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Research

Equipped for success: genomes and metabolomes of the European *Amanita muscaria* are conserved in its novel South African range

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Summary

- Plants and soils have been moved around the world for centuries, but invasive mushrooms receive scant attention. The *Amanita muscaria* species complex was introduced to South Africa in the context of forestry, but its origins, ecology and recent evolution are unstudied.
- We sequenced the genomes of 24 Northern and Southern Hemisphere *A. muscaria*, built phylogenies and reconstructed its South African history. We identified the biosynthetic gene clusters (BGCs) encoding specialized metabolites (SMs). We subsequently extracted mushrooms' metabolites and used mass spectrometry data to group SMs into unique molecular families (MFs). We tested metabolites for bioactivity against diverse microbes and animals.
- We identify Europe as the origin of South African *A. muscaria*. A highly conserved group of BGCs is found in nearly all European and African genomes, and only 13 of 273 MFs are unique to South Africa. Metabolites extracted from all mushrooms kill nematodes, while microbes and flies appear unaffected.
- The nearly global distribution of the fly agaric results from multiple introductions of a single European clade to the Southern Hemisphere. Despite its long history in South Africa, the fun-

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gus has not lost any of its BGCs, suggesting a conservation of function(s) across multiple continents.

Introduction

Humans have moved plants and soils among continents for centuries, resulting in the co-introduction of fungal pathogens and symbionts (Anagnostakis, 1987; Dickie *et al.*, 2010; Cleary *et al.*, 2016). Fungi mediate basic ecological processes (Treseder & Lennon, 2015), and when introduced fungi become invasive, they can dramatically alter local ecosystems (Anagnostakis, 1987; Desprez-Loustau *et al.*, 2007; Rossman, 2009; Boyd *et al.*, 2013). We follow Policelli & Nuñez (2025) and define an invasive fungus as a non-native species able to 'disperse, survive, and reproduce in multiple locations across a range of habitats'. Interest in invasive nonpathogenic fungi is growing (Pringle *et al.*, 2009; Dickie *et al.*, 2010; Vargas *et al.*, 2019; Milani *et al.*, 2022), but most research continues to focus on the biology and impacts of introduced and invasive pathogens. As mutualist and decomposer fungi also spread across the globe (Vellinga *et al.*, 2009; Berch *et al.*, 2017; Vargas *et al.*, 2019; Pildain *et al.*, 2021; Veerabahu *et al.*, 2025), discovering the mechanisms enabling their successful invasions and documenting their impacts on native ecosystems emerge as key research priorities.

Among the most intensively studied nonpathogenic fungal invasions are co-invasions involving ectomycorrhizal (ECM) fungi and plant species of *Pinus* (family Pinaceae). Pines have been introduced across the Southern Hemisphere for commercial forestry, reforestation and afforestation. These non-native trees now cover more than 6 million hectares in countries, including South Africa, Argentina, Brazil, Chile and New Zealand (Rouget *et al.*, 2001; Simberloff *et al.*, 2010; Dickie *et al.*, 2014; Brancatelli *et al.*, 2020). Species like *Pinus contorta*, *P. elliottii*, *P. patula*, and *P. radiata* have spread beyond forestry plantations and into natural forest and grassland biomes, including into biodiversity hotspots, for example, South Africa's fynbos (Richardson & Higgins, 1998; van Wilgen & Richardson, 2012). Invading pines can reduce water availability, suppress native plant and fungal species, and disrupt ecosystem services(Le Maitre *et al.*, 2002; Dickie *et al.*, 2014; Nuñez *et al.*, 2017; Brewer *et al.*, 2018; Sapsford *et al.*, 2022).

Pines are obligately associated with ECM fungi, a symbiosis enabling the trees to obtain essential nutrients and water (Smith & Read, 2008; Nuñez *et al.*, 2009; Dickie *et al.*, 2010). Historically, plantings of pines in the Southern Hemisphere failed until soils were imported to supply their symbionts (Mikola, 1970), and introduced ECM fungi have subsequently facilitated invasions of pine trees (Hayward *et al.*, 2015; Policelli *et al.*, 2019, 2023). Despite the critical role of ECM fungi in pine invasions and the potential for ECM fungi to invade and harm native forests on their own (Dickie *et al.*, 2017), the ecological and evolutionary dynamics of invading ECM fungi remain poorly understood, perhaps because invading mutualists are perceived as unequivocally beneficial (but see Schwartz *et al.*, 2006). In fact, ECM fungi may function across a spectrum of mutualism and parasitism (Karst *et al.*, 2008). Efforts to elucidate the ecology of ECM fungi are further exacerbated by their ephemeral and often cryptic biology (Wang *et al.*, 2023), leaving us with incomplete, often inaccurate historical records of species' native ranges (Pringle & Vellinga, 2006). Strategies combining historical research with modern 'omics methods offer a novel approach to identify the drivers of ECM fungal invasions.

Invasion biology often invokes interactions among species as a mechanism driving spread, and fungal interactions are often hypothesized to be mediated by specialized metabolites (SMs; Drott *et al.*, 2017; Keller, 2019; Tannous *et al.*, 2023). Although ECM fungi produce a plethora of SMs (Schüffler, 2018; Walton, 2018; Obermaier & Müller, 2020; Mudbhari *et al.*, 2024) and must routinely interact with pathogens, predators and competitors, interactions between introduced ECM fungi and local organisms are rarely considered. However, the toxins of at least one invasive ECM fungus, *Amanita phalloides*, appear to be evolving dynamically in its invasive range (Drott *et al.*, 2023). This finding and the ubiquity of fungal SMs raise questions about the translation of the 'enemy release' and 'novel weapons' hypotheses, often invoked to explain the success of non-native plants and animals, to invasive fungi (Blossey & Nötzold, 1995; Torchin *et al.*, 2003; Callaway & Ridenour, 2004; Liu & Stiling, 2006). The 'enemy release' hypothesis proposes introduced species succeed by escaping natural enemies, while the 'novel weapons' hypothesis suggests they spread by producing compounds against which native species have no resistance (Gillett, 1962; Callaway & Ridenour, 2004).

The ecological roles and evolution of SMs remain poorly understood, even in iconic ECM species like *Amanita muscaria sensu lato* (s.l.; Geml *et al.*, 2006), the famous red-and-white-spotted 'fly agaric' known for its psychoactive properties (Camazine, 1983; Michelot & Melendez-Howell, 2003) and for the folklore suggesting it can repel or kill flies (Lumpert & Kreft, 2016). *A. muscaria* produces ibotenic acid and muscimol using a single biosynthetic pathway (Størmer *et al.*, 2004; Su *et al.*, 2023), and muscimol is a potent $GABA_A$ receptor agonist (Johnston, 2014), a feature which contributes to its distinctive biological effects. Native to nearly all temperate and boreal biomes of the Northern Hemisphere, the *A. muscaria* species complex includes multiple cryptic lineages associated with diverse plants, including pines. While some of these cryptic species are now formally recognized (e.g. *Amanita persicina*; Tulloss *et al.*, 2015), the true number of cryptic species within the *A. muscaria* species complex remains unknown. For simplicity, we refer to all as-yet unnamed lineages as *A. muscaria*. Introduced to South America, Australia and Africa, *A. muscaria* forms novel ECM partnerships with native trees in Columbia (*Quercus*

humboldtii; (Vargas *et al.*, 2019), Chile (*Nothofagus*) (Márquez Parraguez, 2024); and Australia (*Nothofagus* and *Allocasuarina*) (Fuhrer, 1992; Dunk *et al.*, 2012; Lebel *et al.*, 2024)). Relatively little is known about its introductions across Africa (Vellinga *et al.*, 2009), but its South African history is particularly well documented (Box 1), offering a rare opportunity to study the impact of *A. muscaria*'s introduction on the fungus's evolution and production of SMs.

We sequenced the genomes of 24 *A. muscaria* s.l. mushrooms (9 South African, 11 European, 2 Australian and 2 Californian) and 1 outgroup (*Amanita pantherina*) to address three hypotheses: First, South African *A. muscaria* would be most closely related to a European lineage of the fungus. Our hypothesis is based on the history of *A. muscaria* in South Africa (Box 1). Second, a release from antagonistic interactions found in Europe but absent in South Africa would relax selection on SMs, enabling the fungus to invest more energy into growth and reproduction and potentially causing the loss of SM genes or gene function. Third, adaptation in the invasive range would result in shifts in metabolic profiles and impact the bioactivity of extracts against laboratory model bacteria, fungi, nematodes, mosquitoes and flies.

Box 1. The history of *Amanita muscaria* in South Africa

Doidge (1950) lists a number of early collections of the fungus; by 1874, *A. muscaria* was growing with pines in and around Cape Town (Fig. 1). By 1945, it was growing in what are now named as the provinces of Mpumulanga to the north and Free State in the east. She identifies *A. muscaria* as growing with 'shrubs', pine trees and 'near living roots of *Quercus* sp.' (Doidge, 1950 p. 553).

The history of *A. muscaria* is linked to the history of Europeans on the continent. Early European colonists did not find the timber species native to South Africa suitable for their uses, and they introduced fast-growing pines. Any plant moved to South Africa by European colonists would have been accompanied by fungi in its tissues, and plants may also have been moved as seedlings growing in soil, enabling the introduction of soil fungi. Doidge (1950) succinctly describes the dynamic of plants arriving to South Africa with Europeans:

> 'The history of plant introduction into South Africa begins in 1651, with the arrival of Johan van Riebeeck to establish a settlement at the Cape; van Riebeeck was accompanied by a gardener… To provide timber, sacks of acorns were sent from Holland and kernels of stone and cluster pines from Italy; these were planted in quantity and flourished amazingly… the majority being species of Pinus, Eucalypts and poplars…' Doidge (1950, pp. 15–16)

Mikola (1970) goes on to describe the subsequent deliberate export of soils from South Africa to enable plantation forestry elsewhere, including in Zimbabwe and Kenya (Mikola, 1970; Zimbabwe discussed as Rhodesia). For example, three tons of soil from the Transvaal were imported to establish pine plantations in Eswatini (formerly Swaziland), and apparently *A. muscaria* was moved as well, 'The commonest mushrooms in Usutu Forest of Swaziland [now Eswatini] in March 1967 were… *Laccaria laccata*, *Amanita muscaria*, *Boletus edulis*, *Rhizopogon roseolus*'. (Mikola, 1970).

The fungus is acknowledged as a European introduction in the most recent scientific literature on the *Amanita* of South Africa (Reid & Eicker, 1991) and in a current field guide (Goldman & Gryzenhout, 2019). While *A. muscaria* may have been introduced with European 'stone and cluster pines' (Doidge, 1950), in modern South African forestry plantations, it associates with pines from other continents, for example Mexican *Pinus patula* and Californian *P. radiata*.

Materials and Methods

Historical records of South African *Amanita muscaria*

Historical records of *Amanita muscaria* s.l. from South Africa (from before 1945) were obtained from (Doidge, 1950). To compare the historical records with the current distribution of the fungus, we downloaded all publicly available South African records from the Global Biodiversity Information Facility (GBIF) on 24 July 2023 (GBIF, 2023). We looked at the photographs linked to each record to identify surrounding tree species. Because every record suggested an association of *A. muscaria* with *Pinus* spp., we extracted information about plantation forestry land cover within South Africa from the 2022 South African National Land Cover dataset (Department of Forestry, Fisheries and Environment). We mapped the historical and current records of *A. muscaria*. We note that historical records often use older place names for both the country and its provinces, for example Transvaal for Gauteng or Mpumalanga.

Mushroom collections

We sequenced genomes from a total of 24 *A. muscaria* and 1 *Amanita pantherina* mushrooms collected from South Africa, Australia, Europe and North America (Table 1). South African (n = 9) and Australian (n = 2) specimens were collected from pine plantations while European *A. muscaria* (n = 11), *A. pantherina* (n = 1) and Californian (n = 2) mushrooms were collected from native forests (Table 1; Supporting Information Fig. S1). Specimens were dried within 1–2 d using conventional food driers at low heat (less than 35°C). Our sampling scheme was designed to contextualize South African *A. muscaria* by integrating new data within published phylogenies, primarily Geml *et al.* (2008); a comprehensive survey of the global species complex was beyond our scope. We targeted as many South African specimens as possible, European specimens from three countries (based on what we found and could collect), and other specimens from additional clades identified by Geml *et al.* (2008), for example Clade I, represented by our two Californian mushrooms. Out of curiosity, we also sequenced two Australian specimens. Before sequencing, all specimens were stored as dried mushrooms at room temperature in either the Pringle or Vilgalys Laboratory fungaria at the University of Wisconsin–Madison and Duke University, respectively.

Genome extraction, sequencing, assembly, annotation and quality control

DNA extraction and sequencing High molecular weight DNA (HMW-DNA) was extracted from samples of dried mushroom

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Table 1 Collection metadata for collected mushrooms.

Specimen ID	Collection date	Location	Region	Country	Latitude	Longitude	Species	Habitat	Host	Collector
11662	27 February 2019	Bulwer	KwaZulu-Natal	South Africa	29.857681 S	29.710906 E	*A. muscaria*	Plantation	*Pinus patula*	J. Roux
11663	27 February 2019	Bulwer	KwaZulu-Natal	South Africa	29.857681 S	29.710906 E	*A. muscaria*	Plantation	*Pinus patula*	J. Roux
11664	27 February 2019	Bulwer	KwaZulu-Natal	South Africa	29.857681 S	29.710906 E	*A. muscaria*	Plantation	*Pinus patula*	J. Roux
11665	6 March 2019	Bulwer	KwaZulu-Natal	South Africa	29.857681 S	29.710906 E	*A. muscaria*	Plantation	*Pinus patula*	J. Roux
11666	25 April 2019	Kranskop / Greytown	KwaZulu-Natal	South Africa	N/A	N/A	*A. muscaria*	Plantation	*Pinus elliottii*	I. Greyling
11667	25 April 2019	Kranskop / Greytown	KwaZulu-Natal	South Africa	N/A	N/A	*A. muscaria*	Plantation	*Pinus elliottii*	I. Greyling
11668	25 April 2019	Kranskop / Greytown	KwaZulu-Natal	South Africa	N/A	N/A	*A. muscaria*	Plantation	*Pinus elliottii*	I. Greyling
11669	25 April 2019	Kranskop / Greytown	KwaZulu-Natal	South Africa	N/A	N/A	*A. muscaria*	Plantation	*Pinus elliottii*	I. Greyling
11670	25 April 2019	Kranskop / Greytown	KwaZulu-Natal	South Africa	N/A	N/A	*A. muscaria*	Plantation	*Pinus elliottii*	I. Greyling
Roed_3	12 September 2019	Østfold	Østlandet	Norway	59.4237 N	10.5937 E	*A. muscaria*	Native habitat	Not recorded	S.L. Harrow
Sogn_5	15 September 2019	Oslo	Østlandet	Norway	59.9822 N	10.7415 E	*A. muscaria*	Native habitat	Not recorded	S.L. Harrow
Skrap_3	17 September 2019	Oslo	Østlandet	Norway	59.8657 N	10.8562 E	*A. muscaria*	Native habitat	Not recorded	S.L. Harrow
Nes_1	17 September 2019	Akershus	Østlandet	Norway	59.8666 N	10.5181 E	*A. muscaria*	Native habitat	Not recorded	S.L. Harrow
Frag_1	18 September 2019	Oslo	Østlandet	Norway	59.9820 N	10.6742 E	*A. muscaria*	Native habitat	Not recorded	S.L. Harrow
Ring_1	6 October 2019	Zurich	Zurich	Switzerland	47.3617 N	8.4853 E	*A. muscaria*	Native habitat	Not recorded	S.L. Harrow
Wirz_3	7 October 2019	Nidwalden	Nidwalden	Switzerland	46.9053 N	8.3696 E	*A. muscaria*	Native habitat	Not recorded	S.L. Harrow
Gril_1	7 October 2019	Nidwalden	Nidwalden	Switzerland	46.9010 N	8.3571 E	*A. muscaria*	Native habitat	Not recorded	S.L. Harrow
Kara_3	7 October 2019	Zurich	Zurich	Switzerland	47.3617 N	8.4853 E	*A. muscaria*	Native habitat	Not recorded	K. O'Keefe
Nagy_Heves_A	12 October 2013	Heves	Heves	Hungary	47.317313 N	19.941566 E	*A. muscaria*	Native habitat	*Picea abies or Betula pendula*	L. Nagy
Nagy_Heves_B	12 October 2013	Heves	Heves	Hungary	47.317313 N	19.941566 E	*A. muscaria*	Native habitat	*Picea abies or Fagus sylvatica*	L. Nagy
20031	17 December 2021	Point Reyes National Seashore	California	United States	38.0519 N	122.8310 W	*A. muscaria*	Native habitat	Not recorded	Wang/Pringle
20045	19 December 2021	Point Reyes National Seashore	California	United States	37.9702 N	122.7304 W	*A. muscaria*	Native habitat	Not recorded	Wang/Pringle
NzAUS95	27 May 2019	Railton	Tasmania	Australia	41.375431 S	146.385042 E	*A. muscaria*	Plantation	*Pinus radiata*	Vilgalys/ Henderson/ Uehling
Aus332	31 Mar 2018	Penrose	NSW	Australia	34.630017 S	150.211625 E	*A. muscaria*	Plantation	*Pinus radiata*	Vilgalys/ Henderson/ Uehling
Nes_pan3	17 September 2019	Akershus	Østlandet	Norway	59.8666 N	10.5181 E	*A. pantherina*	Native habitat	Not recorded	S.L. Harrow

tissues using previously published protocols involving a solution of 25 : 24 : 1 phenol : chloroform : isoamyl alcohol (Bok *et al.*, 2005; Nickles *et al.*, 2023). Sequencing was performed with an Illumina NovaSeq 6000 platform with the aim of obtaining 6 million reads per sample to achieve a coverage of 30× or higher. We estimated the genome size as 44 Mb.

Genome assembly, annotation and quality analyses Raw sequencing data were trimmed with TRIMMOMATIC v.0.36 (Bolger *et al.*, 2014) with LEADING:20 TRAILING:20 SLIDINGWINDOW:4:25 MINLEN:50, and genomes were assembled with SPADES v.3.11.1 (Bankevich *et al.*, 2012; Prjibelski *et al.*, 2020) using default parameters. Assemblies were filtered to keep only the contigs/scaffolds larger than 500 bp. Assembled genomes were annotated with AUGUSTUS v.3.4.0 using models prebuilt for *Laccaria bicolor* (Stanke *et al.*, 2008; Hoff & Stanke, 2013). Genome statistics of each genome were generated using seqtk (Li, 2012) and Benchmarking Universal Single-Copy Orthologs (BUSCO; Simao *et al.*, 2015) completeness was determined using BUSCO v.3.0.2 with the agaricales_odb10 database (Manni *et al.*, 2021).

Before examining the assembled genomes, basic assembly metrics were determined using seqtk. To screen for potential fungal contamination, duplicate BUSCO genes were identified with BUSCO (v.3.0.2) run in genome mode using the Fungi Odb10 database with default settings. Only one genome showed a single duplicated BUSCO gene, suggesting all genomes were free from fungal contaminants. The first 200 nucleotides of each contig from each fully assembled genome were then used to query NCBI's prokaryote nucleotide database (accessed on 27 July 2023) in a local BLASTn search with default settings using the NCBI's BLAST+ software package (v.2.6.0; Altschul *et al.*, 1990; McGinnis & Madden, 2004). Contigs with matches showing an e-value less than 1e-5 were considered contaminated and removed from each genome. Genome quality metrics were then recomputed with seqtk. The decontaminated genomes were used for all subsequent analyses (Dataset S1).

Generating and filtering SNP predictions Short reads were quality controlled with BBMAP v.38.32 (Bushnell, 2015) and aligned to the NCBI reference genome (accession GCA_001691765.1) using BWA MEM v.0.7.17 (Li & Durbin, 2009). Variants were called using GENOME ANALYSIS TOOLKIT v.4.0.12.0 (McKenna *et al.*, 2010) using parameters and hard filters identical to Drott *et al.* (2023). Single-nucleotide polymorphism (SNP) data were filtered using VCFTOOLS (v.0.1.13; Danecek *et al.*, 2011), such that only biallelic sites were retained. Split decomposition was employed to reconstruct the phylogenetic tree using the filtered SNP dataset to identify potential errors or artifacts in the dataset (Methods S1).

Clone correction and population genetics

Clone correction To test whether different specimens are clones of each other, that is mushrooms generated from a single mycelium, or are distinct genetic individuals, we estimated pairwise kinships using KING-robust (Manichaikul *et al.*, 2010) using the function snpgdsIBDKING implemented in the R library SNPRelate.

Genetic differentiation and estimation of gene flow Pairwise *F*-statistics (F_{ST}) and Euclidian distances (*D*) between individuals as well as the identification of genetic variants and private alleles between and within population were calculated or undertaken using the dartR package in R (Gruber *et al.*, 2018). Tajima's *D* was estimated in 5000-bp sliding windows using VCFTOOLS (v.0.1.13; Danecek *et al.*, 2011) with a subset of Clade II genomes from Europe and South Africa. Nucleotide diversity (π) was calculated across the entire genome using pixy (Korunes & Samuk, 2021) and heterozygosity was calculated across all variant sites using VCFTOOLS.

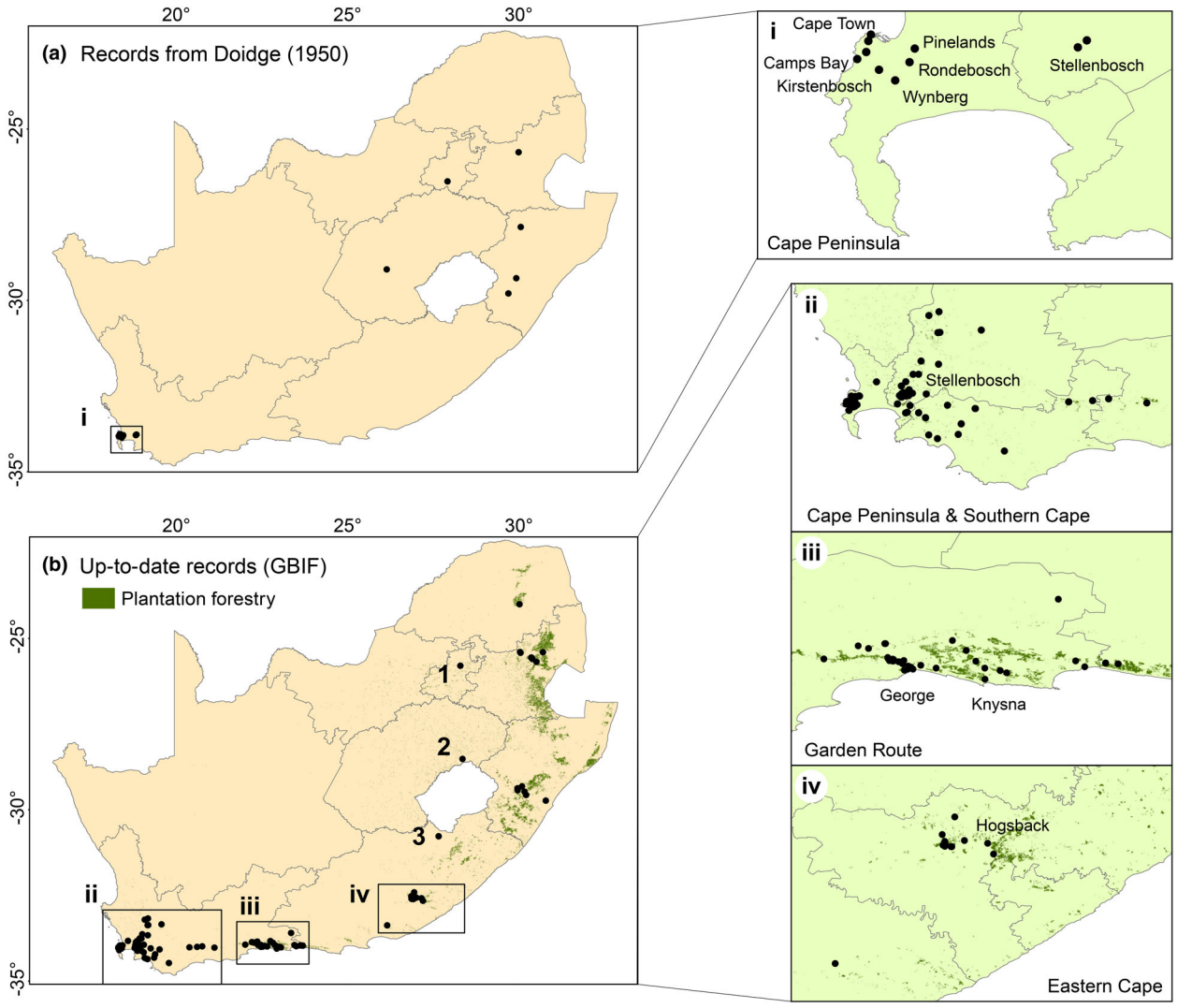
Phylogenomic analyses

Nomenclature of the *A. muscaria* species complex and closely related species and downloading of publicly available *A. muscaria* gene markers We contextualized newly sequenced genomes using sequences of the internal transcribed spacer (ITS), □-tubulin (β-tub), nuclear large ribosomal subunit (LSU) and translation elongation factor 1-alpha (TEF-1α) genes from three key publications (Oda *et al.*, 2004; Geml *et al.*, 2008; Vargas *et al.*, 2019; Dataset S2). Specimen ID codes from Oda *et al.* (2004) were shortened to only include the numeric ID. The taxonomy and nomenclature of *A. muscaria* s.l. are complex, and we used published literature to develop naming protocols.

Following recent precedent (Vargas *et al.*, 2019), we used Geml *et al.*'s (2008) clade labels to identify our specimens. While taxonomists used to consider *A. muscaria* as a complex of subspecific varieties existing under a single species concept and identifiable on the basis of cap color (Miller & Jenkins, 1978), when specimens with different cap colors were integrated into single phylogenies using DNA sequence data, new species concepts emerged (Oda *et al.*, 2004; Geml *et al.*, 2006). Geml *et al.* (2008) represent the last major phylogenetic revision of the taxa, but instead of naming clades as species, Geml *et al.* (2008) chose to label distinct clades with roman numerals. We use the same numbering system for our specimens and for the specimens associated with already published data (Oda *et al.*, 2004; Geml *et al.*, 2008; Vargas *et al.*, 2019), with two exceptions: after Geml *et al.* (2008) was published, *A. persicina* was formally recognized as a species, and we use its name (Tulloss *et al.*, 2015). We also use the name *A. regalis* because it was formally published in a previous revision of the species complex (Neville & Poumarat, 2004) and is supported by the later phylogenetic evidence (Geml *et al.*, 2008).

We used the same logic to identify specimens associated with other public data. Any specimen originally classified as and submitted to GenBank as one of the widely recognized and currently accepted infraspecific taxa of *A. muscaria* was given the working designation *A. muscaria* s.l. and later identified using Geml *et al.*'s (2008) clade labels. For our purposes, *A. muscaria* s.l. includes *A. muscaria* var. *guessowii*, *A. muscaria* var. *muscaria* and

A. muscaria var. *flavivolvata*. However, if a specimen had a GenBank accession for ITS locus sequence data and was submitted as *Amanita regalis* (or *A. muscaria* var. *regalis*) or *Amanita persicina* (or *A. muscaria* var. *persicina*), we used the modern nomenclature (Tulloss & Yang, 2010), in other words the names *A. regalis* or *A. persicina*. These two taxa are easily differentiated from



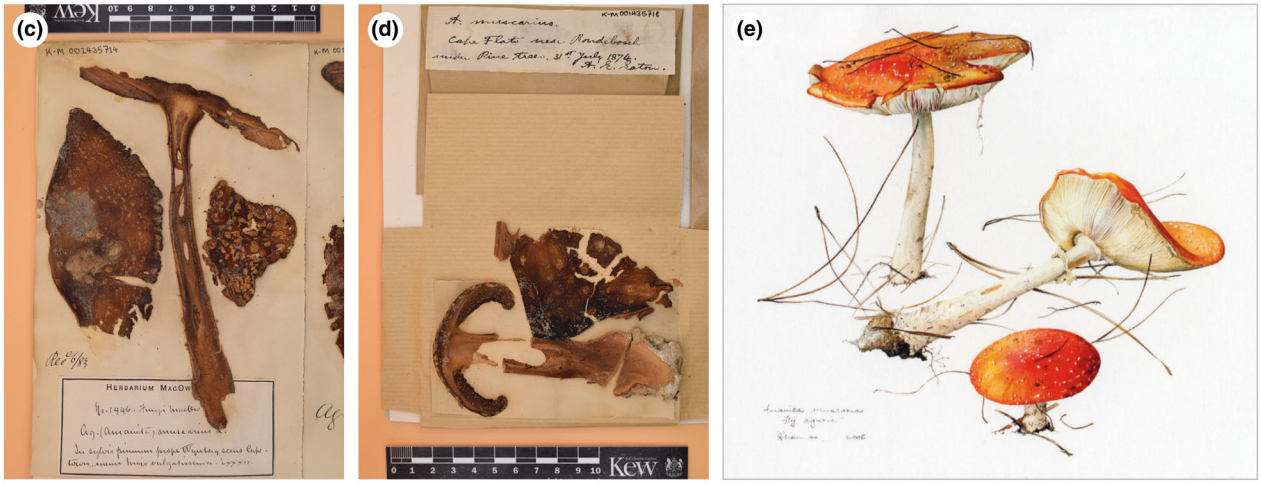


Fig. 1 Records of *Amanita muscaria* in South Africa. Occurrence data illustrate the spread of *A. muscaria* in South Africa over the last century. Panel (a) plots historical records up until 1945 as compiled by Doidge (1950). Insert (i) is an enlargement of the Cape region. Panel (b) plots contemporary records as of 24 July 2023, also illustrating plantation forestry land use. Inserts are enlargements of (ii) Cape Town and the southern Cape, (iii) the Garden Route and (iv) the Eastern Cape. Numbered occurrences (1–3) are not associated with plantation forestry and instead record *A. muscaria* associated with pine trees in urban areas as in (1) Pretoria or (2) Clarens, or in rural areas as in (3), where pines are often used as windbreaks or to adorn the edges of farm roads. Panels (c) and (d) are images of *A. muscaria* specimens from Wynberg and Rondebosch in Cape Town collected in 1883 and 1874, respectively. Both records provide valuable information about associations with plants, for example 'under Pine trees' in panel (d). Panel (e) is an image from a card bought at Kirstenbosch Botanical Gardens in Cape Town in 2023, reflecting widespread awareness of the fungus in South Africa (image copyright Vicki Thomas (Aristea Publications) and used with her kind permission).

A. muscaria s.l. on the basis of gross morphology. Specimens classified as *A. pantherina* were kept as such. For the two specimens lacking an ITS accession, the metadata associated with the LSU accession was used instead. All collection location data were extracted from the publication corresponding to each given collection.

Species tree construction using multi-loci barcodes We aligned ITS (n = 189), β-tub (n = 102), LSU (n = 98) and TEF-1α (n = 52) sequences with MAFFT v.7.511 (Katoh & Standley, 2013) with an automated model selection (L-INS-i, FFT-NS-i and FFT-NS-2) and adjustment of reverse complements. Alignments were trimmed with TRIMAL v.1.4.rev15 (Capella-Gutierrez *et al.*, 2009) with the gappyout parameter. Once trimmed, alignments for all four regions were used to construct a maximum likelihood phylogeny using an edge-linked partition model with IQ-TREE multicore v.2.2.0 (Minh *et al.*, 2020), with the optimal substitution model determined by MODELFINDER (Kalyaanamoorthy *et al.*, 2017) and 1000 rapid bootstraps.

Specialized metabolite biosynthetic gene cluster prediction and characterization

Genome mining for specialized metabolite BGCs To identify canonical SM biosynthetic gene clusters (BGCs; i.e. those with experimentally vetted class-defining core biosynthetic genes (termed backbone genes), e.g. polyketide (PK) BGCs, which are a canonical class of SM defined by polyketide synthase (PKS) backbone genes). Fungal ANTISMASH (v.5; Blin *et al.*, 2019) was run on all of the genomes using the default settings. However, specimen 11 662 was erroneously omitted from genome mining analyses. Closely related terpene or PKS BGCs were networked into gene cluster families (GCFs) using BIG-SCAPE v.1.0.1 (Navarro-Munoz *et al.*, 2020). An optimal networking cutoff of 0.3 was determined across a testing range of 0.1 to 0.6 (Figs S2, S3).

We ran CBLASTER (v.1.3.18; Gilchrist *et al.*, 2021) using a previously characterized ibotenic acid BGC sequence as the query to identify the noncanonical ibotenic acid BGC (Obermaier & Müller, 2020), which is made up of the genes (from left to right) *iboA*, *iboF*, *iboD*, *iboC*, *iboG1*, *iboH* and *iboG2*. We further investigated the distribution of genes in the ibotenic acid BGC using reciprocal best-hit blast analysis. Briefly, characterized protein sequences were used to replace corresponding protein predictions of IboA, IboC, IboD, IboF, IboG1, IboG2 and IboH (Kohler *et al.*, 2015) in the file containing the proteome of mushroom Gril_1. The resulting proteome was queried against a dataset of all annotated publicly available protein sequences downloaded from NCBI in the spring of 2023 with methods similar to those described previously (Drott *et al.*, 2020). Any two hits that were within 30 kb of each other were considered physically clustered. The presence of a putative ortholog was mapped to a modified version of the whole-kingdom phylogeny from Nickles *et al.* (2023) using GGTREE (Yu *et al.*, 2017).

We searched for the presence of amatoxin-encoding 'MSDIN' genes and the toxin-processing *popB* gene using methods and publicly available scripts from (Drott *et al.*, 2023), as detailed in Methods S1.

Phylogeny of Agaricomycete polyketide synthase proteins A representative query protein was selected from the two conserved PKSs found within the *A. muscaria* genomes. Each genome's protein was BLASTP (Altschul *et al.*, 1990; Schaffer *et al.*, 2001) searched against every annotated Agaricomycetes genome publicly available on NCBI as of 1 December 2022 (n = 292 genomes) with an e-value cutoff of $1e^{-5}$. Only the top hit for each BLAST search was retained for further analysis. A gene tree of resulting sequences was constructed with the same methodology used to construct the species tree. To confirm whether the observed PKS duplication was unique to the *A. muscaria* species complex, we also conducted a targeted analysis of the *Amanita* genus. Genes encoding all PKS enzymes were identified from the six available non-*muscaria Amanita* genomes. The resulting 18 PKS protein sequences were used to construct a gene tree with the same methodology described to construct the species tree. Domain analysis revealed a single hybrid nonribosomal peptide synthetase (NRPS)-PKS protein in *A. inopinata* (KAF8636077.1), which was subsequently used to root the tree.

Metabolomics for chemical identification of metabolites

Methanolic extraction of metabolites We extracted metabolites from nearly every dried mushroom that had its genome sequenced (n = 22); the two Australian specimens (Aus332 and NzAUS95) were not included in metabolomics because there were insufficient tissues left. Two additional North American (20031-a and 20 045-b) and one additional South African (11671) mushrooms whose genomes were not sequenced were assayed, bringing the total number of metabolite extracts to 26 (*A. muscaria*: 25 and *A. pantherina*: 1; Table S1).

All samples were imaged before extraction (Fig. S1). Mushroom caps were pulverized and extracted overnight in 20–100 ml (depending on the volume of the mushroom tissue) of high-performance liquid chromatography (HPLC)-grade methanol. Extracts were filtered with a 0.2 µm syringe filter and evaporated to dryness on a rotary evaporator. Compounds were

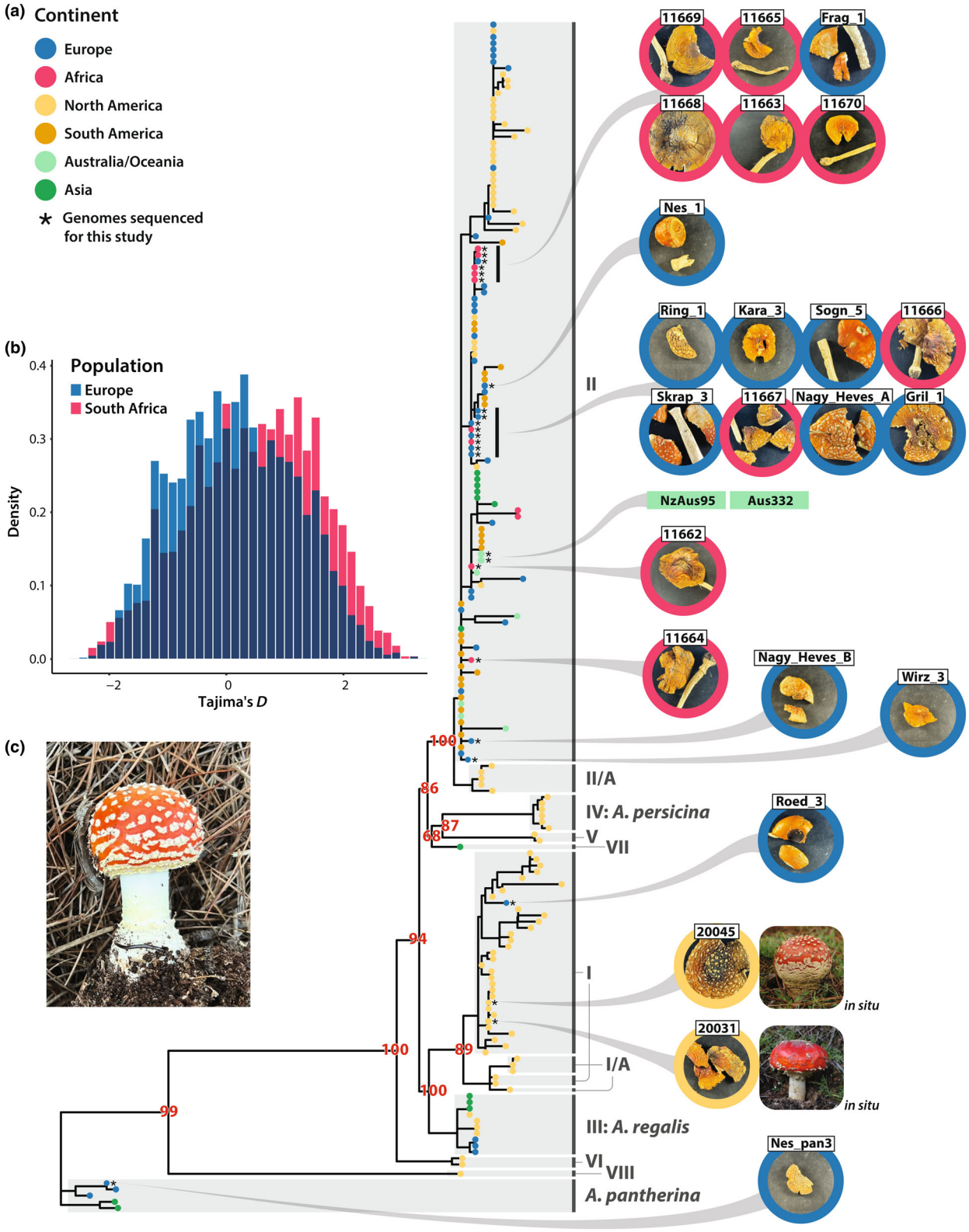


Fig. 2 Phylogenetic species reconstruction of 184 *Amanita muscaria* and 5 *Amanita pantherina* mushrooms. (a) Phylogeny constructed from an edge-linked partition model of ITS, LSU, β-tub and TEF-1α gene sequences. Clades are labeled with either the numbers used in Geml *et al*. (2008) or as *Amanita persicina* or *Amanita regalis*; many of the labels from Geml *et al*. (2008); e.g. clade VIII) represent undescribed taxa. Our phylogeny groups two specimens from Clade I in Geml *et al*. (2008) with Clade I/A. The tree is rooted with *A. pantherina*. Bootstrap support values are shown in red. Each mushroom's continent of origin is indicated by tip color, and available images of our sequenced mushrooms are displayed to the right of the phylogeny. Colors of the circles surrounding individual images mark the mushroom's continent of origin. (b) The distribution of Tajima's *D* was estimated from 5000-bp windows across the reference genome using biallelic single-nucleotide polymorphisms (SNPs) from the European (blue) and South African (red) Clade II genomes sequenced by us. (c) A photograph of *A. muscaria* collected near pine trees on a golf course (Simola Golf and Country Estate) in 2023 in the Western Cape province of South Africa.

resuspended at 1 mg ml^{-1} in methanol or dimethylsulfoxide (DMSO) for chemical analysis or biological assays, respectively.

UHPLC–MS/MS analysis Mushroom cap extracts were characterized on Ultra-high-performance liquid chromatography coupled with tandem mass spectrometry (UHPLC–MS/MS) using only spectroscopic grade solvents. Data were acquired using a Thermo Fisher Scientific Q Exactive Orbitrap mass spectrometer (Waltham, MA, USA) coupled to a Vanquish UHPLC (Waltham, MA, USA) operated in positive ionization mode. For all runs, we used a Waters XBridge BEH-C18 column (2.1 mm × 100 mm, 1.7 µm) and added 0.05% formic acid to our spectroscopic grade acetonitrile and water (flow rate 0.2 ml min^{-1}). The 37 min screening gradient method for the samples is as follows: Starting at 10% organic for 5 min, followed by a linear increase to 90% organic over 20 min, another linear increase to 98% organic for 2 min, holding at 98% organic for 5 min, decreasing back to 10% organic for 3 min, and holding at 10% organic for the final 2 min.

Global Natural Product Social Molecular Networking (GNPS) analysis To construct a molecular network, the raw mass spectra were converted to the mzXML format using the RawConverter software (v.1.2.01., The Scripps Research Institute; He *et al*., 2015). We used the web-based server to run a GNPS analysis with the default parameters provided by the platform: Min pairs cos = 0.7, network TopK = 10, max connected components size = 100, min matched fragment ions = 6, min cluster size = 2 (Wang *et al*., 2016). A molecular network was created with a precursor ion mass tolerance of 2.0 Da and fragment ion mass tolerance of 0.5 Da. The resulting molecular network was visualized in Cytoscape (v.3.10.2.; Shannon *et al*., 2003).

Preparing metabolites for bioactivity screening Every crude extract was tested against a range of laboratory model organisms available to us at the University of Wisconsin–Madison. Organisms included the pathogenic bacterium Methicillin-resistant *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa* and the pathogenic fungus *Candida auris*. Because experiments involving invertebrates are more complex, crude extracts from only a subset of the samples were used in tests against the nematodes *Caenorhabditis elegans* and *Brugia* spp., and the dipterans *Aedes aegypti* and *Musca domestica*. For nematode screening, we used three extracts from each region (United States, South Africa and Europe) in addition to extracts from the outgroup, *A. pantherina*. For insect screening, we used one sample from each of the three regions in addition to the outgroup (Table S1). Crude extracts were redissolved in methanol (MeOH) to test for antimicrobial activity or DMSO for invertebrate assays. Details of bioactivity assays are provided in Methods S1. Our focus on model organisms was a practical choice and enables basic insights into bioactivity; future tests with species and populations of South African antagonists are needed.

Results

South African *A. muscaria* derive from a single European species

Amanita muscaria has long been associated with imported pines (Box 1; Fig. 1), and its history in South Africa is remarkably well recorded. The earliest herbarium specimen dates to 1874 (Box 1), but historical records suggest the fungus was already established by then. Today, it is common in planted forests, cities and on rural farms (Fig. 1).

To investigate the origin of South African *A. muscaria*, we reconstructed phylogenies of *A. muscaria* using data from our own genomes and public repositories (Dataset S2); the topologies of created trees were largely in agreement with minor differences (Methods S1; Figs S4, S5). The nine South African individuals we sequenced belong to Clade II of the *A. muscaria* complex (Fig. 2a), a lineage also including specimens from Australia and South America, where *A. muscaria* is also introduced and spreading (Vargas *et al*., 2019). Clade II broadly corresponds to *A. muscaria var. muscaria*, widespread across Eurasia and into Alaska.

Site-frequency spectrum analyses support a historical introduction of *A. muscaria* into South Africa. European Clade II individuals show a near-zero value (Fig. 2b), while South African individuals display a positive shift, consistent with the loss of rare alleles during a founder event. The European mushrooms consistently have higher nucleotide diversity (0.00166) and mean heterozygosity (0.0799 ± 0.0132; Table S2) as compared to the South African population (0.00118 and 0.0684 ± 0.0045, respectively). Although sampling differences might influence results, the results align with historical records and phylogenetic inferences. Additionally, although the South African population shows signs of a bottleneck, the population remains diverse and is sexual: no clones were detected in either population and kinships

are typically below 0.2 (Dataset S3). However, one pair of mushrooms from a single *P. elliottii* plantation in KwaZulu-Natal has a higher kinship (*c.* 0.33), suggesting some level of inbreeding, perhaps between multiple monokaryotic mycelia of a shared parent.

Roed_3 was the only European individual we sequenced falling into Clades I-I/A. Clades I-I/A represent a complex of two cryptic taxa widely distributed throughout North America. Interestingly, mitochondrial DNA sequences placed Roed_3 closer to Clade II than Clades I-I/A, a pattern we speculate as reflecting a hybridization event (Figs S4, S5).

Specialized metabolite gene clusters are conserved across geographic regions and phylogenetic clades

AntiSMASH predicted between six and eight BGCs per genome, all of which were Type-1 PKSs or isoprenoid(s) biosynthesis (terpene) backbone enzymes, with the exception of a single NRPS found in the South African specimen 11 667. All genomes, including the *A. pantherina* outgroup, contained the full ibotenic acid BGC (Figs 3a, S6, S7) as defined previously (Obermaier & Müller, 2020). While the products of most of these *A. muscaria* BGCs are unknown, we confirmed the ibotenic acid BGC produced muscimol in each of the *A. muscaria* mushrooms we sequenced, regardless of phylogenetic grouping or geographic origin. Muscimol was also made by the *A. pantherina* outgroup (Nes_pan3 in Fig. S8).

BGCs were networked into the GCFs (Figs 3b, S2, S3) likely to produce identical or closely related SMs (Navarro-Munoz *et al.*, 2020; Bağcı *et al.*, 2025). All *A. muscaria* genomes encoded two PKSs, with both copies (referred to as PKS1.1 and PKS1.2; Fig. 3c) grouping into the same GCF, termed PK GCF-1. The terpene BGCs, which showed more variability in copy number, were grouped into two major GCFs termed terpene GCF-1 and terpene GCF-2. Terpene GCF-1 was universally present as a single copy in every *A. muscaria* genome but was absent in the outgroup. By contrast, the copy number of terpene GCF-2 varied from 1 to 3 across samples, with no synteny observed among the neighboring BGC genes (Fig. 3c). The final group, termed terpene GCF-'other' (Fig. 3), represents all terpenes whose predicted BGC locus was found only in a single isolate. Variation in terpene gene copy number may reflect genome fragmentation or incomplete assemblies. However, expansions of terpenes are common across the Basidiomycota. A thorough analysis of these BGCs is beyond our scope but emerges as an interesting direction for future research. Only a small fraction of genes in antiSMASH-predicted BGCs housed domains associated with SM in model Ascomycetes, suggesting a different biosynthetic logic in *A. muscaria* – either through stand-alone backbone enzymes or from the recruitment of novel tailoring genes.

Although *A. muscaria* has been reported to produce lethal amatoxins (Faulstich & Cochet-Meilhac, 1976), we found no genomic or chemical evidence – using bioinformatic searches, relaxed BLAST queries and direct metabolite analysis – to support these claims (see Fig. S1).

Conserved PKS enzymes in *A. muscaria* originated from duplication in a common ancestor of the species complex

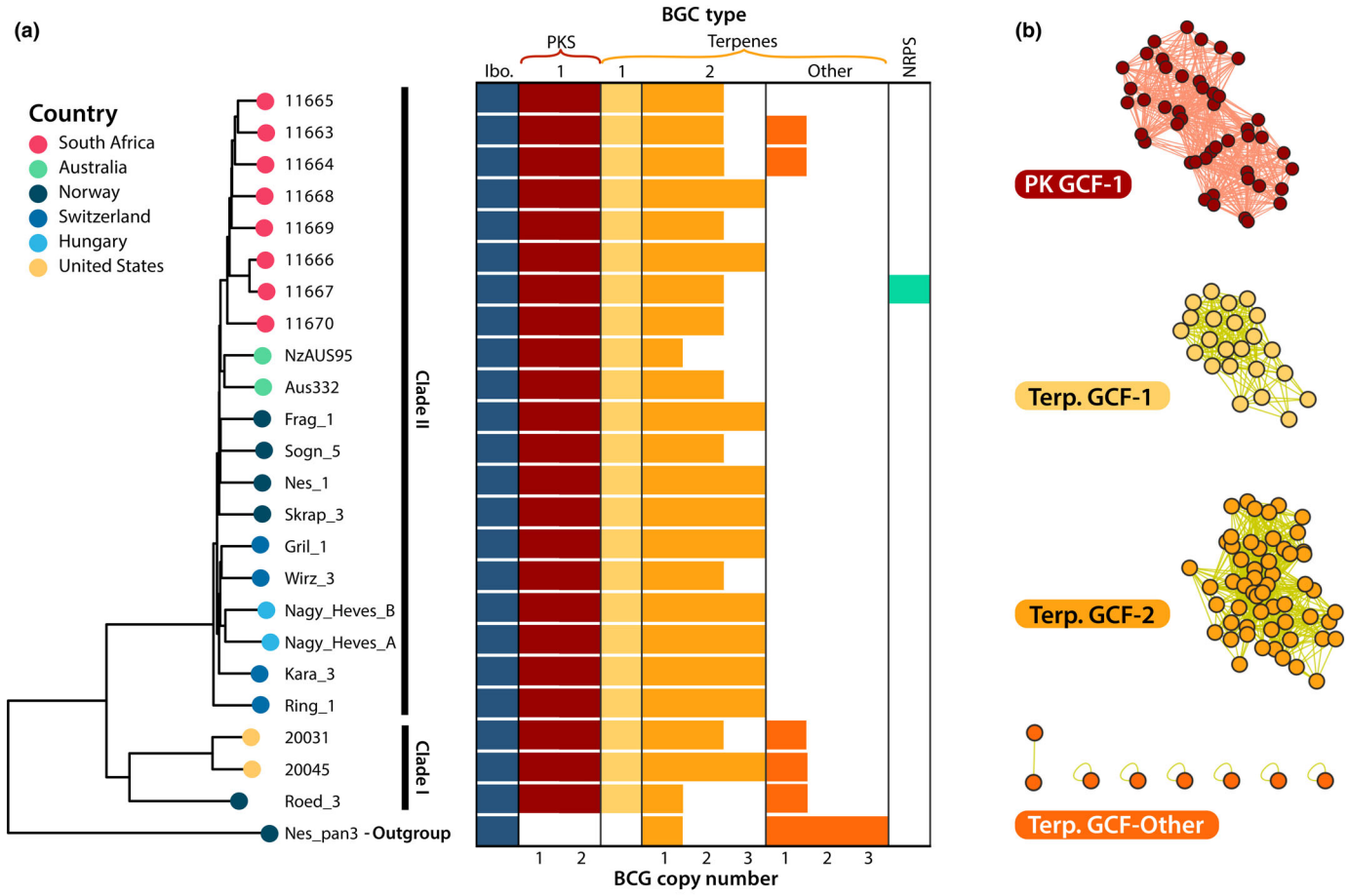
The strong similarity between the two copies of PKS enzymes in all *A. muscaria* genomes led us to hypothesize the copies arose from a recent duplication event. In a phylogenetic analysis, all of *A. muscaria*'s PKS sequences form a monophyletic clade, while all other PKS sequences from other *Amanita* spp. share more ancient ancestry (Fig. 4b). A targeted follow-up analysis confirms the PKS duplication as unique to *A. muscaria* (Fig. S9). This analysis also revealed an interesting and distinct evolutionary history of the PKS enzyme in *A. inopinata*, a lineage which has independently duplicated the ancestral nonreducing PKS and acquired both a reducing PKS and a hybrid NRPS-PKS (Fig. S9). The lack of *trans*-species representation in the duplicated *A. muscaria* PKS clade is strong evidence for the duplication having occurred relatively recently, likely in a common ancestor of the *A. muscaria* species complex.

To test whether the duplication of PKS-1.1 and PKS1.2 enabled functional divergence, we compared domain variation between the two copies and a sampling of highly similar PKS enzymes across diverse fungi. The PKS1.2 backbone in all *A. muscaria* genomes, regardless of geographic region or clade, has lost its C-terminal thioesterase (TE) domain (Fig. 4a). This result is not an annotation error; we used tBLASTn to verify that the coding sequence for this domain was only present in the genomic DNA associated with PKS1.1. The C-terminal domain is typically involved in the chain release and cyclization of PK products (Tang *et al.*, 2019). While most PKS hits from closely related *Amanita* species were found in single copies within their genomes and retained the C-terminal thioesterase, a distinct PKS clade – from *Amanita brunnescens* – showed a similar pattern of duplication and loss, suggesting the pattern has evolved multiple times in the genus (Fig. S9).

The domain structure of *A. muscaria*'s PKS enzymes closely mirrors the structure of known orsellinic-acid-producing PKSs in other Agaricomycetes (Lackner *et al.*, 2013; Braesel *et al.*, 2017) and in the Ascomycete *Aspergillus nidulans* (Schroeckh *et al.*, 2009; Fig. 4a). While these findings may suggest *A. muscaria* is producing an orsellinic-acid-like compound, a similarly strong hit to a PKS in the *Aspergillus fumigatus* genome associated with production of a melanin intermediate (Fujii *et al.*, 2000; Brakhage & Liebmann, 2005) raises some doubt about the true end-product of the *A. muscaria* PKS enzymes.

GNPS-based untargeted mass spectrometry analysis suggests the *A. muscaria* metabolome is highly conserved across native and novel ranges

GNPS analysis identified 273 unique molecular families (MFs) across all spectra (Fig. S10) including both primary and specialized metabolic features (Fig. 5a; see the Materials and Methods section). Of the 273 MFs, 215, including all MFs with at least 11 nodes, were detected in every geographic region. The largest MF (MF-21), comprising 100 unique nodes, was predicted to belong to a primary metabolism phospholipid family. We identified 25



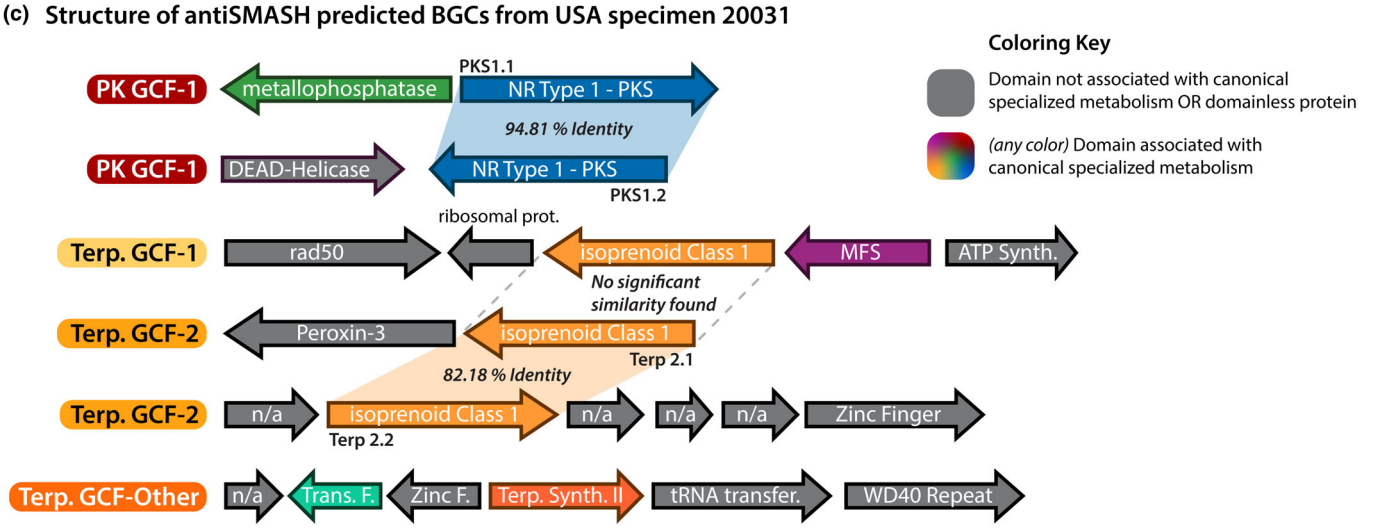


Fig. 3 Specialized metabolism (SM) in *Amanita muscaria* genomes. (a) Numbers of biosynthetic gene clusters (BGCs) per genome, sorted by type based on backbone enzymes. Gene copy numbers for each BGC locus also depicted. The maximum likelihood phylogeny of the isolates was generated using single-nucleotide polymorphism (SNP) data and rooted with *A. pantherina* (Nes_pan3). The profiles of our South African genomes appear similar to the profiles of our European genomes. (b) Gene cluster family (GCF) networks of the two major SM classes within the genomes: polyketide synthase (PKS) and terpenes (Terp.). Nodes represent a single BGC from an individual genome and edges between nodes connect related nodes. Networked nodes represent closely related clusters likely producing the same or similar products (i.e. a GCF). (c) Domain and genetic architecture of the antiSMASH-predicted BGC loci using Californian sample 20 031-a (Clade I) as an example; the panel illustrates the unique architectures of each GCF. Genes are depicted approximately to scale. All major GCFs were found in similar copy numbers across *A. muscaria* populations, suggesting the retention of chemical potential among groups.

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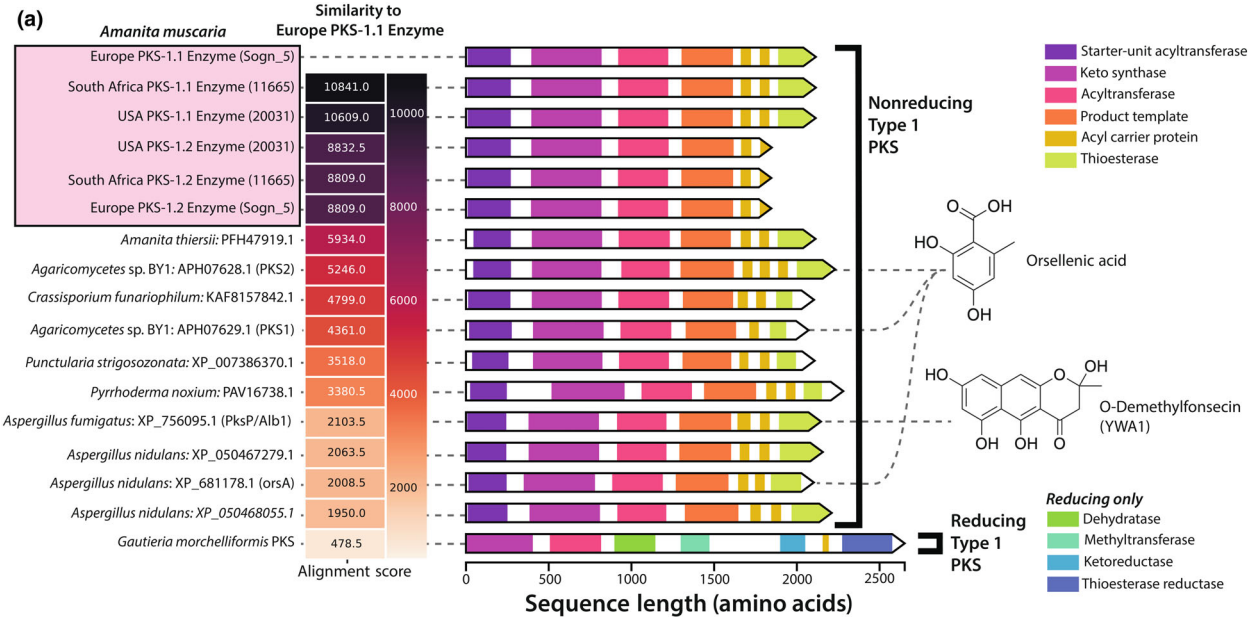
MFs unique to *A. muscaria* Clade II samples (South Africa and Europe) and 13 MFs specific to South African *A. muscaria*. The largest South Africa-specific MF (MF-9) was made up of 10 unique nodes (Fig. 5b). These nodes had precursor *m/z* values between 900 and 1200, with no matches to known library entries or previously characterized metabolites.



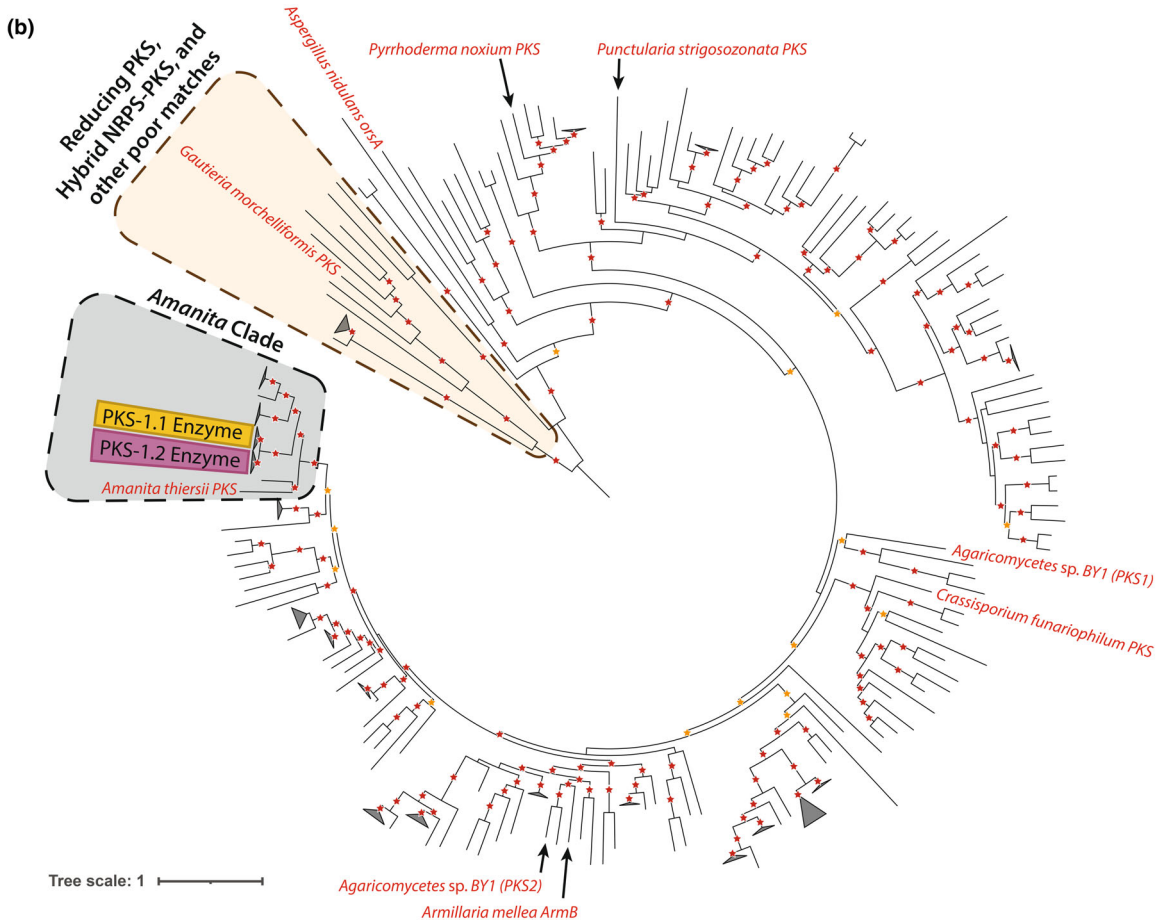


Fig. 4 Analysis of the Type 1 polyketide synthase (PKS) gene duplication found in *Amanita muscaria*. (a) Comparison of the two Type I PKS enzymes found in the PK GCF-1 gene cluster family (termed PKS-1.1 and PKS-1.2), including copies from European, South African and Californian *A. muscaria* genomes (highlighted in pink box). Left to right: Similarity scores were calculated by aligning each PKS sequence to the Sogn_5 PKS-1.1 backbone enzyme. The domain architecture of each PKS is color-coded. Known metabolites associated with specific PKSs are linked by dashed lines to the corresponding structures on the right. (b) Midpoint-rooted phylogeny of 244 Agaricomycete PKS proteins with additional hand-selected PKS proteins (e.g. reducing and nonreducing PKSs, *orsA* from *Aspergillus nidulans*). The tree was midpoint-rooted to facilitate clade visualization. Clades with an average branch length of less than 0.3 to their leaves were collapsed and displayed as triangles sized proportionally to the number of collapsed leaves. The monophyletic clade containing all *Amanita* PKS proteins is shaded in gray. A detailed analysis of proteins from the genus is provided in Supporting Information Fig. S9. The clades for *A. muscaria* PKS-1.1 and PKS-1.2 are highlighted in yellow and purple, respectively. Nodes with > 80% and > 90% support are indicated with orange and red stars, respectively. A subset of the proteins used to build the phylogeny are labeled for reference.

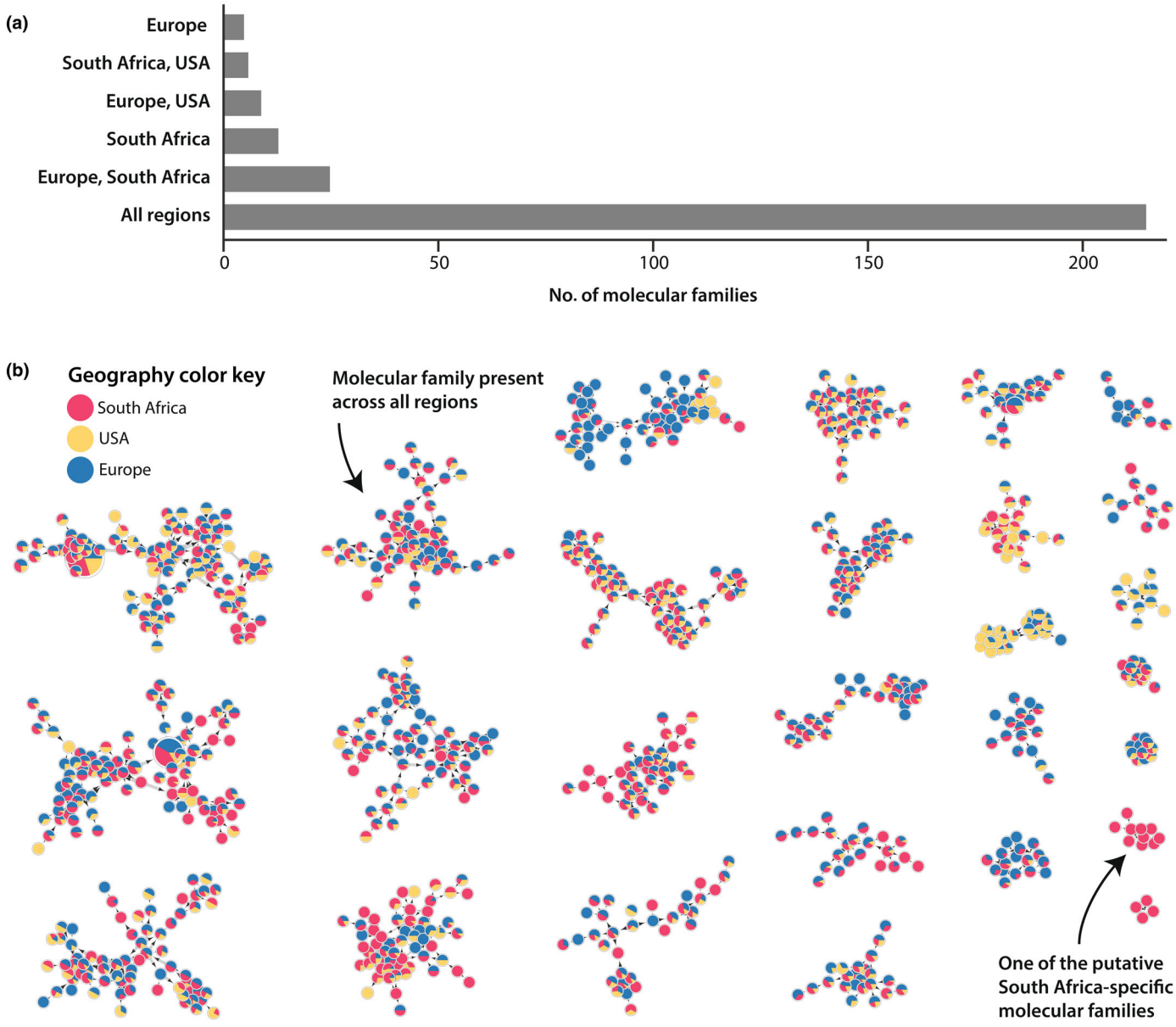


Fig. 5 Metabolome of *Amanita muscaria* contextualized by geography. (a) Count of molecular families (MF) grouped by geographic region. (b) Network visualization of a subset of MFs. Node size reflects the total precursor intensity. Each node includes a pie chart marking the geographic regions where mushrooms with that MF were located. The full network of all detected MFs is in Supporting Information Fig. S10. Most MFs are found in all regions and comparatively few MFs are found only in South Africa.

A. muscaria extracts reveal biological activity/inactivity, with no population-specific patterns

To understand the potential of *A. muscaria* metabolites to have broad-spectrum activity against bacteria, filamentous fungi and yeasts, with potential relevance for ecological inferences and drug discovery, we performed disk-diffusion assays against MRSA (Gram-positive; Fig. S11), *Pseudomonas aeruginosa* (Gram-negative; Fig. S12) and *Candida auris* (a *Saccharomyces* yeast; Fig. S13). None of these organisms were inhibited by extracts (Fig. 6). In contrast to the microbial assays, extracts from nine geographically representative *A. muscaria* samples and one

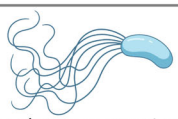
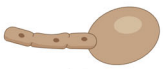
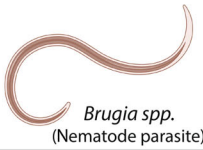
Organism	No. of extracts tested (*n*)			Bioactivity (Strong / Mild / None)	Lowest concentration with bioactivity	Highest concentration tested (mg l^{-1})	Bioactivity assay	Supporting figure (with all results)
	Europe	SA	USA					
 *MRSA* (Gram positive pathogen)	12	10	4		n/a	50	Disc diffusion assay	S11
 *Pseudomonas aeruginosa* (Gram negative pathogen)	12	10	4		n/a	50	Disc diffusion assay	S12
 *Candida auris* (Yeast pathogen)	12	10	4		n/a	50	Disc diffusion assay	S13
 *Caenorhabditis elegans* (Nematode)	4	3	3		50 µg l^{-1}	1	96-well liquid assays	S14
 *Brugia spp.* (Nematode parasite)	4	3	3		10 µg l^{-1}	1	96-well liquid assays	S14
 Adult *Musca domestica* (Housefly)	1	1	1		n/a	1	Administered by cotton wick	S15
 First/Late Instar *Aedes aegypti* (Yellow fever mosquito)	1	1	1	First Late	1 mg l^{-1}	1	24-well liquid assays	S15
 Adult *Aedes aegypti* (Yellow fever mosquito)	1	1	1		n/a	1	Administered by cotton wick	S15

Fig. 6 Bioactivities of *Amanita muscaria* extracts against Methicillin-resistant *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa*, *Candida auris*, *Caenorhabditis elegans*, *Brugia* spp., *Musca domestica* and *Aedes aegypti*. Columns from left to right are the organisms tested, number of extracts (mushrooms) tested in each experiment (SA = South Africa, USA = California), the viability of tested organisms when confronted with extracts, the concentrations used, the method of inoculation and a reference to Supporting Information with full results.

A. pantherina sample all strongly inhibited the growth of the clade V nematode *Caenorhabditis elegans* (Figs 2, S14). Extracts also immobilized the Clade III parasitic nematodes *Brugia pahangi* and *Brugia malayi*, and assays with *B. malayi* reveal that the impact of extracts on *B. malayi* was similar to heat-killed controls (Fig. S14). Insect assays revealed a more selective pattern: while adult *Musca domestica* and *Aedes aegypti* were unaffected by the extracts, first-instar *A. aegypti* larvae were highly susceptible. In general, bioactivity declined as animals reached later developmental stages (Fig. S15). While the model organisms available to us may be unlikely to interact with *A. muscaria* in nature, our findings suggest there is variation in dipteran susceptibility, even despite the presence of $GABA_A$ receptors in flies. Our data also suggest potential bioactivity may be limited to early developmental stages in certain species. We note we commonly find dipteran eggs laid among the gills of *A. muscaria* (Drott, Pringle and Stokes, pers. obs.). None of the bioactivities observed were different among the mushrooms collected from different continents. While our data suggest broad nematicidal activity, important differences may exist between the models we used and the populations of species interacting with *A. muscaria* in South Africa and elsewhere.

Discussion

The mushrooms of the fungus *A. muscaria* s.l. are famous, featured in settings ranging from Super Mario™ to treatises of Hindu texts. Despite its prominent cultural status, the human-mediated dispersal of this fungus is poorly documented. In this study, we used genomics and metabolomics to investigate the history and evolution of *A. muscaria* following its introduction to South Africa. Our phylogenetic analyses reveal South African *A. muscaria* populations belong to Clade II, a cryptic lineage of European origin. Historical records suggest the fungus was likely introduced more than 150 yr ago, perhaps with pine seedlings brought by European settlers to Cape Town. Its subsequent spread was facilitated by the expansion of pine forestry in southern Africa, and South African *A. muscaria* now grow with pines imported from other continents, including the Mexican *P. patula*. The fungus is sexual, and its populations in South Africa are genetically diverse, with multiple distinct individuals present within single forest stands. Despite its extended history in South Africa, the specialized metabolome of *A. muscaria* remains highly conserved, with gene clusters encoding muscimol, terpene and PK products present in all genomes. Bioactivity assays with laboratory model organisms reveal the potent nematicidal effects of the fungus's metabolites, suggesting their potential (as yet untested) role in mediating ecological interactions.

The European Clade II lineage found in South Africa is also the lineage introduced to Colombia (Vargas *et al.*, 2019) and Australia (Lebel *et al.*, 2024; Fig. 2a). Remarkably, the global dispersal of *A. muscaria* appears to involve just one of its many cryptic species (and we note many of the cryptic species await formal naming, e.g. the currently unnamed species of Clades I-I/A (Geml *et al.*, 2008), which include our two Californian genomes). Europe has long been suspected as the source of Australian *A. muscaria*, but we are the first to identify Clade II as the specific lineage introduced there. More intensive sampling will be needed to pinpoint the exact European source(s) of South African *A. muscaria*, and additional sequencing may reveal whether other clades (or after they are named, species) within the complex have been introduced elsewhere in the country. However, based on available data, Clade II appears to be the primary lineage driving invasions, suggesting underlying genetic, ecological or historical factors contributing to its spread.

The fungus may also have been introduced to South Africa from another invasive range, for example, Colombia or Australia. But historical records suggest the most parsimonious explanation for the *A. muscaria* in South Africa is a direct introduction(s) from Europe (Box 1; Fig. 1). Interestingly, several of our phylogenies (but not all) suggest South African *A. muscaria* share their most recent common ancestry with Australian populations, a pattern which may reflect a shared European source population (Figs S4, S5).

Kinship analyses indicate no two South African mushrooms are clones, although many mushroom pairs are closely related; the data are consistent with ongoing sexual reproduction. While invasive fungal populations often exhibit shifts in mating systems or reproductive modes (Wang *et al.*, 2023), the conservation of sexual reproduction and mating type diversity in South Africa suggests either a weak genetic bottleneck or continued admixture from multiple introductions.

The introduction of *A. muscaria* into South Africa is also reflected in site-frequency spectra, where a positive shift in Tajima's *D* estimates among Clade II mushrooms (Fig. 2b) is consistent with a founder event followed by insufficient time and/or evolution to regenerate rare alleles. A similar pattern has been observed in *A. phalloides* populations introduced to California from Europe (Drott *et al.*, 2023). Other population genetic statistics, including Fst estimates, also suggest South African populations are differentiated from their European counterparts (Fig. S16a). Loci with the highest Fst scores encode genes associated with oxidoreductase activity, metal binding and cell membranes – functions previously linked to host and environmental stress responses in fungi (Fig. S16b; Staerck *et al.*, 2018; Feng *et al.*, 2023). Further research is needed to clarify the significance of the differences.

Despite the different ecological contexts of Europe, South Africa and Australia, all sequenced *A. muscaria* genomes housed a similar complement of BGCs, including the ibotenic acid BGC (Figs 3, S6, S7), an expanded set of terpenes and two PKS gene clusters (Fig. 4). Research on the ecology of *A. muscaria* has focused primarily on its ecological niche; the ECM symbiosis has one of its origins in the *Amanita* genus (Wolfe *et al.*, 2012). Although little is known about the role of SMs in the ECM symbiosis, we identified an expansion of an orsellinic-acid-like PKS ortholog maintained across populations (Figs 3, 4; how the duplicate genes are expressed remains a target for future research). One of these orthologs has lost a TE domain, suggesting that the mature product is not cyclized or may depend on other enzymes for processing, similar to lovastatin biosynthesis (Xu *et al.*, 2013). Orsellinic acid, found in several basidiomycetes (Lackner

et al., 2012), has derivatives with potential herbicidal properties (Peres *et al.*, 2009), which we speculate may influence fungal–plant interactions. Orsellinic-acid-sesterpene hybrids occur in some fungi, resulting in highly diverse bioactivities (Gao *et al.*, 2023). *Trans*-BGC interactions are increasingly being discovered (Won *et al.*, 2022) but are not well understood, particularly in Basidiomycete lineages; we speculate that such epistatic interactions modulate the chemical diversity associated with the conserved terpene BGCs in *A. muscaria*.

The conservation of primary and SMs across introduced and native populations provides no evidence for relaxed selection (Fig. S17) as might be expected if metabolic resources were being reappropriated to other pathways (Blossey & Nötzold, 1995). Instead, our findings raise the possibility of conserved functional interactions with communities in both native and introduced ranges. Perhaps imported European soils harbored antagonistic microbes and invertebrates now interacting with *A. muscaria* in South Africa, or perhaps South African *A. muscaria* have equivalent kinds of interactions with native African antagonists. It is also possible there has not been enough time for genomic signatures of relaxed selection to emerge, although SMs are often subject to strong selection and are readily lost, causing their highly patchy distribution across taxa (Robey *et al.*, 2021).

While *A. muscaria*'s core BGCs are highly conserved, we also identified 13 MFs unique to South African populations. Slight differences in mushroom handling after collection and differences among the microbial communities inhabiting mushrooms may have impacted identified metabolites, but we speculate these novel MFs represent metabolic innovations influencing interactions in the novel range. None of the consensus spectra of the South African MFs matched to public reference libraries in GNPS, additional evidence they represent truly novel metabolites evolving in the invasive range. The differentiation of metabolic space between fungal populations is often slight, but it can sometimes be defined by the production of a small subset of ecologically important metabolites (Drott *et al.*, 2021).

The association between *A. muscaria* and fly-killing dates back centuries, and both historical and modern accounts suggest insecticidal properties. The traditional recipe for using *A. muscaria* to kill flies involves soaking *A. muscaria* in milk or water (Lumpert & Kreft, 2016). As recently as 2021, Carboué & Lopez (2021) cited *A. muscaria* extracts as having fly-killing bioactivity. However, skepticism about its ability to kill insects has persisted for just as long; as early as 1779, French botanist Jean Bulliard challenged claims of insecticidal activity and noted flies appeared unaffected by the mushroom (Wasson, 1969). Some folklore suggests *A. muscaria* may act as an attractant rather than a direct toxin, luring flies to consume the mushroom before they ultimately drown in the *A. muscaria*-soaked milk.

Our findings align with historical skepticism – while *A. muscaria* extracts strongly suppressed nematodes (*C. elegans* and *Brugia* spp.) and inhibited mosquito larvae, they had no detectable effect on any adult dipterans (or bacteria or yeast; Fig. 6). Our experiments targeted model organisms, but the models serve as a robust proxy for detecting broad-spectrum bioactivity. The strong nematicidal properties of *A. muscaria* reproduced by us have previously been attributed to its SMs ibotenic acid and muscimol (Johnston, 2014). Both are known agonists to the $GABA_A$ receptor complex (Johnston, 2014). While we did not discover any effect on adult dipterans, muscimol resistance has been documented among mycophagous species of dipterans but not their frugivorous counterparts (Tuno *et al.*, 2007), emphasizing questions about the susceptibility of relevant antagonists in the introduced range. Our experiments used model organisms as tools, but with these data, future efforts focused on South African antagonists can take a more targeted and informed approach to understanding local interactions.

In the aggregate, our data highlight the genetic and metabolic versatility of *A. muscaria*, a globally invasive ECM fungus. While our results offer insights into the origin of introduced populations, the evolution of the *A. muscaria* lineage, and the genomic signatures of South African introductions (or their lack), these findings also emphasize many unanswered questions: how does *A. muscaria* interact with local antagonists in its novel ranges, and does enemy release or do novel weapons play a role in the spread of the fungus? Do population-specific MFs contribute to its success? Our results offer insights into the global distribution of *A. muscaria*, creating a strong foundation for future research exploring how fungal invasions and co-invasions shape ecosystems across the globe.

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Competing interests

None declared.

Author contributions

Our study stems from a unique collaboration between scientists working in South Africa and the United States, and together, we span diverse countries and career stages. Our collaboration began at a US National Science Foundation-funded workshop (grant no.: 1953299 awarded to JDH and JSB) held at Future Africa (www.futureafrica.science) in November 2022. The workshop initially focused on introducing students to invasion biology and providing bioinformatics training, using *Amanita muscaria* s.l. genomes as a teaching tool. AP and JDH conceived the study, and it was developed fully over the following 14 months. GRN designed and drafted the manuscript, leading the bioinformatic and natural product experiments and analyses. SRF, KMTL, DLN and CKS (listed in reverse alphabetical order as equal co-second authors) contributed substantially to analyses and writing. CB also contributed significantly to analyses and writing. GRN, NPK, JWB and AP sequenced genomes. MAH, AN and AP led the historical research. Y-WW led kinship analyses. GRN, SCP and NPK conducted the metabolic extractions and mass spectrometry analyses. Bioactivity work was designed, executed and analyzed as follows: microbial assays by GRN, CKS and NPK; insect assays by GRN, TKW, HLN, MEMM and KLC; and nematode assays by GRN, KTR and MZ. JDH, MTD and AP contributed to data analysis, interpretation and writing, with MTD and AP leading revisions to interpretations and the manuscript.

All authors (GRN, CKS, DLN, KMTL, SRF, CB, BMA, CPB, JWB, JSB, STB, JPRMC, KLC, LRC, MPAC, CD, TAD, MAH, NPK, KK, FAL, HLN, AN, MAN, MEMM, SCP, NQP, KTR, MS, RV, JMW, Y-WW, BDW, MJW, TKW, TAZ, MZ, JDH, MTD and AP) participated in workshop discussions, contributed to initial analyses and helped edit the final manuscript. CKS, DLN, KMTL, SRF and CB contributed equally to this work.

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Data availability

All genomic data used in our study are either publicly available through NCBI (see the Materials and Methods section and Dataset S2) or were generated by the authors and are made public with our publication. Raw sequencing reads and genome assemblies are available through NCBI BioProject (accession no.: PRJNA1372216). The screening methods used to test bioactivities and raw results are provided in Supporting Information (Figs S11–S15). The Supporting Information also provides details on genome annotation (Methods S1), mitogenome analysis (Methods S1), split decomposition of the SNP dataset (Methods S1) and targeted genome mining for amatoxin-encoding genes (Methods S1). Supporting Datasets include a summary of genome summary statistics (Dataset S1), the gene markers used to generate Fig. 2 (Dataset S2) and the results of our kinship analysis (Dataset S3).

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Dataset S1 Genome summary statistics.

Dataset S2 Full metadata for *Amanita muscaria* data retrieved from prior publications.

Dataset S3 Kinship analyses of the sequenced genomes.

Fig. S1 Dried tissue of each mushroom prior to chemical analyses.

Fig. S2 Determination of optimal cutoff to generate the PKS GCF predictions.

Fig. S3 Determination of optimal cutoff to generate the Terpene GCF predictions.

Fig. S4 Rooted phylogenies from the fully sequenced *Amanita muscaria* genomes.

Fig. S5 Tree compatibility comparisons between different species reconstruction methods.

Fig. S6 A cblaster analysis showing the presence and absence of genes in the ibotenic acid cluster in *Amanita muscaria* and outgroup genomes.

Fig. S7 Whole fungal kingdom phylogeny depicting the number of Reciprocal best-hit BLAST hits to genes in the ibotenic acid gene cluster.

Fig. S8 Quantity of putative muscimol in every sample.

Fig. S9 Comparison of polyketide synthases found in *Amanita* genomes.

Fig. S10 Full GNPS output of every molecular family, including singletons.

Fig. S11 Bioassays of metabolite extracts against Methicillin-resistant *Staphylococcus aureus*.

Fig. S12 Bioassays of metabolite extracts against *Pseudomonas aeruginosa*.

Fig. S13 Bioassays of metabolite extracts against *Candida auris*.

Fig. S14 Nematode bioassay results after treatment with several concentrations of extracts.

Fig. S15 Viability assays in *Aedes aegypti* and *Musca domestica*.

Fig. S16 GO-Term map corresponding to genes that overlapped with 5 kb sliding windows containing at least 100 SNPs and where estimates of Fst corresponded to the right 5% tail of estimates.

Fig. S17 Phylogenetic trees constructed using a codon-aware alignment of the iboF and iboH genes.

Methods S1 Additional information on phylogenomics, BUSCO genes, mitogenomes, bioinformatics and bioactivity assays.

Table S1 Summary of mushrooms used in this study.

Table S2 Summary of heterozygosity in sequenced genomes.

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