# 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, a compound with breakthrough status for treatment of mental health disorders. Wood-degrading species of *Psilocybe*, such as P. *subaeruginosa* and relatives, have high concentrations of psilocybin but are discouraged for clinical production due to a temporary paralytic side effect known as Wood Lover's Paralysis, the cause of which is unknown. We studied *P. subaeruginosa* over its partial distribution in Australia based on genomic analyses of 89 isolates to investigate population structure and species boundaries, examine allelic diversity at psilocybin loci, and test its centre of origin. *Psilocybe subaeruginosa* is structured by geography in Australia, but geographically separated populations are fully sexually compatible. Allelic diversity among populations, such as at mating compatibility loci, is likely a result of genetic drift and minimal gene flow since differentiation from a shared ancestor. Movement of woodchips, mulch, or plants has most likely spread genotypes of *P. subaeruginosa* locally within Australia and to the northern hemisphere. Species from the northern hemisphere, namely *P. azurescens* and *P. cyanescens*, clustered among Australian populations, indicating shared ancestry and supporting a hypothesis these taxa are conspecific with *P. subaeruginosa*. We identified high allelic diversity in genes of the psilocybin metabolic pathway and haplotypes of *P. subaeruginosa* with either one or two putatively functional paralogs of *psiH*, however the functionality of this gene duplication is yet to be determined. Our study provides insights into the evolutionary history and species boundaries of *P. subaeruginosa*, which has a centre of origin in Australasia.

## Introduction

Mushrooms (Agaricomycetes, Basidiomycota) disperse large quantities of wind-borne spores (Dam, 2013), yet populations of many different species are structured by geography (Amend, Garbelotto, Fang, & Keeley, 2010; Branco et al., 2017; Dabao Sun et al., 2023). These geographically isolated populations may form allopatric species if 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, Bakkeren, Sun, Hood, & Giraud, 2017). Mating-compatibility loci have high allele diversity and long allele residence times (Skrede, Maurice, & Kauserud, 2013; van Diepen, Olson, Ihrmark, Stenlid, & James, 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 reproductive isolation needs better understanding to delineate populations from species of Basidiomycota.

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).

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. (2023) showed*psiH* , a gene in the psilocybin pathway that converts tryptamine into 4- hydroxytryptamine (Fricke, Blei, & Hoffmeister, 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. (2023) 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 described from Australia (*P. australiana* , *P. eucalypta* , and *P. tasmaniana* ), New Zealand (*P. makarorae*and *P. weraroa* ), and the northern hemisphere (*P. allenii* ,*P. azurescens* , and *P. cyanescens* ) (Borovička, Noordeloos, Gryndler, & Oborník, 2011; Gotvaldová et al., 2022). 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 (Borovička, Rockefeller, & Werner, 2012; Dennis & Wakefield, 1946), 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* .

## 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, Tas-

mania, 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 Herbarium. Cultures were grown in half-strength potato dextrose broth for three weeks, 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, Lohse, & Usadel, 2014) and assembled with SPAdes v.3.12.0 (Bankevich et al., 2012). Assembled genomes are accessioned in GenBank (Supplementary Table 1). 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*

SNPs were called using kSNP (k=91, min frac = 1) (Gardner, Slezak, & Hall, 2015), from 81 genomes of *P. subaeruginosa* assembled in the present study, five from a past study (McTaggart et al., 2023), two genomes of *P. azurescens* (Dörner et al., 2022; K McKernan et al., 2021), 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 SNPs were visualised 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, Devillard, & Balloux, 2010). The packages vcfR (Knaus & Grünwald, 2017), adegenet (Jombart, 2008), and ggplot2 implemented in R (R_Core_Team, 2014) were used to import an LD-corrected VCF file, cluster populations by k-means clustering and DAPC, and plot results, respectively.

We used the *relatedness* command in vcftools v1.17 (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).

*Analyses of single copy orthologs*

We used OrthoFinder v.1.0.6 (Emms & Kelly, 2019) with a Diamond search (Buchfink, Xie, & Huson, 2015) to identify orthologous groups of genes. All single copy orthologs were aligned with MAFFT (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. (2023) 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 IQTree 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, Nguyen, & von Haeseler, 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 (pi), and Tajima's D across contigs and using populations defined by DAPC analyses. $F_{ST}$, pi, 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, 1L distilled water) and left for three weeks. 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 ×1000 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 were aligned with MAFFT and their homology was confirmed with a search for the most likely tree using IQTree. The coding sequences of genes in the psilocybin pathway, including *psiD, psiM, psiT2, psiH* (paralog 1), *psiT1, psiH*(paralog 2), *psiK, psiR* , 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 vcfools and plotted with ggplot2 in R.

The *psiH* gene family, including pseudogenes, was annotated with exonerate (Slater & Birney, 2005). We aligned all alleles of the*psiH* family with MAFFT, and searched for a maximum likelihood tree with IQTree 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 (K. McKernan et al., 2021). SNPs were called from all mitochondrial contigs using kSNP (k=31, min frac=1), and 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 assembled genomes using the top hit with a BLASTn search (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. weraroa* 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 IQTree 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* and one of *P. cyanescens* from the USA. 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 (Figs 2B, 2C). 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* clustered

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

We visualised relationships among genomes 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 Figure 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. A possible explanation for this is that a parental, dikaryotic genotype has spread long distances via woodchips and mulch in Victoria, facilitated by the perennial nature of *P. subaeruginosa* mycelium. 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 did not expect to see any geographic pattern beyond this given the high allelic diversity and effects of negative frequency 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, and gene flow could occur among geographically limited populations if given the opportunity.

We expected incompatible crosses between haplotypes with identical or near-identical *STE3* alleles (e.g., BRIP75264 x BRIP75266, BRIP75402 x BRIP75297) and different alleles at HD loci. A cross of two near identical haplotypes of siblings in the Bunya population (BRIP75388 x POZ13-2) was successful, which is expected in tetrapolar mating systems (25% of siblings are sexually compatible). However, three crosses against BRIP75297 and a cross of BRIP75266 x POZ16-3 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 $F_{ST}$, pi, and Tajima's D*

We used $F_{ST}$, pi, 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 and 4D), but decreased when the South Australian, Tasmanian, and Victorian population was 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 (pi) varied greatly across populations for these two regions (Fig. 4B and 4E) 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, which may reflect genetic drift since populations became isolated.

Tajima's D was positive across the contigs that contained mating-type loci (Fig. 4C and 4F), 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* with two approaches, namely comparing individual genes annotated by FunAnnotate, and 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 (pi), 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 nucleotide diversity (pi) 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* positions (Fig. S3A). *psiH* 1 formed a strongly supported clade with short branch lengths. *psiH2* appears paraphyletic in respect to*psiH1* , and *psiH3* appears to have originated from*psiH* 2. 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.

*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 17 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.

## Discussion

Psilocybin has breakthrough potential for treatment of mental health disorders, 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 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 (Giessler, 2018), and behave as weedy taxa, occurring in disturbed rather than natural areas (Borovička et al., 2012; Dennis & Wakefield, 1946).

*Psilocybe subaeruginosa* colonises wood chips and leaf litter, and mushrooms from one vicinity often fruit 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 kin 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, phylogenetic analyses, gene flow measured by $F_{ST}$, and mating compatibility. The alternative hypothesis of cryptic species diversity is rejected by evidence of gene flow among sexually compatible populations and shared mating type alleles. 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, 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 are 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, and this genetic diversity is expected in the centre of origin. Dabao Sun et al. (2023) found that differentiated lineages in a fungal taxon at a global scale had complicated species boundaries because sympatric inter-sterile populations could theoretically exchange genetic material by crossing through other

compatible populations. 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 (Geml, Tulloss, Laursen, Sazanova, & Taylor, 2008; James, Moncalvo, Li, & Vilgalys, 2001; Li, Han, Liu, Zhao, & Yang, 2020; M.-Z. 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 more opportunities for allopatric speciation than panmictic organisms that migrate. 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, Gryta, Jargeat, Mouhamadou, & Gardes, 2008; Mi et al., 2016; J. 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; in fungi with dominant asexual stages, isolation generates near clones, in which clonal reproduction is interspersed with infrequent sexual reproduction that maintains species cohesion (Taylor, Hann-Soden, Branco, Sylvain, & Ellison, 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 (Peris et al., 2022; van Diepen et al., 2013). 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 undersampled potentially shared alleles. Allopatric species boundaries may be interrupted given that humans move soils and their accompanying microorganisms, and as shown with magic mushrooms here, species connectivity through mate compatibility persists even in disparate populations with small evidence of 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 homologs 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 also in the *psiH3* position. 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. McTaggart et al. (2023) 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-HT2A has the highest affinity for psilocin and is linked to hallucinogenic effects of magic mushrooms (Glennon, Titeler, & McKenney, 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-HT2A (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, Shimizu, & Tokudome, 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* . Our findings put perspective on what

may be expected in terms of genetic diversity in the 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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**Author Contributions**

Alistair McTaggart, Kelly Scarlett, Caine Barlow, Chris Appleyard, Nigel Fechner, Jan Tilden, David Hass, Snu Voogelbreinder, William Lording, Rhys Lloyd and Louise Shuey contributed to resources; Alistair McTaggart and Timothy James contributed to investigation and analyses; Alistair McTaggart and Timothy James contributed to writing – original draft; Kelly Scarlett, Caine Barlow, Chris Appleyard, Donald Gardiner, Jason Slot, Nigel Fechner, Jan Tilden, David Hass, Snu Voogelbreinder, William Lording, Rhys Lloyd, Louise Shuey and André Drenth contributed to writing – review and editing; Chris Appleyard, Jason Slot, André Drenth and Timothy James contributed to mentoring and supervision.

**Data Accessibility**

Assembled genomes are available under their NCBI accession numbers listed in Table S1.

Fungal cultures are lodged in the Queensland Plant Pathology herbarium (BRIP).

**Tables**

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

**Figure captions**

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 Karen Keats). D. Ellendale, Tasmania. E. Victoria (image courtesy of Tannar Coolhaas). F. Tasmania (image courtesy of Karen Keats). G. kunanyi, Tasmania. H. Ellendale, Tasmania. I. Western Australia (image courtesy of Otto). J. Victoria (image courtesy of Tannar Coolhaas). K. Victoria. L. Bunya Mountains, Queensland.

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. Provenance 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. Figures B and C produced by R packages adegenet, vcfR, and ggplot2.

Fig. 3. 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. Concatenated*STE3* genes at the pheromone/receptor (PR) locus, with compatible (black) and incompatible (red) crosses among connected haplotypes. B. Relationships among concatenated *HD1* and *HD2* genes at the homeodomain (HD) locus. Alleles at mating compatibility loci are mostly private to the sampled populations, which indicates high allelic diversity in Australia.

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 (pi) 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 (pi) 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.

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 (pi) 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 an alignment 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. cyanescens, 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.

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).

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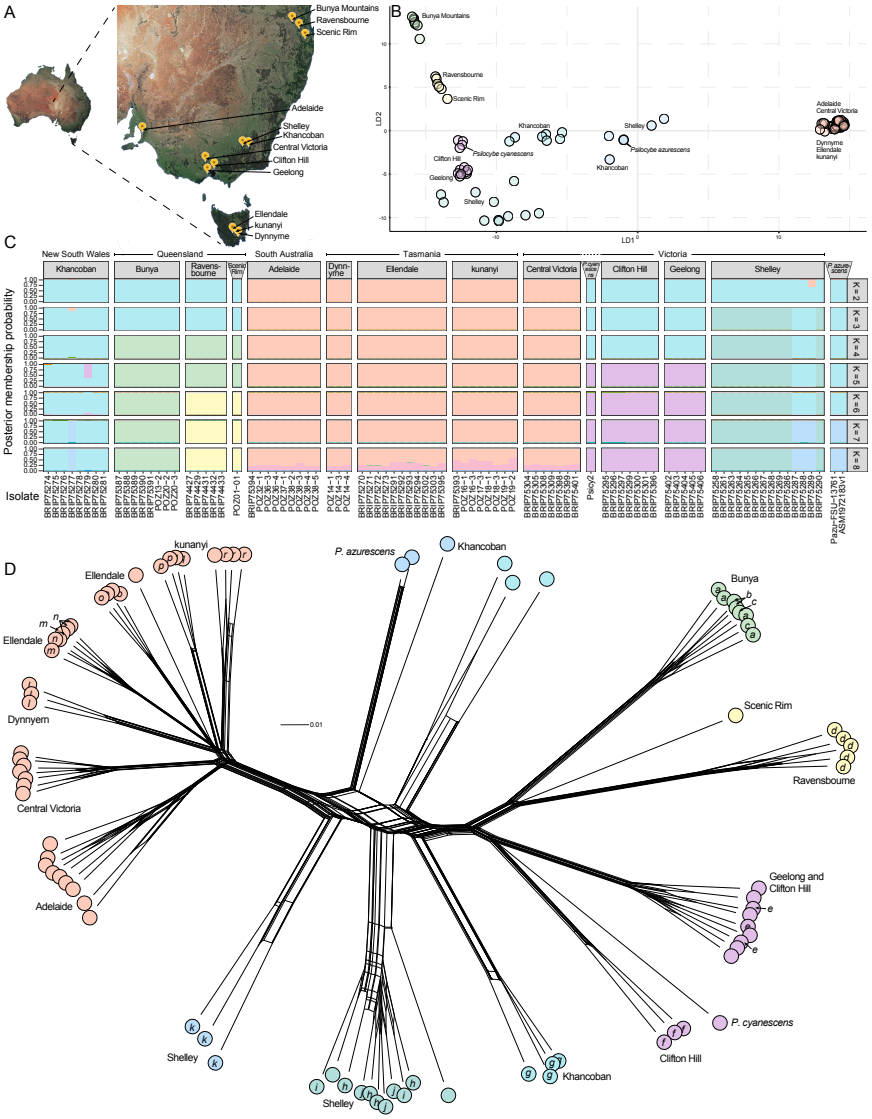

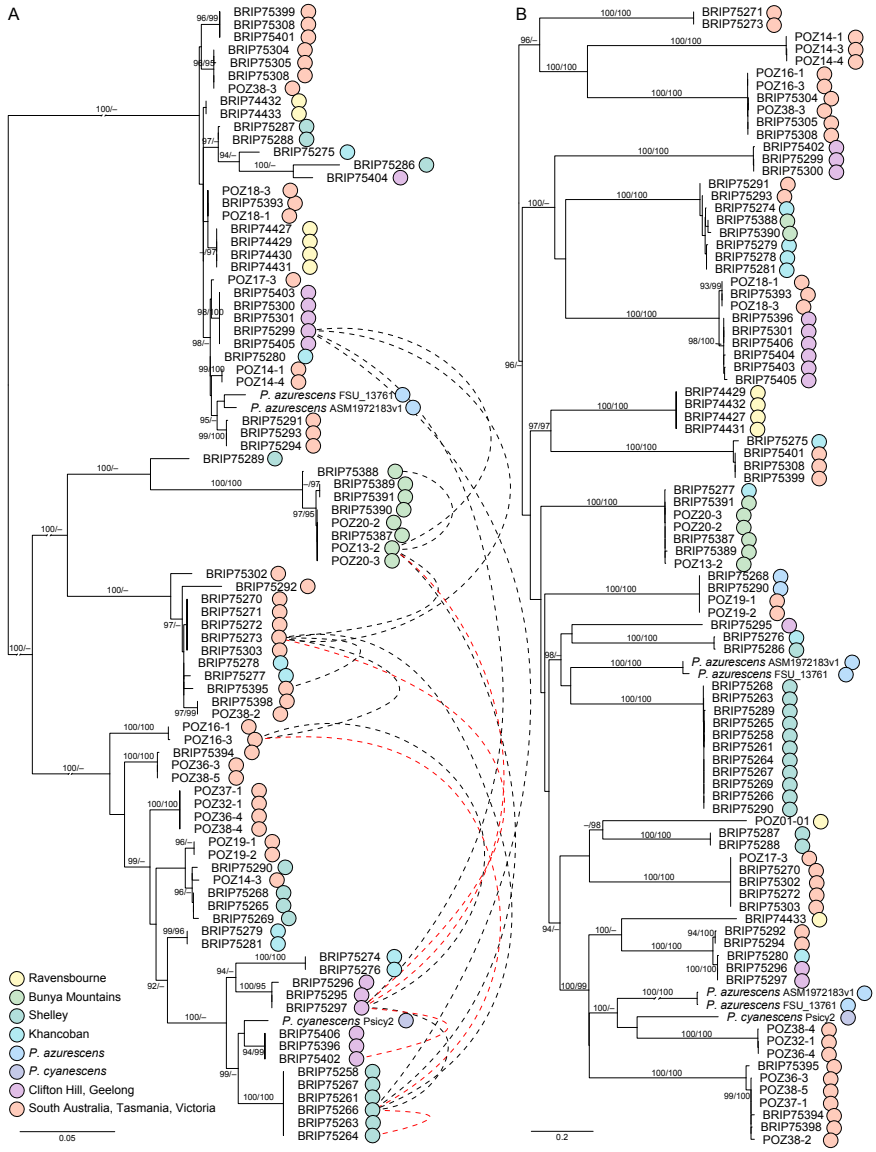

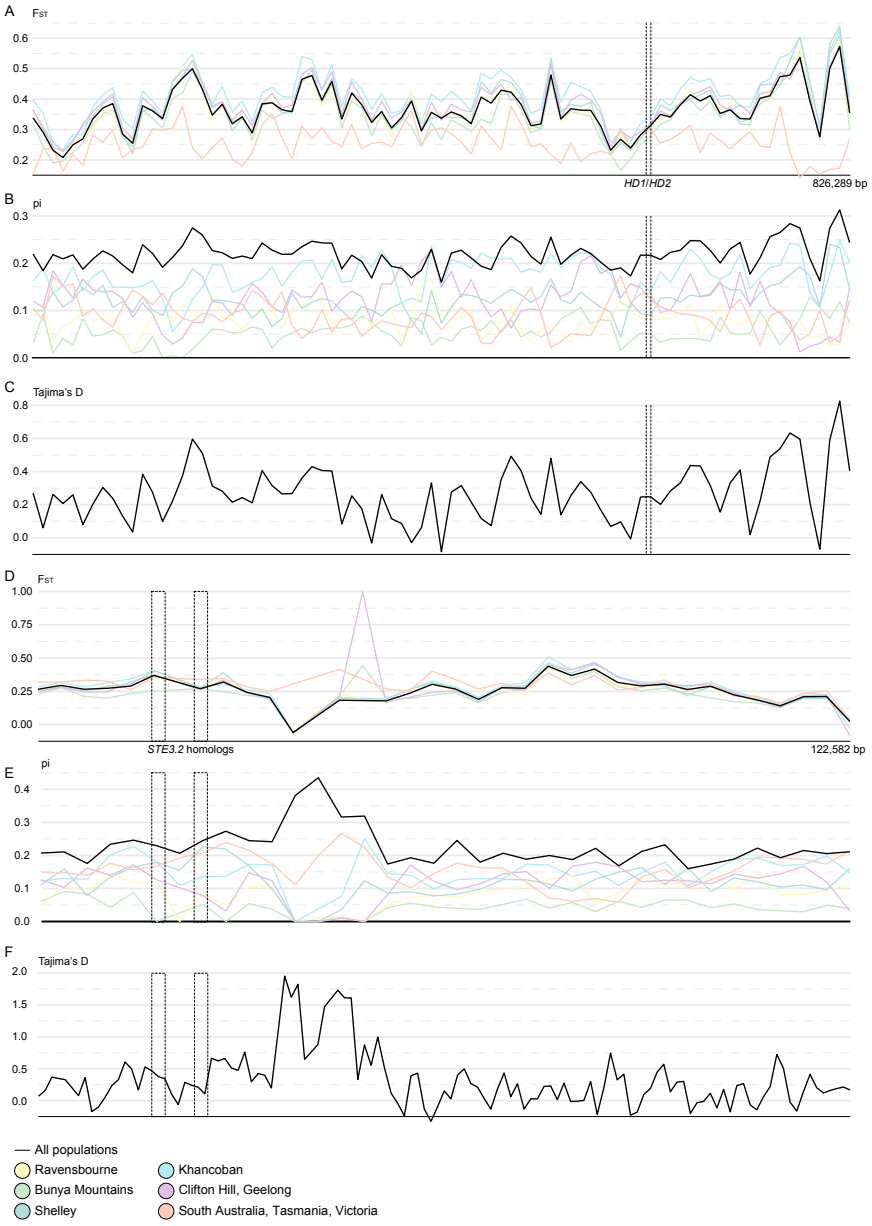

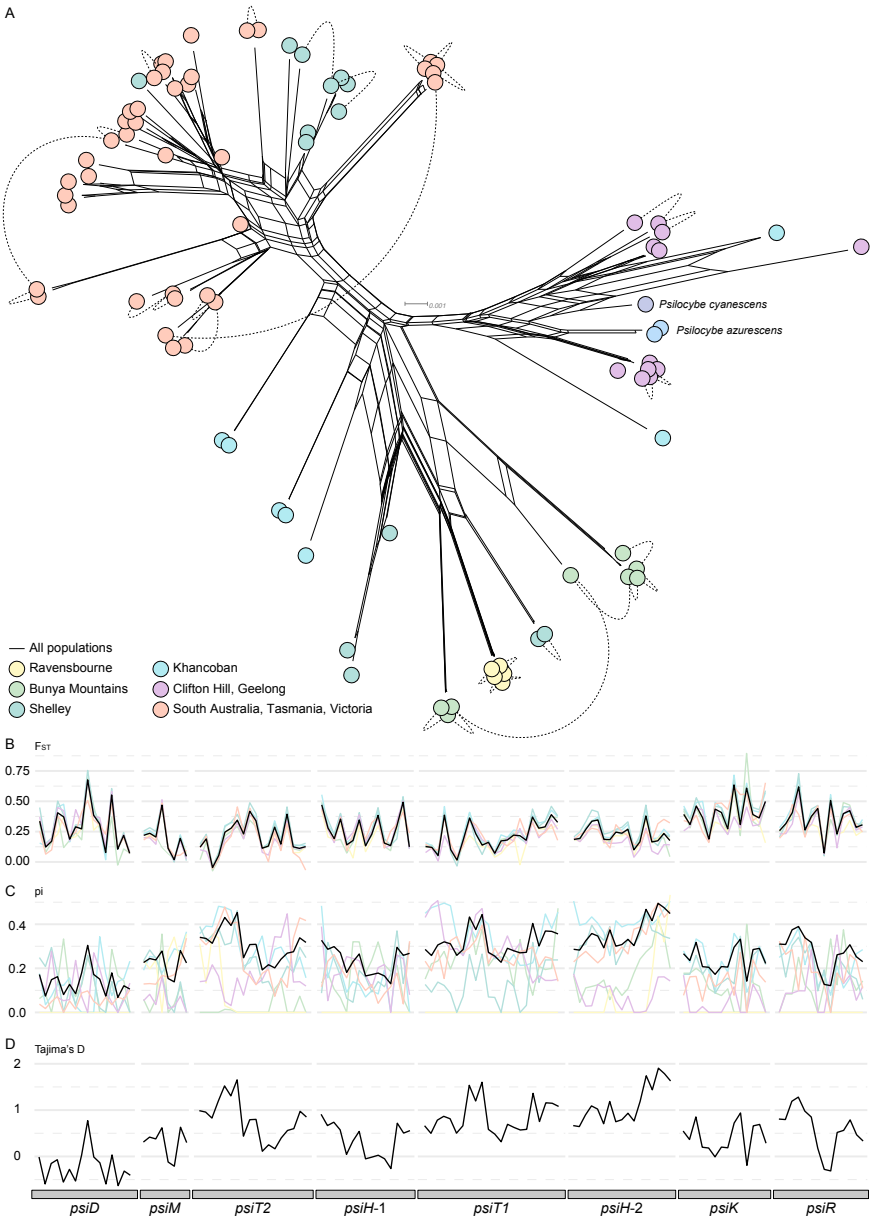

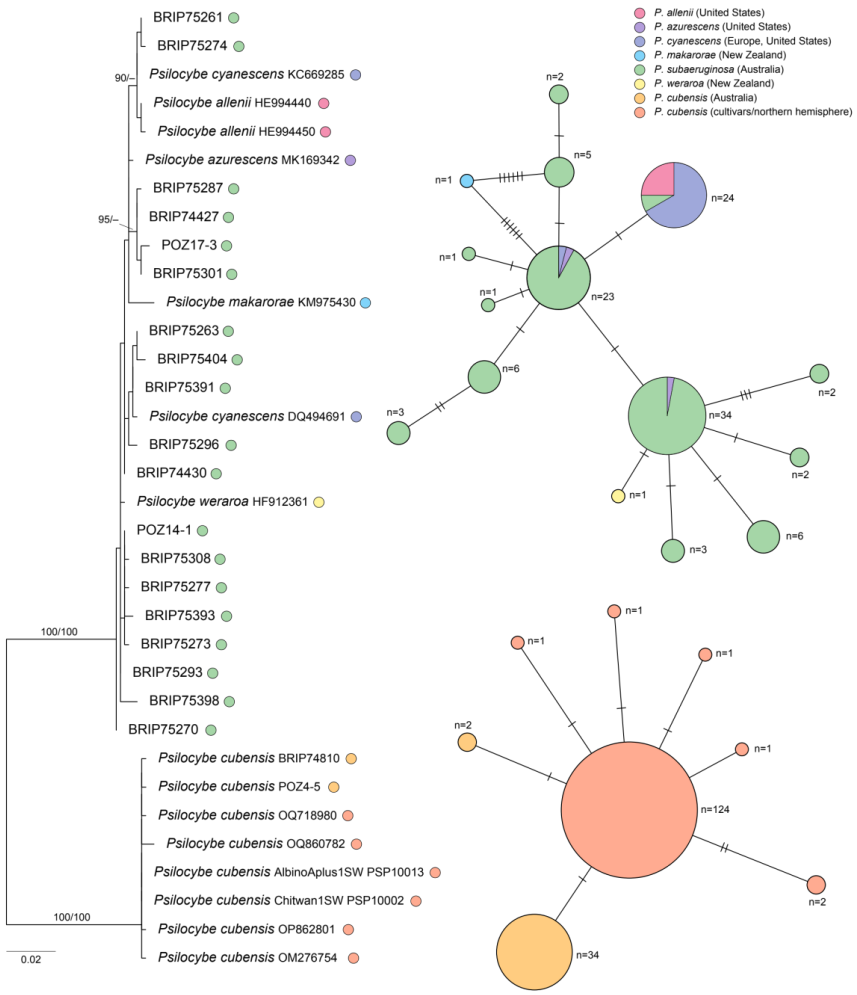

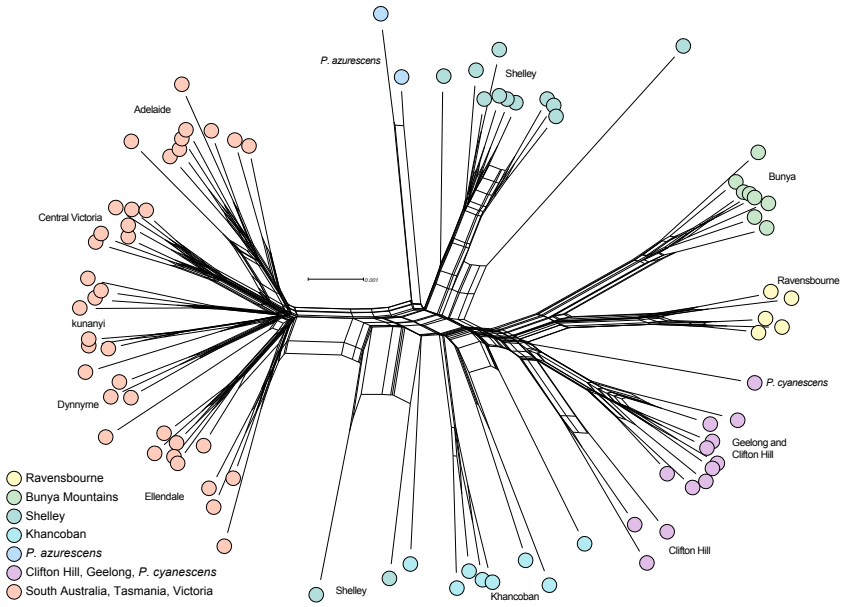

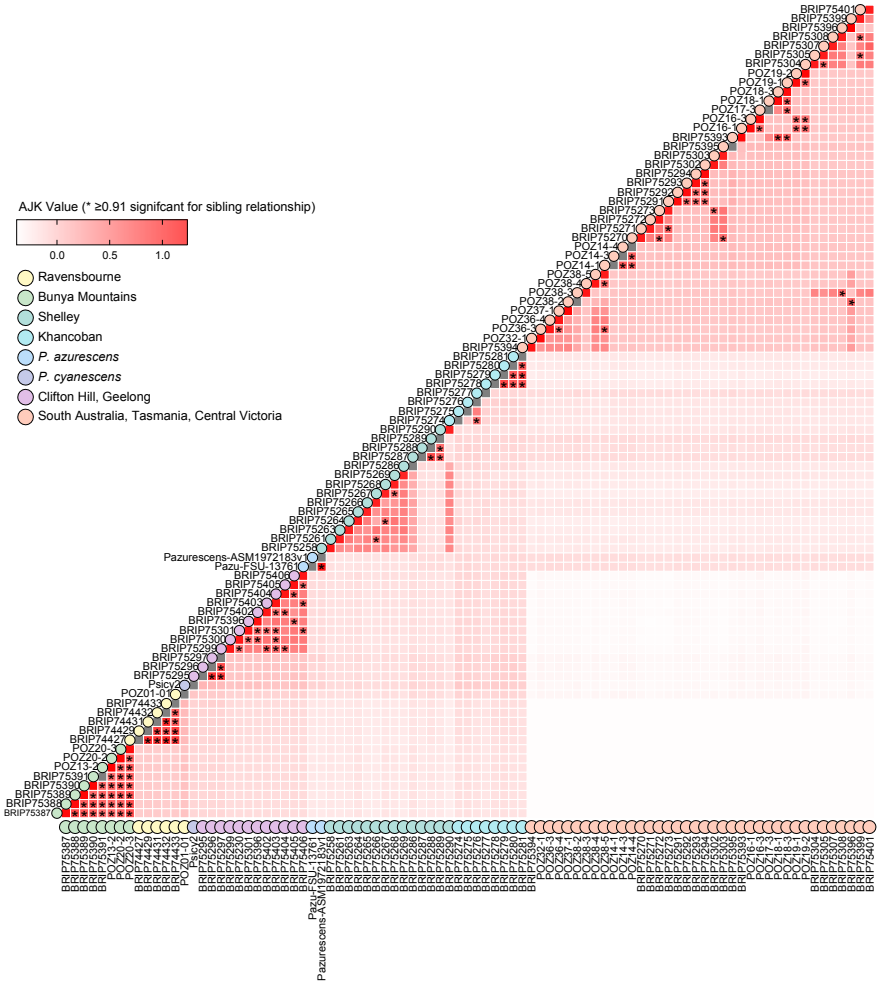

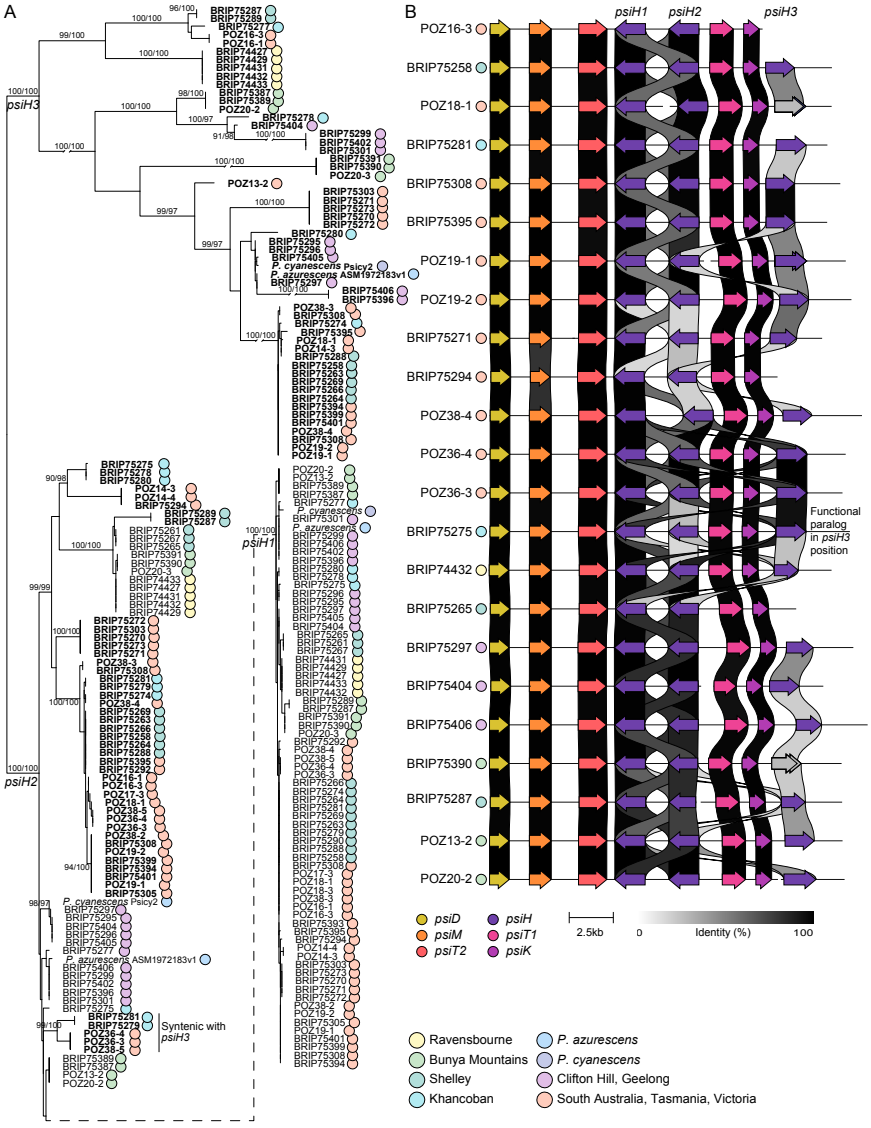

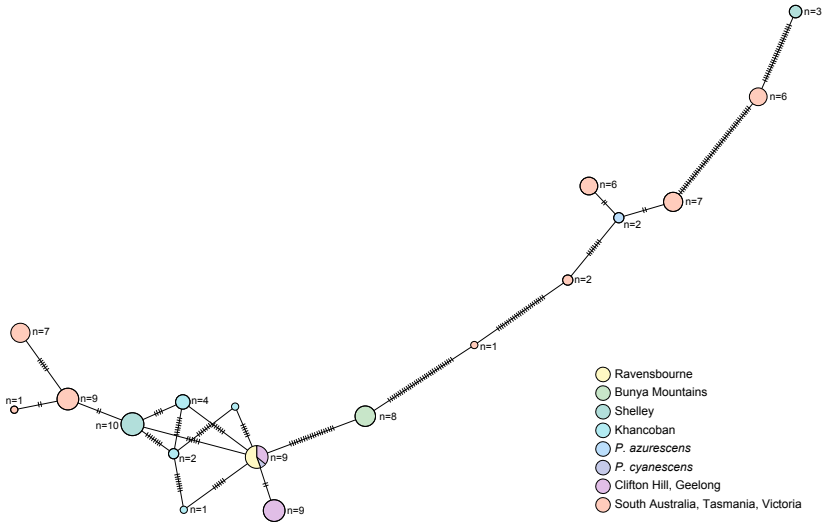


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