magic mushrooms is a blueing reaction, due to oligomers of psilocin that form when psilocybin is dephosphorylated and oxidised after tissue damage (Lenz *et al.* 2020). Psilocin, the active metabolite of psilocybin, binds to serotonin receptors, which are the targets hypothesised to protect magic mushrooms against fungivory by metazoans (Reynolds *et al.* 2018). The etymology behind epithets of wood-degrading magic mushrooms, *e.g.*, *P. azurescens*, *P. cyanescens*, and *P. subaeruginosa*, describes their strong blueing reaction and these mushrooms have some of the highest concentrations of psilocybin (Gotvaldová *et al.* 2022).

Magic mushrooms contain metabolites with breakthrough potential for mental health (Kargbo 2020) and evidence of longer-lasting impact than synthetic psilocybin (Shahar *et al.* 2024). The Oregon Psilocybin Advisory Board discouraged commercialisation of wood-degrading species of *Psilocybe* as in some cases a side effect temporarily paralyses users during a psilocybin experience (Abbas *et al.* 2021). The cause of “wood lover’s paralysis” is unknown (Dörner *et al.* 2022). Dörner *et al.* (2022) and McTaggart *et al.* (2023a) showed *psiH*, a gene in the psilocybin pathway that converts tryptamine into 4-hydroxytryptamine (Fricke *et al.* 2017), is duplicated with up to three homologs in wood-degrading species of *Psilocybe*, compared to a single copy in *P. cubensis*. McTaggart *et al.* (2023a) hypothesised these gene duplications may impact the production of psilocybin and its analogues; however, there is no evidence that links the psilocybin pathway to wood lover’s paralysis, and Dörner *et al.* (2022) suggested the symptoms are likely not linked to tryptamines.

Psilocybe subaeruginosa was described from national parks and natural environments in Australia (Cleland 1927). It has taxonomic priority in a complex of closely related, potentially conspecific species (Chang & Mills 1992, Gießler 2018) described from Australia (*P. australiana* and *P. eucalypta*), New Zealand (*P. makarorae* and *P. weraroa*), and the Northern Hemisphere (*P. allenii*, *P. azurescens*, and *P. cyanescens*) (Borovička *et al.* 2011, Gotvaldová *et al.* 2022). Taxa in the *P. subaeruginosa* species complex were differentiated by morphology, or a phylogenetic species concept based on the ITS region. The morphological diversity of *P. subaeruginosa* in Australia encompasses phenotypes of taxa described in the Northern Hemisphere (Fig. 1). *Psilocybe allenii* and *P. cyanescens* behave as invasive taxa in the Northern Hemisphere, occurring in planted garden beds rather than undisturbed ecosystems, un-recorded before the 1900s (Dennis & Wakefield 1946, Borovička *et al.* 2012), and having low genetic diversity among populations (Gießler 2018).

Psilocybe subaeruginosa has a wide distribution across Australia, occurring from southeast Queensland at its northernmost extent to Tasmania, South Australia, and Western Australia. We examined populations of *P. subaeruginosa* across its eastern distribution in Australia to test hypotheses (i) that it has a centre of origin in Australia, (ii) that it is conspecific with species in the Northern Hemisphere, (iii) that populations in Australia are structured by geography, and (iv) that selection shapes the genes in the psilocybin pathway. To do so, we used population genomics and mating compatibility to compare

relationships among Australian populations and included reference sequences of *P. azurescens* and *P. cyanescens* to test conspecificity with taxa in the Northern Hemisphere. Our study examines how fungal species connectivity is maintained across geographic boundaries and provides new knowledge on the centre of origin of wood-degrading species of *Psilocybe*.

MATERIALS AND METHODS

Specimen collection, culturing, DNA sequencing

We cultured single basidiospores on malt extract agar from spore prints of *P. subaeruginosa* collected by citizen scientists from private land and roadsides in New South Wales, Queensland, South Australia, Tasmania, and Victoria (Australia, Fig. 2A). Sampled spore prints were non-uniform, with some received as an individual pileus and some received as populations of pilei from one location. Sibling haplotypes, those known to come from the same pileus, are listed in Table S1. Cultures are lodged in the Queensland Plant Pathology Fungarium. Cultures were grown in half-strength potato dextrose broth for 3 wk, then sent to the Australian Genome Research Facility (AGRF, Brisbane, Australia) for DNA extraction and high-throughput sequencing. AGRF prepared a Nextera Flex 150PE library that was sequenced on an Illumina HiSeq, which provided sequencing depth of 42–80 times coverage per isolate. A list of 81 specimens cultured, sequenced and assembled from Australia in the current study are provided in Table S1.

Genome assembly and annotation

Raw sequencing reads were trimmed with Trimmomatic v. 0.12 (Bolger *et al.* 2014) and assembled with SPAdes v. 3.12.0 (Bankevich *et al.* 2012). Assembled genomes are accessioned in GenBank (Table S1). FunAnnotate (Palmer & Stajich 2019) was used to annotate all examined genomes using protein models from the annotated *P. cyanescens* reference assembly (Reynolds *et al.* 2018), BUSCO models for *Basidiomycota*, and Augustus models from *Laccaria bicolor* (Martin *et al.* 2008).

SNP calling

Single Nucleotide Polymorphisms (SNPs) were called using kSNP (k = 91, min frac = 1) (Gardner *et al.* 2015), from 81 genomes of *P. subaeruginosa* assembled in the present study, five from a past study (McTaggart *et al.* 2023a), two genomes of *P. azurescens* (McKernan *et al.* 2021, Dörner *et al.* 2022), and a genome of *P. cyanescens* (Reynolds *et al.* 2018) (Table S1). PLINK (Purcell *et al.* 2007) was used to remove SNP sites under linkage disequilibrium across the entire dataset (r^2 cutoff = 0.99). Relationships among individuals were visualised using non-pruned SNP data with a neighbour net in SplitsTree v. 4.14.8 (Huson & Bryant 2005).

Tests for population structure and ancestry

We used Discriminant Analysis of Principal Components (DAPC) and K-means clustering to determine whether there was underlying population structure of *P. subaeruginosa* in Australia and the Northern Hemisphere (Jombart *et al.* 2010). The packages vcfR (Knaus & Grünwald 2017), adegenet (Jombart 2008), and ggplot2 implemented in R (R_Core_Team 2014) were



Fig. 2. Analyses of Australian populations of *Psilocybe subaeruginosa* (n = 85), and *P. azurescens* (n = 2) and *P. cyanescens* (n = 1) from the Northern Hemisphere. **A.** Provenience map of Australian collections used in the study. **B.** 2-dimensional plot of Discriminant Analysis of Principle Components (DAPC) based on 6 757 SNPs with indels and sites under LD removed from the dataset, and individuals coloured based on K = 7 in the barplot of 2C. **C.** DAPC exploring K-values 2–8 based on the same dataset in 2B. Facets reflect geographic sampling from local populations. **D.** SplitsTree network based on 1 555 848 LD-corrected SNPs, including indels, with all genomes treated as haploids. Individuals are coloured by populations defined in DAPC analysis at K = 7. Sibling haplotypes sampled from the same pileus are labelled with the same letter. Edge length reflects genetic difference and reticulation may indicate recombination. B and C produced by R packages adegenet, vcfR, and ggplot2.

used to import an LD-corrected VCF file from PLINK (vcfR), cluster populations by k-means clustering and DAPC (adeigenet), and plot results (ggplot2).

We used the *relatedness* command in vcftools v. 1.17 (Danecek *et al.* 2011), which estimates relationships based on pairwise similarity of genetic markers between individuals due to shared genetic ancestry, as defined as the AJK statistic by Yang *et al.* (2010). We plotted relatedness values in a pairwise heat map using *ggplot2* in R (R_Core_Team 2014) with code generated by ChatGPT, OpenAI.

Analyses of single copy orthologs

We used OrthoFinder v. 1.0.6 (Emms & Kelly 2019) with the default inflation parameter and a Diamond search (Buchfink *et al.* 2015) to identify orthologous groups of genes. All single copy orthologs were aligned with MAFFT v. 7.508 (Katoh & Standley 2013), concatenated with FASconCAT-G (Kück & Longo 2014), and their relationships visualised with a neighbour net in SplitsTree.

Studies of selection, divergence, and diversity at mating compatibility loci

Alleles at mating compatibility loci identified by McTaggart *et al.* (2023a) were searched in gene annotations with BLASTp. *STE3.2* genes and HD genes (*HD1* and *HD2*) at pheromone/receptor (PR) and homeodomain (HD) loci, were aligned with MAFFT, concatenated, and the most likely tree was searched in IQ-TREE v. 2 (Minh *et al.* 2020) with a model test (command -m TEST), 10 000 ultrafast bootstraps and 10 000 approximate likelihood ratio tests (Minh *et al.* 2013).

We called SNPs with kSNP across the longest contigs that contained mating compatibility loci (HD locus: BRIP75299; PR locus: POZ38-3). A lower kmer cutoff was used (kmer cutoff 21) to increase the number of SNPs called across these shorter genomic regions. vcftools (Danecek *et al.* 2011) was used to determine F_{ST} , nucleotide diversity (π), and Tajima's D across contigs and using populations defined by DAPC analyses. F_{ST} , π , and Tajima's D were plotted across HD and PR contigs with 10 000 and 3 500 base pair windows using ggplot2 in R.

Mating compatibility was tested based on clades recovered in phylogenetic relationships of the PR locus. Pieces of culture, 1 × 1 cm, were placed adjacently on rice water agar (333 g rice, 20 g sucrose, 15 g agar, 1 L distilled water) and left for 3 wk. Presence or absence of clamp connections, as an indication of mate compatibility, was determined from hyphae sampled at the interaction zone under a light microscope at ×1 000 magnification.

Allelic diversity at psilocybin loci and mitochondria

tBLASTn was used to identify contigs that contained the psilocybin gene cluster and BLASTp was used to search annotated assemblies based on genes in the psilocybin pathway identified in wood-loving species of *Psilocybe* (Reynolds *et al.* 2018). Amino acid sequences of psilocybin genes annotated by FunAnnotate, including *psiD*, *psiM*, *psiT2*, *psiH* (paralog 1), *psiT1*, *psiH* (paralog 2), *psiK*, *psiR* were aligned with MAFFT and the homology of the *psiH* genes was confirmed with a search for the most likely tree using IQ-TREE. The coding sequences of these same genes in the psilocybin pathway were aligned

with MAFFT, concatenated with FASconCAT-G, and visualised with a neighbour net in SplitsTree. F_{ST} , nucleotide diversity, and Tajima's D were calculated across coding sequences of the psilocybin locus using vcftools and plotted with ggplot2 in R with code generated by ChatGPT, OpenAI.

We used exonerate (Slater & Birney 2005) for targeted annotation of *psiH* genes to avoid errors from automated annotation, including annotation of pseudogenes or erroneous open reading frames. We aligned all paralogs and orthologs of the *psiH* family with MAFFT and searched for a maximum likelihood tree with IQ-TREE v. 2. We used Clinker (Gilchrist & Chooi 2021) to align representative genotypes of the entire psilocybin locus.

Mitochondrial contigs of *P. subaeruginosa* were identified using a BLASTn search against the mitochondrial genome (NW_025952838) of the *P. cubensis* reference assembly (McKernan *et al.* 2021). SNPs were called from all mitochondrial contigs using kSNP ($k = 31$, min frac = 1); a lower k-mer value was used to call SNPs from the mitochondrial genome to increase the number of potential sites in a smaller dataset. Relationships were visualised with a haplotype network using PopART (Leigh & Bryant 2015) with sites masked that had more than 5 % missing data.

Intraspecific diversity of the ITS region

The ITS region was extracted directly from all studied genomes using the top hit with a BLASTn search of the type sequence of *P. cyanescens* (GenBank NR_111478; Borovička *et al.* 2011) (command -outfmt '6 sseq' -max_target_seqs 1) and aligned with ITS sequences of *P. allenii*, *P. azurescens*, *P. cyanescens*, *P. makarorae*, *P. subaeruginosa*, and *P. werarora* from GenBank using MAFFT. Sequences of *P. cubensis* were used as an outgroup, based on its sister group relationship with *P. cyanescens* (Bradshaw *et al.* 2022). We searched for the maximum likelihood tree in IQ-TREE v. 2 based on an alignment that contained one representative of each ITS sequence type and used a phylogenetic hypothesis to explore whether the ITS region was monophyletic at species rank in *P. subaeruginosa* and related taxa. We used PopART to visualise the distribution of ITS genotypes across the entire ITS dataset.

RESULTS

Analyses of SNPs and genes clustered wood-degrading magic mushrooms from the Northern Hemisphere among Australian populations

We included 86 haplotypes from at least 28 separate mushrooms in Australia, with some haplotypes collected as populations and not associated with a single pileus. Our sampling covered 12 sites in eastern Australia (Fig. 2A) and included two reference genomes of *P. azurescens* from Europe (Pazu-FSU-13761) and the USA (Pazurescens) and one of *P. cyanescens* from the USA (Pscy2). kSNP called 1 580 296 SNPs, and we tested for population structure based on ancestry of 6 757 LD-corrected SNPs, which was a subset that excluded all sites that contained indels or missing data (Fig. 2B, C). Mixed ancestry within sites and among known siblings appeared in DAPC analyses beyond $K = 7$ (Fig. 2C). DAPC analyses showed populations were admixed in geographic locations. *Psilocybe azurescens* and *P. cyanescens*

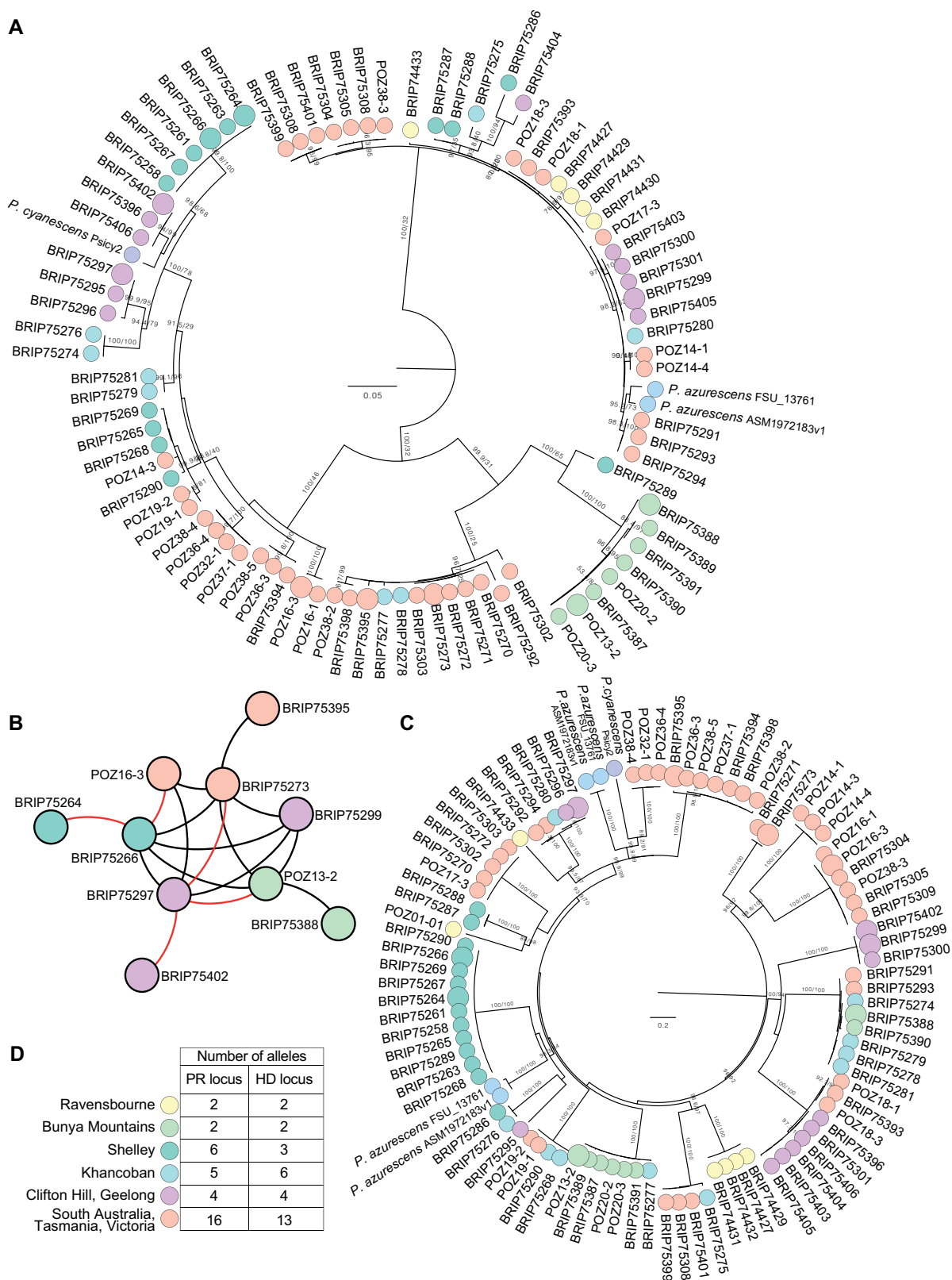


Fig. 3. Allelic diversity, compatibility, and phylograms from maximum likelihood searches based on translated, aligned, concatenated genes at mating-compatibility loci. Individuals are coloured based on structured populations in Fig. 2C. **A.** Maximum likelihood phylogeny of concatenated *STE3* genes at the pheromone/receptor (PR) locus. **B.** Compatible (black) and incompatible (red) crosses, based on formation of clamp connections, between isolates from different populations of *P. subaeruginosa*. **C.** Maximum likelihood phylogeny of concatenated *HD1* and *HD2* genes at the homeodomain (HD) locus. **D.** Allelic diversity at mating compatibility loci in populations of *P. subaeruginosa*. Alleles at mating compatibility loci are mostly private to the sampled populations, which indicates high allelic diversity in Australia.

clustered with *P. subaeruginosa* in 2D plots (Fig. 2B) and had recent shared ancestry with Australian populations (Fig. 2C).

We visualised relationships among isolates of *P. subaeruginosa* with SplitsTree neighbour networks based on 1 555 848 LD-corrected SNPs, including indels (Fig. 2D), and 194 aligned protein coding genes (76 076 amino acids, Fig. S1) identified by OrthoFinder. Relationships recovered by SNPs and genes were congruent. *Psilocybe azurescens* and *P. cyanescens* clustered among Australian populations.

Most individuals in populations from Bunya, Clifton Hill, Ellendale, kunanyi, Ravensbourne, and Shelley were sampled as siblings that could be linked to the same pileus (Fig. 2D). We used the AJK statistic (Yang *et al.* 2010) to investigate the relatedness of haplotypes within populations (Fig. S2). The observed relatedness suggests that haplotypes in clusters observed in Fig. 2D are as related as known siblings even if they were not from the same pileus. These close relationships are supported by likelihoods of the AJK statistic ≥ 0.91 , which is the lowest likelihood for known siblings sampled from Ellendale, Tasmania.

Groups defined by DAPC and supported by network analyses of SNPs and genes show that *P. subaeruginosa* is structured by geography in Australia. Relationships among groups reflect geographic boundaries, for example, samples east of the Great Dividing Range (which divides the eastern coast of Australia), namely Khancoban, Shelley, Clifton Hill, and Geelong, differed from populations west of the range in South Australia, Tasmania, and central Victoria. Full sib haplotypes sampled from one spore print from Clifton Hill (Fig. 2D e) were completely intermixed with sibling haplotypes (based on genetic distance and the AJK statistic) of other fruiting bodies from planted garden beds in both Clifton Hill and Geelong. Geographic areas with mixed ancestry based on DAPC (Fig. 2C) and genetic distance (Fig. 2D), namely Clifton Hill and Shelley, indicate that pilei were sampled from fruiting mycelia of different genotypes at the same location.

Alleles at mating genes are diverse and haplotypes are sexually compatible across geographically isolated populations

We explored the boundaries of sexual reproduction with a hypothesis that allopatric and sympatric speciation may erode mate compatibility and conspecific populations are sexually compatible. We examined phylogenetic relationships of two concatenated *STE3.2* genes at the pheromone/receptor (PR) locus and *HD1* and *HD2* genes at the homeodomain locus (HD) (Figs 3A, B). We identified approximately 25–30 alleles at PR and HD loci across the Australian population, which we consider high diversity given that many samples are siblings. Alleles at PR and HD loci were rarely shared among geographically distant populations, and we expected to see geographic structure from mating compatibility genes given their high allelic diversity and effects of negative frequency dependent selection, which distributes alleles equally across populations.

Crosses between one haploid culture from the Bunya population were compatible, based on formation of clamp connections, with isolates from the most geographically distant and genetically diverse populations in Tasmania and Victoria. This suggests there are no barriers to reproductive compatibility, even among mating-compatibility loci that have differentiated in populations. We did not have permission to attempt fruiting of crosses and have not confirmed whether dikaryons from crosses could produce mushrooms; compatible fusion of hyphae to

form a dikaryon does not guarantee that a mushroom will be produced and form viable spores.

We expected incompatible crosses between haplotypes with identical or near-identical *STE3* alleles (namely, BRIP75264 × BRIP75266 in the Shelley population) and different alleles at HD loci. However, three crosses against BRIP75297 (a within-population cross of Clifton Hill/Geelong, and against Bunya and Tasmania), and a cross of BRIP75266 × POZ16-3 (Shelley and Tasmania) were incompatible (based on a lack of clamp connections) despite different alleles at PR and HD loci.

Balancing selection across mate compatibility loci in differentiated populations inferred from F_{ST} , π , and Tajima's D

We used F_{ST} , π , and Tajima's D as measures of gene flow and selection and tested whether the contigs that contain mating-type loci were differentiated among populations. F_{ST} was comparable among all populations (Fig. 4A, D), but decreased when the South Australian, Tasmanian, and Victorian population were removed (mean F_{ST} of all populations at the HD locus = 0.36; mean F_{ST} of all populations *sans* South Australian, Tasmanian, and Victorian population = 0.26). This difference in F_{ST} supports gene flow or shared ancestry among the eastern populations of *P. subaeruginosa* in Australia, which are less differentiated from each other than from the South Australia, Tasmanian, and Victorian population.

Nucleotide diversity (π) varied greatly across populations for these two regions (Fig. 4B, E) and was generally low (< 0.2) across the contigs that contained mating-type genes. Mating-compatibility loci did not have higher or lower nucleotide diversity than other parts of the contig. Tajima's D was positive across the contigs that contained mating-type loci (Fig. 4C, F), which is expected when multiple alleles are maintained in populations under balancing selection.

Diversity of alleles at psilocybin loci

We examined variation at the psilocybin gene cluster in *P. subaeruginosa* by comparing aligned, concatenated coding sequences across the entire locus. We used a phylogenetic hypothesis to analyse the putatively functional and non-functional copies of the *psiH* gene family (Fig. S3). There were 178 amino acid differences among haplotypes across the translated alignment, (number of amino acid differences *psiD* = 10, *psiK* = 24, *psiM* = 15, *psiR* = 14, *psiT1* = 35, *psiT2* = 23, *psiH1* = 15, and *psiH2* = 42).

A SplitsTree network of 11 768 nucleotides from concatenated coding sequences of *psiD*, *psiK*, *psiM*, *psiT1*, *psiT2*, *psiR* and two paralogs of *psiH* (*psiH1* and *psiH2*) show that genotypes of psilocybin loci cluster by geographic location (Fig. 5A). The locus is heterozygous in some dikaryotic parents, as siblings from the same parental genotype had different alleles (*e.g.*, the Bunya population in Fig. 5A), and there is possible evidence of recombination within the locus with siblings from Bunya and Shelley sharing three genotypes.

We calculated F_{ST} , nucleotide diversity (π), and Tajima's D index across coding sequence of the psilocybin locus as measures of differentiation between populations, diversity in different populations, and selection. F_{ST} did not vary in comparisons of populations, which may indicate that allelic differences in the psilocybin pathway have occurred by genetic drift in differentiated populations (Fig. 5B). There was high

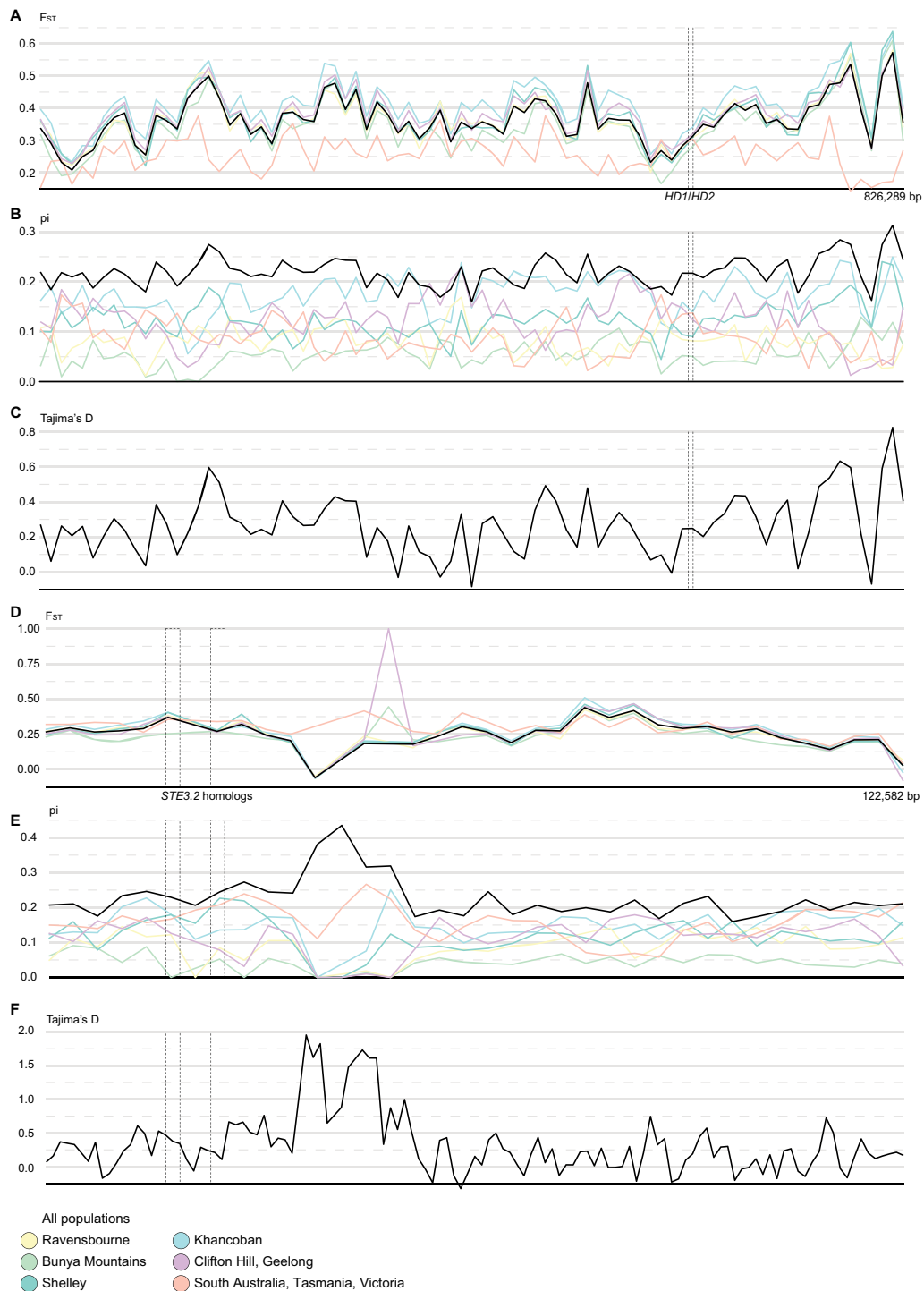


Fig. 4. Measures of differentiation, nucleotide diversity and selection across contigs containing mate compatibility genes among populations of *P. subaeruginosa*. **A–C.** Homeodomain (HD) locus (826 289 base pairs). **D–F.** Pheromone/receptor (PR) locus (122 582 base pairs). **A.** F_{ST} plotted in 10 000 base pair windows of the HD locus as a measure of differentiation among populations. Line colour indicates which population has been removed from the comparison of all populations. F_{ST} values approach 0 in admixed, recombinant populations, and high F_{ST} values may indicate divergence or a lack of recombination. F_{ST} decreases when the South Australian, Tasmanian, and Victorian population is removed from the comparison, which indicates genetic differentiation from populations east of the Great Dividing Range. **B.** Nucleotide diversity (π) plotted across 10 000 base pair windows of the HD locus as a measure of diversity within defined populations. Diversity is low within all populations ($\pi < 0.2$). **C.** Tajima's D index plotted in 10 000 base pair windows across the HD locus as a measure of selection across all populations. Positive values may be a signature of balancing selection, in particular negative frequency dependent selection, in which multiple alleles are maintained in populations and no allele becomes dominant. **D.** F_{ST} plotted in 3 500 base pair windows of the PR locus, with similar levels of differentiation across the locus in all populations. **E.** Nucleotide diversity (π) plotted across 3 500 base pair windows of the PR locus, with divergence among populations driven by diversity in the South Australian, Tasmanian, and Victorian population. **F.** Tajima's D index plotted in 3 500 base pair windows across the PR locus, with support for balancing selection based on positive values comparable to the HD locus.

nucleotide diversity (π) in genes of the psilocybin pathway within and among populations (Fig. 5C), expected under balancing selection. Analyses of Tajima's D index that recovered mostly positive values indicate that some genes of the psilocybin pathway may be under balancing selection, specifically *psiT2*, *psiT1*, and *psiH2*, suggesting that there is some advantage to maintaining multiple alleles (Fig. 5D). *psiH2* had the highest values of the Tajima's D index, which may reflect differential functionality at the locus.

We examined gene diversity of the *psiH* family, annotated with exonerate, with a phylogenetic analysis, which delineated clades closely approximating the *psiH1*, *psiH2*, and *psiH3* paralogs (Fig. S3A). *psiH1* formed a strongly supported clade with short branch lengths. *psiH2* appears paraphyletic in respect to *psiH1*, and *psiH3* appears to have originated from *psiH2*. Alternate topologies in *psiH2* and *psiH3* could reflect recombination among populations or ambiguity in the alignment and splice sites of pseudogenes. All but one ortholog of *psiH3* were considered pseudogenes, and only 29 of 76 isolates annotated for *psiH2* had a putatively functional allele. We plotted gene synteny and identity using Clinker and noted structural variation and differential sequence conservation at *psiH2* and *psiH3* positions (Fig. S3B).

Diversity of alleles at mitochondria

We called 1 334 SNPs in mitochondrial contigs using a k-mer approach ($k = 31$) with kSNP and plotted mitochondrial genotypes in a haplotype network (Fig. S4). There were 18 mitochondrial genotypes across the 9 populations defined by DAPC analyses and 14 geographic locations (including *P. azurescens* and *P. cyanescens*), indicating high mitochondrial diversity. *Psilocybe cyanescens* had a near-identical mitochondrial genotype to populations of *P. subaeruginosa* in Australia, and *P. azurescens* clustered among Australian genotypes differing by as few as two parsimony informative characters.

High intraspecific diversity of the ITS region in *P. subaeruginosa*

We examined whether the ITS region was informative at species rank in *P. subaeruginosa*, as phylogenetic relationships based on the ITS region are used as a proxy for species identification. Phylogenetic relationships were compared across 26 different ITS types, reflective of ITS sequence diversity within and among populations of *P. subaeruginosa* and related taxa identified on GenBank (Fig. 6). We used a haplotype network to visualise the proportions of individuals that shared a particular ITS type. *Psilocybe subaeruginosa* was paraphyletic in the ITS region with respect to *P. allenii*, *P. azurescens*, *P. cyanescens*, *P. makarorae*, and *P. weraroa*. Based on parsimony informative characters, ITS sequences of *P. allenii*, *P. azurescens*, and *P. cyanescens* clustered with samples of *P. subaeruginosa* from Australia, whereas *P. makarorae* and *P. weraroa* had unique ITS sequences not sampled from Australia. *Psilocybe cubensis*, sampled from eight ITS-types in 166 individuals, is a sister taxon to *P. subaeruginosa* (Bradshaw *et al.* 2022) and most sequences cluster with cultivars (exemplified by the AlbinoAplus sequence, Fig. 6). These sequences of *P. cubensis* are representative of naturalised and cultivated populations rather than diversity in its centre of origin, which is currently unknown.

DISCUSSION

Psilocybin has breakthrough potential for treatment of mental health disorders (Kargbo 2020), and as momentum builds in the clinical landscape, knowledge of diversity in magic mushrooms will impact development of natural medicines. Our results suggest that *Psilocybe subaeruginosa* originated in Australasia as evidenced by its widespread distribution in natural areas, high allelic diversity of mating genes, high genetic diversity at mitochondria and psilocybin loci, and high phenotypic diversity. Mushrooms in the *P. subaeruginosa* species complex were likely introduced to the Northern Hemisphere, as hypothesised by Giebler (2018), through movement of plants, soil, or wood chips, as they cluster among Australian populations in analyses of genetic diversity based on SNPs or genes, have low genetic diversity in their invaded areas (Giebler 2018), and behave as weedy taxa, occurring in disturbed rather than natural areas (Dennis & Wakefield 1946, Borovička *et al.* 2012).

Psilocybe subaeruginosa colonises wood chips and leaf litter, and mushrooms from one vicinity often sporulate from the same mycelial genotype, based on sibling relationships supported by the AJK statistic and genetic distance within populations defined by DAPC. We studied 86 haplotypes across eastern Australia, although the overall effective sample size is reduced as many haplotypes were siblings, and greater genetic diversity is expected with wider sampling, potentially including founding genotypes of *P. azurescens* and *P. cyanescens*. Mushrooms collected from geographically different areas that were recovered as siblings support that *P. subaeruginosa* spreads as a saprotrophic invader of garden beds. Genotypes of *P. subaeruginosa* likely persist perennially, and anecdotal evidence from citizen scientists in the present study shows that fruiting sites are re-visited to collect mushrooms, likely with the same genotype, year after year. This contrasts with *P. cubensis*, in which genotypes are ephemeral, with mycelia disappearing after manure is degraded, akin to annual plants.

Our findings indicate that *P. subaeruginosa* is one taxon rather than a complex of species, supported by evidence from population analyses based on SNPs, reticulation/coalescence in network analyses based on single copy genes, low levels of nucleotide diversity across the entire population, shared alleles at mating compatibility and psilocybin loci, and mating compatibility tested by formation of clamp connections. A null hypothesis that these taxa are one species is not rejected by phylogenetic or biological criteria, and if cryptic species were described, they could reflect populations derived from the same dikaryotic mycelium or partially isolated populations with minimal gene flow. Low phenotypic diversity or a fixed phenotype in populations from the Northern Hemisphere is likely caused by underlying low genetic diversity from an invasion event (Gladioux *et al.* 2015), as *P. azurescens* and *P. cyanescens* share close ancestry with *P. subaeruginosa* in all analyses. Additional species described in this taxonomic complex are likely phenotypic/geographic variants and con-specific with *P. subaeruginosa*. Taxonomic synonyms may be useful to describe invasive populations, such as *P. cyanescens* in Europe. However, these taxa have an origin from Australia and could be considered subpopulations of *P. subaeruginosa*. The ITS region is intraspecifically variable in the Australian population, including among haplotypes from the same dikaryotic parent, and is not a suitable genetic marker to differentiate species from populations in *P. subaeruginosa*. Dabao Sun *et al.* (2023) found

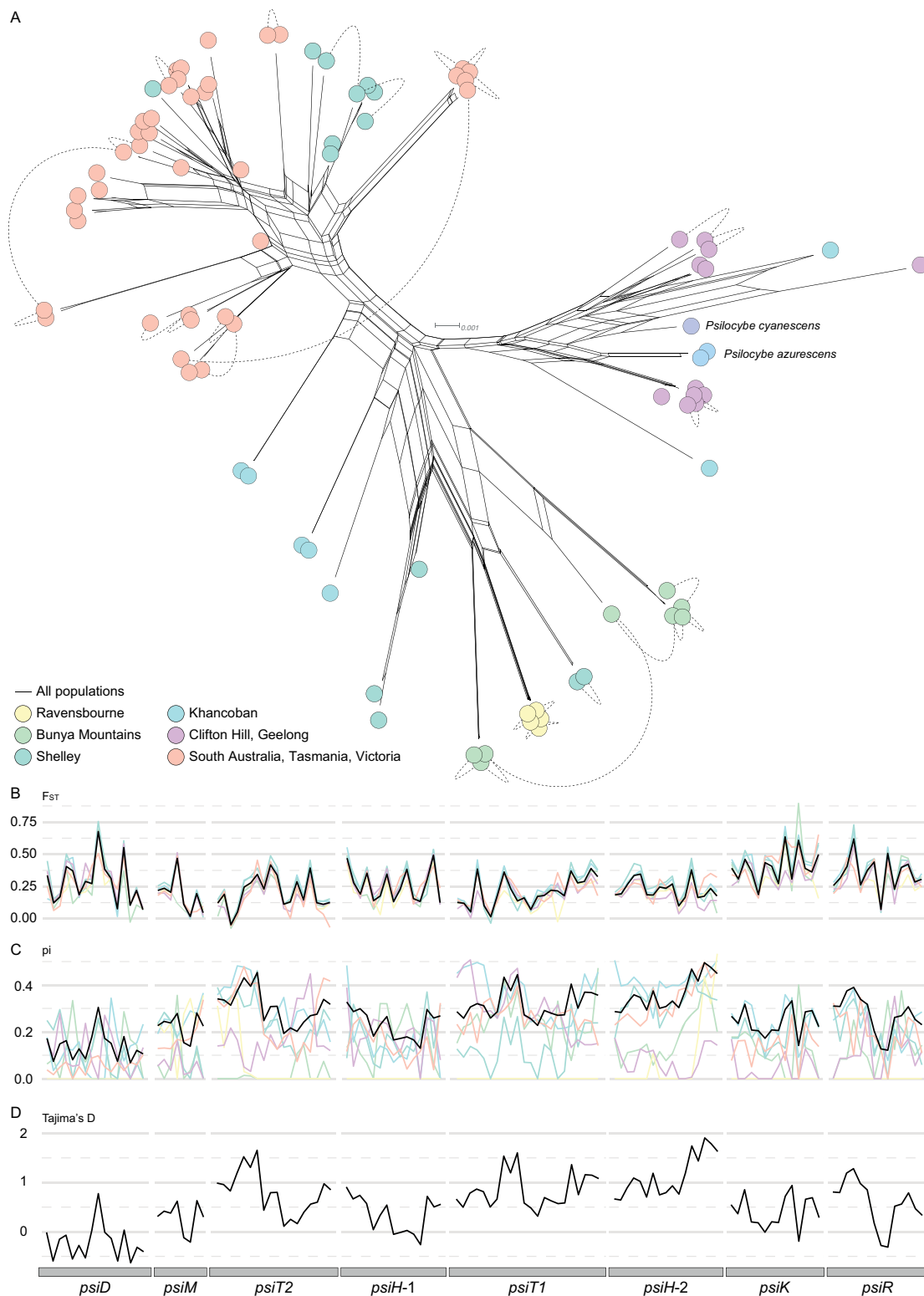


Fig. 5. Analyses of genetic diversity at the psilocybin locus. **A.** SplitsTree network of aligned coding sequences of *psiD*, *psiK*, *psiM*, *psiT1*, *psiT2*, *psiR* and two paralogs of *psiH* (*psiH1* and *psiH2*). Individuals are coloured based on structured populations in Fig. 1A. Sibling isolates sampled from the same pileus are linked by dashed lines. Siblings that have different alleles at psilocybin loci indicate heterozygosity in the parental genotype. Siblings with more than two genotypes (in populations from Bunya and Shelley) may reflect recombination within the psilocybin locus. **B.** F_{ST} plotted across individual genes as a measure of differentiation among populations at the psilocybin locus. F_{ST} is comparable among populations. **C.** Nucleotide diversity (π) plotted across individual genes as a measure of genetic diversity at the psilocybin locus. The Khancoban population is the most genetically diverse at the psilocybin locus relative to other populations. **D.** Tajima's D index plotted across individual genes as a measure of selection at the psilocybin locus. Most genes have neutral to positive values of Tajima's D, which indicates balancing selection or maintenance of diversity at the psilocybin locus. Measures of genetic diversity were plotted in 50-base pair windows of all genes except *psiM*, which used a 30-base pair window.

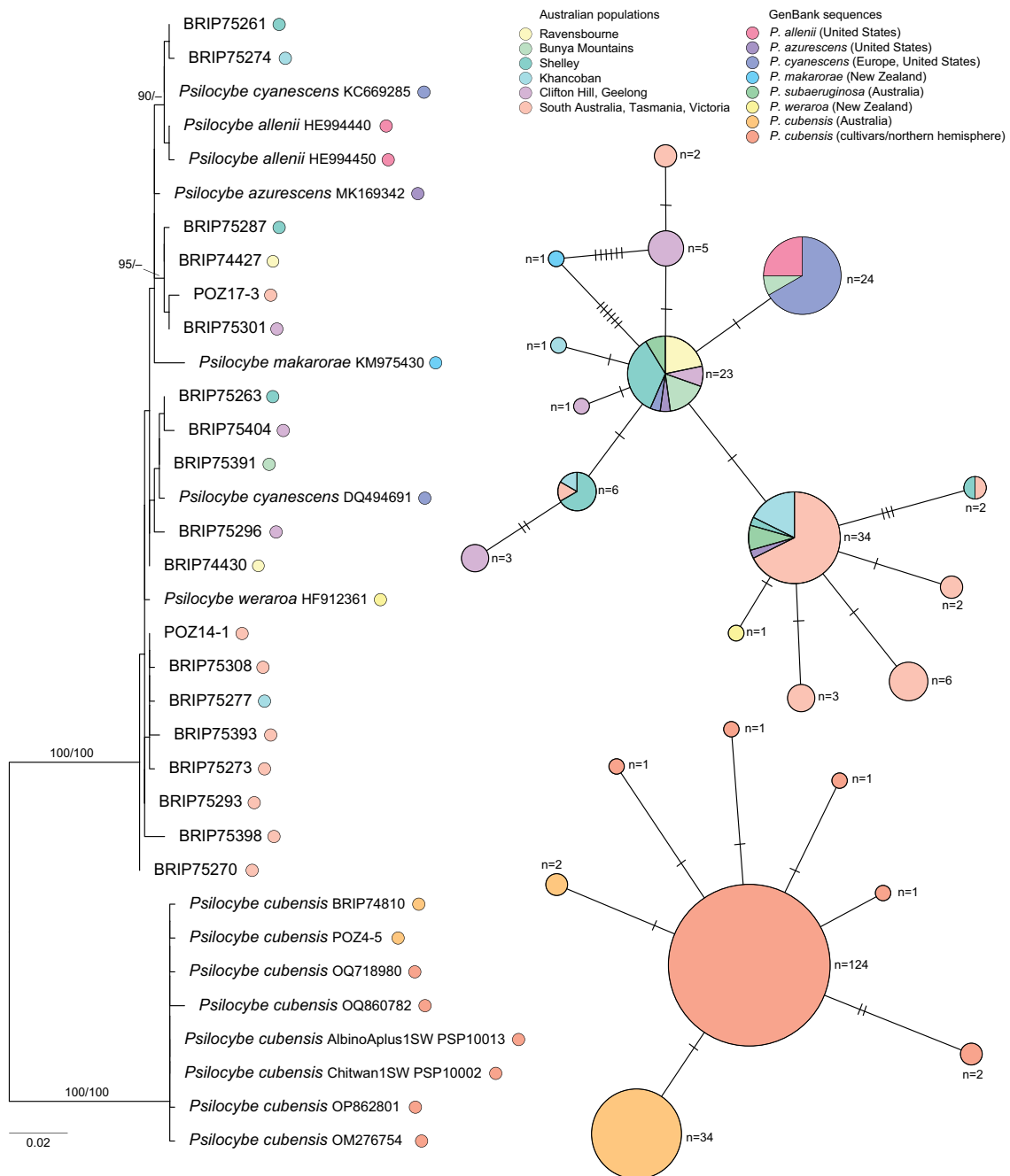


Fig. 6. Phylogram from a maximum likelihood search of 26 ITS types of *Psilocybe subaeruginosa* and related taxa, showing a sister relationship to *P. cubensis*. Sequences were obtained from genomes in the present study, and from GenBank for sequences of *P. allenii*, *P. azurescens*, *P. cyaneoscens*, *P. makarorae*, and *P. weraroa*. Sequence abundance for each ITS type is shown in the adjacent haplotype networks for *P. subaeruginosa* and *P. cubensis*, with dashes indicating the number of parsimony informative sites between ITS types. The ITS region is intraspecifically variable in *P. subaeruginosa*, which is paraphyletic with respect to other related taxa. Isolates with a sibling relationship may differ at the ITS region, indicating it is an unsuitable marker for phylogenetic resolution at species rank.

that differentiated lineages in a fungal taxon at a global scale had complicated species boundaries because sympatric closely-related species could be inter-sterile due to reinforcement whereas allopatric populations may appear to be highly compatible but genetically isolated species. More crosses in the case of *P. subaeruginosa* will be needed to determine if any reproductive isolation exists, however, most populations show some degree of mating compatibility.

Mushrooms produce copious spores that are wind dispersed (Dam 2013), and allopatric speciation of mushrooms has occurred at the scale of continental geographic boundaries (James *et al.* 2001, Geml *et al.* 2008, Li *et al.* 2020, Zhang *et al.* 2023). Our study, which found isolated populations of *P. subaeruginosa* on mountain ranges in Australia, may add to evidence that mushrooms limited by their available habitat and spore dispersal, by nature, have opportunities for allopatric

speciation. For example, Amend *et al.* (2010) found montane populations of *Tricholoma matsutake* were isolated based on topography, with mountain ranges a barrier to gene flow. Another study found that the ectomycorrhizal species *Suillus brevipes* was structured into subpopulations within North America due to isolation by and on mountain ranges (Branco *et al.* 2017). The mean level of population differentiation we report here from haploid genomes, $F_{ST} = 0.36$, may be high compared to other taxa, yet, these values vary considerably across species of mushroom (Carriconde *et al.* 2008, Mi *et al.* 2016, Zhang *et al.* 2022). This level of differentiation suggests that spatial populations of *P. subaeruginosa* have had sufficient time to show the effects of isolation within their centre of origin. Why some species show strong intracontinental population substructure while others do not is uncertain and highlights how little we understand fungal niche breadth, gene flow, distribution, and the temporal and geographic scale of the centre of origin.

Isolation and infrequent gene flow lead to divergence and speciation (Peterson & Hughes 1999). Isolation of fungi with dominant asexual stages generates near clones, in which clonal reproduction is interspersed with infrequent sexual reproduction that maintains species cohesion (Taylor *et al.* 2015). In obligate outcrossing fungi, mating compatibility loci maintain species connectivity because allelic diversity benefits compatibility, and MAT genes diverge but maintain key amino acids at functional sites (van Diepen *et al.* 2013, Peris *et al.* 2022). Populations of *P. subaeruginosa* were sexually compatible, and slight differences at mating compatibility loci among populations may be caused by genetic drift and isolation, or alternatively, we under-sampled potentially shared alleles. Allopatric species boundaries may be interrupted given that humans move soils and their accompanying microorganisms, and as shown by others (Vilgalys & Johnson 1987, Aanen *et al.* 2000, Dettman *et al.* 2003, Moncalvo & Buchanan 2008, Menolli Jr *et al.* 2022) and with magic mushrooms here, species connectivity through mate compatibility persists even in disparate populations with small opportunity for gene flow.

Psilocybin loci were genetically different within and among populations of *P. subaeruginosa* in Australia, whether from allelic diversity, or potential differences in presence or absence of functional paralogs of *psiH*. Some haplotypes contained two putatively functional paralogs of the *psiH* gene family (*psiH1* and *psiH2*), whereas others contained one (*psiH1*). One isolate, BRIP75275 has a putative functional *psiH* in the *psiH3* position, but a pseudogene in the *psiH2* position. That sequence groups at the base of the *psiH2* clade with several pseudogenes that are syntenic with *psiH3*. Analyses of F_{ST} and Tajima's D indicated that differentiation of the psilocybin locus among populations may be a result of genetic drift, such as from a founder effect in isolated populations, and populations maintain allelic diversity through balancing selection. A longstanding knowledge gap is whether psilocybin binds to serotonin receptors in metazoans to attract, repel, or manipulate, and genetic diversity in the centre of origin of *P. subaeruginosa* may be a resource to answer this evolutionary question. McTaggart *et al.* (2023a) found the psilocybin locus was homozygous in five siblings of *P. subaeruginosa*, however, with increased sampling, we show heterozygosity in dikaryons at the psilocybin locus.

Allelic differences of genes in the psilocybin pathway may increase/decrease metabolism of tryptamines, and the ratios of psilocybin and its analogues may differ among genotypes. Humans have at least 14 types of serotonin receptors; 5-HT_{2A} has the highest affinity for psilocin and is linked to hallucinogenic effects of magic mushrooms (Glennon *et al.* 1984, Lee & Roth 2012, Madsen *et al.* 2019). The suite of tryptamines produced by magic mushrooms in the psilocybin pathway may have different affinities for types of serotonin receptors beyond 5-HT_{2A} (Glatfelter *et al.* 2022). We put forward the hypothesis that alternate allelic combinations at paralogs of *psiH* may cause wood lover's paralysis by producing a derivative of tryptamine that agonises peripheral serotonin receptors, such as those linked to Parkinson's disease (Ohno *et al.* 2013).

High genetic diversity of all examined alleles/loci in the centre of origin of *P. subaeruginosa* contrasts with low diversity in naturalised and cultivated populations of *P. cubensis* (McTaggart *et al.* 2023b). Our findings put perspective on what may be expected in terms of genetic diversity in the unknown centre of origin of *P. cubensis*. Mitochondrial diversity and allelic diversity at mating loci was variable between and among all examined populations, as should be expected in the centre of origin of *P. cubensis*.

Our study shows that *P. subaeruginosa* is a widespread and invasive mushroom with a centre of origin in Australia. Geographically limited populations are sexually compatible, although there is little evidence of contemporary gene flow, with mitochondria, mating genes, and alleles at psilocybin loci differentiated among populations. *Psilocybe subaeruginosa* produces high concentrations of psilocybin and is a commercially attractive species if the cause of wood lover's paralysis can be determined and excluded for safe clinical use.

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Data availability statement: Assembled genomes are available under their NCBI accession numbers listed in Table S1. Raw data are made publicly available through BioPlatforms Australia. Fungal cultures are lodged in the Queensland Plant Pathology herbarium (BRIP). https://data.bioplatforms.com/organization/fungi?ext_search_by=&q=BPAOPS-1356

Conflict of interest: Alistair McTaggart is an owner of Psymbiotika, a company that grows magic mushrooms commercially.

REFERENCES

- Aanen DK, Kuyper T, Mes THM, *et al.* (2000). The evolution of reproductive isolation in the ectomycorrhizal *Hebeloma crustuliniforme* aggregate (*Basidiomycetes*) in northwestern Europe: a phylogenetic approach. *Evolution* **54**: 1192–1206.
- Abbas A, Carter A, Jeanne T, *et al.* (2021). *Oregon Psilocybin Advisory Board Rapid Evidence Review and Recommendations*. Oregon Psilocybin Advisory Board.
- Amend A, Garbelotto M, Fang Z, *et al.* (2010). Isolation by landscape in populations of a prized edible mushroom *Tricholoma matsutake*. *Conservation Genetics* **11**: 795–802.
- Bankevich A, Nurk S, Antipov D, *et al.* (2012). SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *Journal of Computational Biology* **19**: 455–477.
- Bolger AM, Lohse M, Usadel B (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* **30**: 2114–2120.
- Borovička J, Noordeloos ME, Gryndler M, *et al.* (2011). Molecular phylogeny of *Psilocybe cyanescens* complex in Europe, with reference to the position of the secotioid *Weraroa novae-zelandiae*. *Mycological Progress* **10**: 149–155.
- Borovička J, Rockefeller A, Werner PG (2012). *Psilocybe allenii*—a new bluing species from the Pacific Coast, USA. *Czech Mycology* **64**: 181–195.
- Bradshaw AJR-CV, Awan AR, Furci G, *et al.* (2022). Phylogenomics of the psychoactive mushroom genus *Psilocybe* and evolution of the psilocybin biosynthetic gene cluster. *Proceedings of the National Academy of Sciences USA* **121**: e2311245121.
- Branco S, Bi K, Liao H-L, *et al.* (2017). Continental-level population differentiation and environmental adaptation in the mushroom *Suillus brevipes*. *Molecular Ecology* **26**: 2063–2076.
- Buchfink B, Xie C, Huson DH (2015). Fast and sensitive protein alignment using DIAMOND. *Nature Methods* **12**: 59–60.
- Carriconde F, Gryta H, Jargeat P, *et al.* (2008). High sexual reproduction and limited contemporary dispersal in the ectomycorrhizal fungus *Tricholoma scalpturatum*: new insights from population genetics and spatial autocorrelation analysis. *Molecular Ecology* **17**: 4433–4445.
- Chang YS, Mills AK (1992). Re-examination of *Psilocybe subaeruginosa* and related species with comparative morphology, isozymes and mating compatibility studies. *Mycological Research* **96**: 429–441.
- Cleland JB (1927). Australian fungi: notes and descriptions - No. 6. *Transactions and Proceedings of the Royal Society of South Australia* **51**: 298–306.
- Coelho MA, Bakkeren G, Sun S, *et al.* (2017). Fungal Sex: The *Basidiomycota*. *Microbiology Spectrum* **5**: 3.
- Dabao Sun L, David P, Jørn Henrik S, *et al.* (2023). Reticulate evolution and rapid development of reproductive barriers upon secondary contact pose challenges for species delineation in a forest fungus. *bioRxiv* 2023.2009.2018.558330.
- Dam N (2013). Spores do travel. *Mycologia* **105**: 1618–1622.
- Danecek P, Auton A, Abecasis G, *et al.* (2011). The variant call format and VCFtools. *Bioinformatics* **27**: 2156–2158.
- Dennis RWG, Wakefield EM (1946). New or interesting British fungi. *Transactions of the British Mycological Society* **29**: 141–166.
- Dettman JR, Jacobson DJ, Turner E, *et al.* (2003). Reproductive isolation and phylogenetic divergence in *Neurospora*: comparing methods of species recognition in a model Eukaryote. *Evolution* **57**: 2721–2741.
- Dörner S, Rogge K, Fricke J, *et al.* (2022). Genetic survey of *Psilocybe* natural products. *Chembiochem* **23**: e202200249.
- Emms DM, Kelly S (2019). OrthoFinder: phylogenetic orthology inference for comparative genomics. *Genome Biology* **20**: 238.
- Fricke J, Blei F, Hoffmeister D (2017). Enzymatic synthesis of psilocybin. *Angewandte Chemie International Edition* **56**: 12352–12355.
- Gardner SN, Slezak T, Hall BG (2015). kSNP3.0: SNP detection and phylogenetic analysis of genomes without genome alignment or reference genome. *Bioinformatics* **31**: 2877–2878.
- Geml J, Tulloss RE, Laursen GA, *et al.* (2008). Evidence for strong inter- and intracontinental phylogeographic structure in *Amanita muscaria*, a wind-dispersed ectomycorrhizal basidiomycete. *Molecular Phylogenetics and Evolution* **48**: 694–701.
- Gießler A (2018). *Psilocybe cyanescens* in Germany: ecology and taxonomy of an invasive neomycete. PhD dissertation. Göttingen University, Germany.
- Gilchrist CLM, Chooi YH (2021). clinker & clustermap.js: automatic generation of gene cluster comparison figures. *Bioinformatics* **37**: 2473–2475.
- Gladieux P, Feurtey A, Hood ME, *et al.* (2015). The population biology of fungal invasions. *Molecular Ecology* **24**: 1969–1986.
- Glatfelter GC, Pottier E, Partilla JS, *et al.* (2022). Structure-activity relationships for psilocybin, baeocystin, aeruginascin, and related analogues to produce pharmacological effects in mice. *ACS Pharmacology & Translational Science* **5**: 1181–1196.
- Glennon RA, Titeler M, McKenney JD (1984). Evidence for 5-HT₂ involvement in the mechanism of action of hallucinogenic agents. *Life Sciences* **35**: 2505–2511.
- Gotvaldová K, Borovička J, Hájková K, *et al.* (2022). Extensive collection of psychotropic mushrooms with determination of their tryptamine alkaloids. *International Journal of Molecular Sciences* **23**: 14068.
- Huson DH, Bryant D (2005). Application of phylogenetic networks in evolutionary studies. *Molecular Biology and Evolution* **23**: 254–267.
- James TY, Moncalvo J-M, Li S, *et al.* (2001). Polymorphism at the ribosomal DNA spacers and its relation to breeding structure of the widespread mushroom *Schizophyllum commune*. *Genetics* **157**: 149–161.
- Jombart T (2008). adegenet: a R package for the multivariate analysis of genetic markers. *Bioinformatics* **24**: 1403–1405.
- Jombart T, Devillard S, Balloux F (2010). Discriminant analysis of principal components: a new method for the analysis of genetically structured populations. *BMC Genetetics* **11**: 94.
- Kargbo RB (2020). Psilocybin therapeutic research: the present and future paradigm. *ACS Medicinal Chemistry Letters* **11**: 399–402.
- Katoh K, Standley DM (2013). MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Molecular Biology and Evolution* **30**: 772–780.
- Knaus BJ, Grünwald NJ (2017). vcfr: a package to manipulate and visualize variant call format data in R. *Molecular Ecology Resources* **17**: 44–53.
- Kück P, Longo GC (2014). FASconCAT-G: extensive functions for multiple sequence alignment preparations concerning phylogenetic studies. *Frontiers in Zoology* **11**: 81.
- Lee HM, Roth BL (2012). Hallucinogen actions on human brain revealed. *Proceedings of the National Academy of Sciences USA* **109**: 1820–1821.
- Leigh JW, Bryant D (2015). Popart: full-feature software for haplotype network construction. *Methods in Ecology and Evolution* **6**: 1110–1116.
- Lenz C, Wick J, Braga D, *et al.* (2020). Injury-triggered blueing reactions of *Psilocybe* “magic” mushrooms. *Angewandte Chemie International Edition* **59**: 1450–1454.
- Li J, Han L-H, Liu X-B, *et al.* (2020). The saprotrophic *Pleurotus ostreatus* species complex: late Eocene origin in East Asia, multiple dispersal, and complex speciation. *IMA Fungus* **11**: 10.

- Madsen MK, Fisher PM, Burmester D, *et al.* (2019). Psychedelic effects of psilocybin correlate with serotonin 2A receptor occupancy and plasma psilocin levels. *Neuropsychopharmacology* **44**: 1328–1334.
- Martin F, Aerts A, Ahren D, *et al.* (2008). The genome of *Laccaria bicolor* provides insights into mycorrhizal symbiosis. *Nature* **452**: 88–92.
- McKernan K, Kane L, Helbert Y, *et al.* (2021). A whole genome atlas of 81 *Psilocybe* genomes as a resource for psilocybin production. *F1000Research* **10**: 961.
- McKernan K, Kane LT, Crawford S, *et al.* (2021). A draft reference assembly of the *Psilocybe cubensis* genome. *F1000Research* **10**: 281.
- McTaggart AR, James TY, Slot JC, *et al.* (2023a). Genome sequencing progenies of magic mushrooms (*Psilocybe subaeruginosa*) identifies tetrapolar mating and gene duplications in the psilocybin pathway. *Fungal Genetics and Biology* **165**: 103769.
- McTaggart AR, McLaughlin S, Slot JC, *et al.* (2023b). Domestication through clandestine cultivation constrained genetic diversity in magic mushrooms relative to naturalized populations. *Current Biology* **33**: 5147–5159.
- Menolli Jr N, Sánchez-Ramírez S, Sánchez-García M, *et al.* (2022). Global phylogeny of the Shiitake mushroom and related *Lentinula* species uncovers novel diversity and suggests an origin in the Neotropics. *Molecular Phylogenetics and Evolution* **173**: 107494.
- Mi F, Zhang Y, Yang D, *et al.* (2016). Evidence for inbreeding and genetic differentiation among geographic populations of the saprophytic mushroom *Trogia venenata* from southwestern China. *PLoS ONE* **11**: e0149507.
- Minh BQ, Nguyen MAT, von Haeseler A (2013). Ultrafast approximation for phylogenetic bootstrap. *Molecular Biology and Evolution* **30**: 1188–1195.
- Minh BQ, Schmidt HA, Chernomor O, *et al.* (2020). IQ-TREE 2: new models and efficient methods for phylogenetic inference in the genomic era. *Molecular Biology and Evolution* **37**: 1530–1534.
- Moncalvo J-M, Buchanan PK (2008). Molecular evidence for long distance dispersal across the Southern Hemisphere in the *Ganoderma applanatum-australe* species complex (*Basidiomycota*). *Mycological Research* **112**: 425–436.
- Ohno Y, Shimizu S, Tokudome K (2013). Pathophysiological roles of serotonergic system in regulating extrapyramidal motor functions. *Biological and Pharmaceutical Bulletin* **36**: 1396–1400.
- Palmer J, Stajich JE (2019). nextgenusfs/funcannotate: funcannotate v1.5.3 (1.5.3). *Zenodo* DOI: <https://doi.org/10.5281/zenodo.2604804>
- Peris D, Lu DS, Kinneberg VB, *et al.* (2022). Large-scale fungal strain sequencing unravels the molecular diversity in mating loci maintained by long-term balancing selection. *PLoS Genetics* **18**: e1010097.
- Peterson RH, Hughes KW (1999). Species and speciation in mushrooms. *BioScience* **49**: 440–452.
- Purcell S, Neale B, Todd-Brown K, *et al.* (2007). PLINK: a tool set for whole-genome association and population-based linkage analyses. *American Journal of Human Genetics* **81**: 559–575.
- R_Core_Team (2014) *R: A language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing.
- Reynolds HT, Vijayakumar V, Gluck-Thaler E, *et al.* (2018). Horizontal gene cluster transfer increased hallucinogenic mushroom diversity. *Evolution Letters* **2**: 88–101.
- Shahar O, Botvinnik A, Schwartz A, *et al.* (2024). Effect of chemically synthesized psilocybin and psychedelic mushroom extract on molecular and metabolic profiles in mouse brain. *Molecular Psychiatry*: doi.org/10.1038/s41380-024-02477-w.
- Skrede I, Maurice S, Kausrud H (2013). Molecular characterization of sexual diversity in a population of *Serpula lacrymans*, a tetrapolar basidiomycete. *G3 Genes/Genomes/Genetics* **3**: 145–152.
- Slater GS, Birney E (2005). Automated generation of heuristics for biological sequence comparison. *BMC Bioinformatics* **6**: 31.
- Stamets P (1996). *Psilocybin mushrooms of the world: an identification guide*. Ten Speed Press, Berkeley, California, USA.
- Taylor JW, Hann-Soden C, Branco S, *et al.* (2015). Clonal reproduction in fungi. *Proceedings of the National Academy of Sciences USA* **112**: 8901–8908.
- van Diepen LTA, Olson Å, Ihrmark K, *et al.* (2013). Extensive trans-specific polymorphism at the mating type locus of the root decay fungus *Heterobasidion*. *Molecular Biology and Evolution* **30**: 2286–2301.
- Vilgalys RJ, Johnson JL (1987). Extensive genetic divergence associated with speciation in filamentous *Fungi*. *Proceedings of the National Academy of Sciences USA* **84**: 2355–2358.
- Yang JA, Benyamin B, McEvoy BP, *et al.* (2010). Common SNPs explain a large proportion of the heritability for human height. *Nature Genetics* **42**: 565–569.
- Zhang J, Shen N, Li C, *et al.* (2022). Population genomics provides insights into the genetic basis of adaptive evolution in the mushroom-forming fungus *Lentinula edodes*. *Journal of Advanced Research* **38**: 91–106.
- Zhang M-Z, Xu J-P, Callac P, *et al.* (2023). Insight into the evolutionary and domesticated history of the most widely cultivated mushroom *Agaricus bisporus* via mitogenome sequences of 361 global strains. *BMC Genomics* **24**: 182.

Supplementary Material: <http://fuse-journal.org/>

Fig. S1. SplitsTree network based on 76 076 amino acids aligned from 194 single copy orthologs in *Psilocybe subaeruginosa* and relatives. Branch length is informative for genetic distance between points, reticulation is indicative of recombination, homoplasy, or incomplete lineage sorting.

Fig. S2. Heatmap of sibling relationships based on pairwise comparisons of the AJK statistic. Several of the relationships are known siblings, and based on likelihood values of the AJK statistic, pairwise relationships ≥ 0.91 are an indication that isolates are siblings.

Fig. S3. Evolution of *psiH* paralogs in the psilocybin locus in *Psilocybe subaeruginosa*. **A.** Maximum likelihood tree of positional orthologs of the three *psiH* paralogs extracted from cluster loci using exonerate. Bold alleles are annotated as pseudogenes, and all sequences in the *psiH3* clade were pseudogenes. Isolate BRIP75275 has a predicted functional paralog in the *psiH3* position and a predicted pseudogene in the *psiH2* position. **B.** Clinker plot of synteny and relatedness of genes in the psilocybin metabolic pathway. Darker connections between plots indicate higher percent nucleotide identity. *psiH2* is likely a duplicated copy of *psiH1*, and *psiH3* is likely a duplication of *psiH2*.

Fig. S4. Haplotype network of SNP diversity in the mitochondrial genome based on 1 334 SNPs. Sibling populations shared the same mitochondrial genotype (e.g., populations from Bunya, Ravensbourne, and Clifton Hill and Geelong).

Table S1. Specimen details and GenBank accession numbers for all examined genomes.