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

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All genomic data analyzed in this study were generated by the authors. Raw reads and assemblies are publicly available through NCBI BioProject [###]. Other data and methods enabling replication are provided with this manuscript and in the Supporting Information (which contains Supporting Methods, Tables, and Figures).

For Peer Review

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

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KEYWORDS

Biosynthetic gene clusters, bioactivity, invasive mushrooms, invasive ectomycorrhizal fungi, Global Natural Product Social Molecular Networking (GNPS) analysis, fly agaric, nematicidal, primary or specialized metabolism, secondary metabolites, South Africa

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

WORD COUNTS

Introduction: 1,483 → 1,057

Box 1: 516 → 384

Materials and Methods: 2,612

Results: 3,166 → 1,586 (figure captions 792 → 712).

Discussion: 1,418

No. of Tables: 2

No. of Figures: 5

Supporting Information: 2,977

1 INTRODUCTION

2 Humans have moved plants and soils among continents for centuries, resulting in the co-
3 introduction of fungal pathogens and symbionts (Anagnostakis, 1987; Dickie *et al.*, 2010;
4 Cleary *et al.*, 2016). Fungi mediate basic ecological processes (Treseder & Lennon, 2015) and
5 when introduced fungi become invasive, they can dramatically alter local ecosystems
6 (Anagnostakis, 1987; Desprez-Loustau *et al.*, 2007; Rossman, 2009; Boyd *et al.*, 2013). Interest
7 in invasive nonpathogenic fungi is growing (Pringle *et al.*, 2009; Dickie *et al.*, 2010; Vargas *et*
8 *al.*, 2019; Milani *et al.*, 2022), but most research continues to focus on the biology and impacts
9 of introduced and invasive pathogens. As mutualistic fungi spread across the globe (Vellinga *et*
10 *al.*, 2009; Berch *et al.*, 2017; Vargas *et al.*, 2019; Pildain *et al.*, 2021), discovering the
11 mechanisms enabling their successful invasions and documenting impacts on native ecosystems
12 emerge as key research priorities.

13
14 The most intensively studied plant-fungal co-invasions involve fungi and plant species of *Pinus*
15 (family Pinaceae). Pines have been introduced across the Southern Hemisphere for commercial
16 forestry, reforestation, and afforestation. These non-native trees now cover more than 6 million
17 hectares in countries including South Africa, Argentina, Brazil, and New Zealand (Rouget *et al.*,
18 2001; Simberloff *et al.*, 2010; Dickie *et al.*, 2014; Brancatelli *et al.*, 2020). Species like *Pinus*
19 *elliottii*, *P. patula*, and *P. radiata* have spread beyond forestry plantations and into natural forest
20 and grassland biomes, including into biodiversity hotspots, for example, South Africa's fynbos
21 (Richardson & Higgins, 1998; van Wilgen & Richardson, 2012). Invading pines can reduce
22 water availability, suppress native plant and fungal species, and disrupt ecosystem services (Le
23 Maitre *et al.*, 2002; Dickie *et al.*, 2014; Nuñez *et al.*, 2017; Brewer *et al.*, 2018; Sapsford *et al.*,
24 2022).

25
26 Pines are obligately associated with ectomycorrhizal (ECM) fungi, a symbiosis enabling the trees
27 to obtain essential nutrients and water (Smith & Read, 2008; Nuñez *et al.*, 2009; Dickie *et al.*,
28 2010). Historically, plantings of pines in the Southern Hemisphere failed until soils were
29 imported to supply their symbionts (Mikola, 1970), and introduced ECM fungi have
30 subsequently facilitated invasions of pine trees (Hayward *et al.*, 2015; Policelli *et al.*, 2019;
31 Policelli *et al.*, 2023). Despite the critical role of ECM fungi in pine invasions and the potential
32 for ECM fungi to invade and harm native forests on their own (Dickie *et al.*, 2017), the
33 ecological and evolutionary dynamics of invading ECM fungi remain poorly understood, perhaps
34 because invading mutualists are perceived as unequivocally beneficial (but see Schwartz *et al.*
35 2006). In fact, ECM fungi may function across a spectrum of mutualism and parasitism (Karst *et*
36 *al.*, 2008). Efforts to elucidate the ecology of ECM fungi are further exacerbated by their
37 ephemeral and often cryptic biology (Wang *et al.*, 2023), leaving us with incomplete, often
38 inaccurate historical records of species' native ranges (Pringle & Vellinga, 2006). Strategies
39 combining historical research with modern 'omics methods offer a novel approach to identify the
40 drivers of ECM fungal invasions.

41
42 Invasion biology often invokes interactions among species as a mechanism driving spread, and
43 fungal interactions are often hypothesized to be mediated by specialized metabolites (SMs)
44 (Drott *et al.*, 2017; Keller, 2019; Tannous *et al.*, 2022). Although ECM fungi produce a plethora
45 of SMs (Schüffler, 2018; Walton, 2018; Obermaier & Müller, 2020; Mudbhari *et al.*, 2024) and
46 must routinely interact with pathogens, predators, and competitors, interactions between

47 introduced ECM fungi and local organisms are rarely considered. However, the toxins of at least
 48 one invasive ECM fungus, *Amanita phalloides*, appear to be evolving dynamically in its invasive
 49 range (Drott *et al.*, 2023). This finding and the ubiquity of SMs raise questions about the
 50 translation of the “enemy release” and “novel weapons” hypotheses, often invoked to explain
 51 the success of non-native plants and animals, to invasive fungi (Blossey & Nötzold, 1995;
 52 Torchin *et al.*, 2003; Callaway & Ridenour, 2004; Liu & Stiling, 2006). The “enemy release”
 53 hypothesis proposes introduced species succeed by escaping natural enemies, while the “novel
 54 weapons” hypothesis suggests they spread by producing compounds against which native species
 55 have no resistance (Gillett, 1962; Callaway & Ridenour, 2004).

56
 57 The ecological roles and evolution of SMs remain poorly understood, even in iconic ECM
 58 species like *Amanita muscaria* sensu lato (s.l.) (Geml *et al.*, 2006), the famous red-and-white-
 59 spotted “fly agaric” known for its psychoactive properties (Camazine, 1983; Michelot &
 60 Melendez-Howell, 2003) and for the folklore suggesting it can repel or kill flies (Lumpert &
 61 Kreft, 2016). *A. muscaria* produces ibotenic acid and muscimol using a single biosynthetic
 62 pathway (Størmer *et al.*, 2004; Su *et al.*, 2023), and muscimol is a potent GABA_A receptor
 63 agonist (Johnston, 2014), a feature which contributes to its distinctive biological effects. Native
 64 to nearly all temperate and boreal biomes of the Northern Hemisphere, the *A. muscaria* species
 65 complex includes multiple cryptic lineages associated with diverse plants, including pines. While
 66 some of these cryptic species are now formally recognized (for example, *A. persicina* (Tulloss *et*
 67 *al.*, 2015)), the true number of cryptic species within the *A. muscaria* species complex remains
 68 unknown. For simplicity, we refer to all as-yet unnamed lineages as *A. muscaria*. Introduced to
 69 South America, Australia, and Africa, *A. muscaria* forms novel ECM partnerships with native
 70 trees in Columbia (*Quercus humboldtii*; (Vargas *et al.*, 2019), Chile (*Nothofagus*; (Márquez
 71 Parraguez, 2024) and Australia (*Nothofagus* and *Allocasuarina*; (Fuhrer, 1992; Dunk *et al.*,
 72 2012; Lebel *et al.*, 2024). Relatively little is known about its introductions across Africa
 73 (Vellinga *et al.*, 2009), but its South African history is particularly well documented (Box 1),
 74 offering a rare opportunity to study the impact of *A. muscaria*’s introduction on the fungus’s
 75 evolution and production of SMs.
 76

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

86

87 **BOX 1: The history of *Amanita muscaria* in South Africa**

88 Doidge (Doidge, 1950) lists a number of early collections of the fungus; by 1874 *A. muscaria* was
 89 growing with pines in and around Cape Town (Fig. 1). By 1945 it was growing in what are now named as

91 the provinces of Mpumalanga to the north and Free State in the east. She identifies *A. muscaria* as
 92 growing with “shrubs”, pine trees, and “near living roots of *Quercus* sp.” (Doidge 1950 p. 553).

93
 94 The history of *A. muscaria* is linked to the history of Europeans on the continent. Early European
 95 colonists did not find the timber species native to South Africa suitable for their uses, and they introduced
 96 fast-growing pines. Any plant moved to South Africa by European colonists would have been
 97 accompanied by fungi in its tissues, and plants may also have been moved as seedlings growing in soil,
 98 enabling the introduction of soil fungi. Doidge (1950) succinctly describes the dynamic of plants arriving
 99 to South Africa with Europeans,

100
 101 “The history of plant introduction into South Africa begins in 1651, with the arrival of Johan van
 102 Riebeeck to establish a settlement at the Cape; van Riebeeck was accompanied by a gardener...
 103 To provide timber, sacks of acorns were sent from Holland and kernels of stone and cluster pines
 104 from Italy; these were planted in quantity and flourished amazingly... the majority being species
 105 of *Pinus*, Eucalypts and poplars...” Doidge (1950 pp. 15-16)

106
 107 Mikola (1970) goes on to describe the subsequent deliberate export of soils from South Africa to enable
 108 plantation forestry elsewhere, including in Zimbabwe and Kenya (Mikola 1970; Zimbabwe discussed as
 109 Rhodesia). For example, three tons of soil from the Transvaal were imported to establish pine plantations
 110 in Eswatini (formerly Swaziland), and apparently *A. muscaria* was moved as well, “The commonest
 111 mushrooms in Usutu Forest of Swaziland [now Eswatini] in March 1967 were... *Laccaria laccata*,
 112 *Amanita muscaria*, *Boletus edulis*, *Rhizopogon roseolus*.” (Mikola, 1970).

113
 114 The fungus is acknowledged as a European introduction in the most recent scientific literature on the
 115 *Amanita* of South Africa (Reid & Eicker, 1991) and in a current field guide (Goldman & Gryzenhout,
 116 2019). While *A. muscaria* may have been introduced with European “stone and cluster pines” (Doidge,
 117 1950), in modern South African forestry plantations it associates with pines from other continents, for
 118 example Mexican *Pinus patula* and Californian *P. radiata*.

120 121 MATERIALS AND METHODS

122 Historical Records of South African *A. muscaria*

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

133 134 Mushroom Collections

135 We sequenced genomes from a total of 24 *A. muscaria* and 1 *A. pantherina* mushrooms collected
 136 from South Africa, Australia, Europe and North America (Table 1). South African ($n = 9$) and
 137 Australian ($n = 2$) specimens were collected from pine plantations while European *A. muscaria*
 138 ($n = 11$), *A. pantherina* ($n = 1$) and Californian ($n = 2$) mushrooms were collected from native

139 forests (Table 1; Fig. S1). Specimens were dried within 1-2 days using conventional food driers
 140 at low heat (less than 35 °C). Our sampling scheme was designed to contextualize South African
 141 *A. muscaria* by integrating new data within published phylogenies, primarily Geml et al. (2008);
 142 a comprehensive survey of the global species complex was beyond our scope. We targeted as
 143 many South African specimens as possible, European specimens from three countries (based on
 144 what we found and could collect), and other specimens from additional clades identified by
 145 Geml et al. (2008), for example Clade I, represented by our two Californian mushrooms. Out of
 146 curiosity, we also sequenced two Australian specimens. Prior to sequencing, all specimens were
 147 stored as dried mushrooms at room temperature in either the Pringle or Vilgalys Laboratory
 148 fungaria at the University of Wisconsin-Madison and Duke University, respectively.
 149

150 **Table 1: Collection metadata for the specimens collected and analyzed.**

Specimen ID	Collection Date	Location	Region	Country	Latitude	Longitude	Species	Habitat	Host	Collector
11662	27-Feb-19	Bulwer	KwaZulu-Natal	South Africa	29.8077 S	29.76 E	<i>A. muscaria</i>	plantation	Pinus patula	J. Roux
11663	27-Feb-19	Bulwer	KwaZulu-Natal	South Africa	29.8077 S	29.76 E	<i>A. muscaria</i>	plantation	Pinus patula	J. Roux
11664	27-Feb-19	Bulwer	KwaZulu-Natal	South Africa	29.8077 S	29.76 E	<i>A. muscaria</i>	plantation	Pinus patula	J. Roux
11665	6-Mar-19	Bulwer	KwaZulu-Natal	South Africa	29.8077 S	29.76 E	<i>A. muscaria</i>	plantation	Pinus patula	J. Roux
11666	25-Apr-19	Kranskop / Greytown	KwaZulu-Natal	South Africa	28.9667 S	30.8642 E	<i>A. muscaria</i>	plantation	Pinus elliottii	I. Greylung
11667	25-Apr-19	Kranskop / Greytown	KwaZulu-Natal	South Africa	28.9667 S	30.8642 E	<i>A. muscaria</i>	plantation	Pinus elliottii	I. Greylung
11668	25-Apr-19	Kranskop / Greytown	KwaZulu-Natal	South Africa	28.9667 S	30.8642 E	<i>A. muscaria</i>	plantation	Pinus elliottii	I. Greylung
11669	25-Apr-19	Kranskop / Greytown	KwaZulu-Natal	South Africa	28.9667 S	30.8642 E	<i>A. muscaria</i>	plantation	Pinus elliottii	I. Greylung
11670	25-Apr-19	Kranskop / Greytown	KwaZulu-Natal	South Africa	28.9667 S	30.8642 E	<i>A. muscaria</i>	plantation	Pinus elliottii	I. Greylung
Roed_3	12-Sep-19	Østfold	Østlandet	Norway	59.4237 N	10.5937 E	<i>A. muscaria</i>	native habitat	Not recorded	S.L. Harrow
Sogn_5	15-Sep-19	Oslo	Østlandet	Norway	59.9822 N	10.7415 E	<i>A. muscaria</i>	native habitat	Not recorded	S.L. Harrow
Skrap_3	17-Sep-19	Oslo	Østlandet	Norway	59.8657 N	10.8562 E	<i>A. muscaria</i>	native habitat	Not recorded	S.L. Harrow
Nes_1	17-Sep-19	Akershus	Østlandet	Norway	59.8666 N	10.5181 E	<i>A. muscaria</i>	native habitat	Not recorded	S.L. Harrow
Frag_1	18-Sep-19	Oslo	Østlandet	Norway	59.9820 N	10.6742 E	<i>A. muscaria</i>	native habitat	Not recorded	S.L. Harrow
Ring_1	6-Oct-19	Zurich	Zurich	Switzerland	47.3617 N	8.4853 E	<i>A. muscaria</i>	native habitat	Not recorded	S.L. Harrow
Wirz_3	7-Oct-19	Nidwalden	Nidwalden	Switzerland	46.9053 N	8.3696 E	<i>A. muscaria</i>	native habitat	Not recorded	S.L. Harrow
Gril_1	7-Oct-19	Nidwalden	Nidwalden	Switzerland	46.9010 N	8.3571 E	<i>A. muscaria</i>	native habitat	Not recorded	S.L. Harrow
Kara_3	7-Oct-19	Zurich	Zurich	Switzerland	47.3617 N	8.4853 E	<i>A. muscaria</i>	native habitat	Not recorded	K. O'Keefe
Nagy_Heves_A	12-Oct-13	Heves	Heves	Hungary	47.3182 N	13.8414 E	<i>A. muscaria</i>	native habitat	Picea abies or Betula pendula	L. Nagy
Nagy_Heves_B	12-Oct-13	Heves	Heves	Hungary	47.3173 N	13.3415 E	<i>A. muscaria</i>	native habitat	Picea abies or Fagus sylvatica	L. Nagy
20031	17-Dec-21	Point Reyes National Seashore	California	United States	38.0519 N	122.8310 W	<i>A. muscaria</i>	native habitat	Not recorded	Wang/Pringle
20045	19-Dec-21	Point Reyes National Seashore	California	United States	37.9702 N	122.7304 W	<i>A. muscaria</i>	native habitat	Not recorded	Wang/Pringle
NzAUS95	27-May-19	Railton	Tasmania	Australia	41.2231 S	146.236 E	<i>A. muscaria</i>	plantation	Pinus radiata	Vilgalys/Henderson/Uehling
Aus332	31-Mar-18	Penrose	NSW	Australia	34.3748 S	150.1241 E	<i>A. muscaria</i>	plantation	Pinus radiata	Vilgalys/Henderson/Uehling
Nes_pan3	17-Sep-19	Akershus	Østlandet	Norway	59.8666 N	10.5181 E	<i>A. pantherina</i>	native habitat	Not recorded	S.L. Harrow

151 152 153 **Genome Extraction, Sequencing, Assembly, Annotation, and Quality Control**

154 **DNA extraction and sequencing**

155 High molecular weight DNA (HMW-DNA) was extracted from samples of dried mushroom
 156 tissues using previously published protocols involving a solution of 25:24:1
 157 phenol/chloroform/isoamyl alcohol (Bok *et al.*, 2005; Nickles *et al.*, 2023). Sequencing was
 158 performed with an Illumina NovaSeq 6000 platform with the aim of obtaining six million reads
 159 per sample, to achieve a coverage of 30x or higher. We estimated the genome size as 44 Mb.

160 **Genome assembly, annotation, and quality analyses**

161 Raw sequencing data were trimmed with Trimmomatic v0.36 (Bolger *et al.*, 2014) with
 162 LEADING:20 TRAILING:20 SLIDINGWINDOW:4:25 MINLEN:50, and genomes were
 163 assembled with SPAdes v3.11.1 (Bankevich *et al.*, 2012; Prjibelski *et al.*, 2020) using default
 164 parameters. Assemblies were filtered to keep only the contigs/scaffolds larger than 500 bp.
 165 Assembled genomes were annotated with Augustus v3.4.0 using models pre-built for *Laccaria*
 166 *bicolor* (Stanke *et al.*, 2008; Hoff & Stanke, 2013). Genome statistics of each genome were
 167 generated using seqtk (Li, 2013) and Benchmarking Universal Single-Copy Orthologs (BUSCO)
 168 (Simao *et al.*, 2015) completeness was determined using BUSCO v3.0.2 with the
 169 agaricales_odb10 database (Manni *et al.*, 2021).

Prior to examining the assembled genomes, basic assembly metrics were determined using seqtk. To screen for potential fungal contamination, duplicate BUSCO genes were identified with BUSCO (v3.0.2) 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 was then used to query NCBI's prokaryote nucleotide database (accessed July 27, 2023) in a local BLASTn search with default settings using NCBI's BLAST+ software package (v2.6.0) (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 S2).

183 Generating and filtering SNP predictions

Short reads were quality controlled with BBMap v38.32 (Bushnell, 2015) and aligned to the NCBI reference genome (accession GCA_001691765.1) using BWA mem v0.7.17 (Li & Durbin, 2009). Variants were called using Genome Analysis Toolkit v4.0.12.0 (McKenna *et al.*, 2010) using parameters and hard filters identical to Drott *et al.* (2023). Single nucleotide polymorphism (SNP) data were filtered using VCFtools (v0.1.13) (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 (Supplemental Methods).

193 Clone Correction and Population Genetics

194 Clone correction

To test if different specimens are clones of each other, i.e., 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.

199 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 5,000 bp sliding windows using VCFtools (v0.1.13) (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.

208 Phylogenomic Analyses

209 Nomenclature of the *A. muscaria* species complex and closely related species and 210 downloading of publicly available *A. muscaria* gene markers

We contextualized newly sequenced genomes using sequences of the internal transcribed spacer (ITS), β-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;

214 Vargas *et al.*, 2019) (Dataset S1). Specimen ID codes from Oda *et al.* (2004) were shortened to
 215 only include the numeric ID. The taxonomy and nomenclature of *A. muscaria* s.l. is complex,
 216 and we used published literature to develop naming protocols.
 217

218 Following recent precedent (Vargas *et al.*, 2019), we used Geml *et al.*'s (2008) clade labels to
 219 identify our specimens. While taxonomists used to consider *A. muscaria* as a complex of
 220 subspecific varieties existing under a single species concept and identifiable on the basis of cap
 221 color (Miller & Jenkins, 1978), when specimens with different cap colors were integrated into
 222 single phylogenies using DNA sequence data, new species concepts emerged (Oda *et al.*, 2004;
 223 Geml *et al.*, 2006). Geml *et al.* (2008) represents the last major phylogenetic revision of the taxa,
 224 but instead of naming clades as species, Geml *et al.* (2008) chose to label distinct clades with
 225 roman numerals. We use the same numbering system for our specimens and for the specimens
 226 associated with already published data (Oda *et al.*, 2004; Geml *et al.*, 2008; Vargas *et al.*, 2019),
 227 with two exceptions: after Geml *et al.* (2008) was published, *A. persicina* was formally
 228 recognized as a species, and we use its name (Tulloss *et al.*, 2015). We also use the name *A.*
 229 *regalis* because it was formally published in a previous revision of the species complex (Neville
 230 & Poumarat, 2004) and is supported by the later phylogenetic evidence (Geml *et al.*, 2008).
 231

232 We used the same logic to identify specimens associated with other public data. Any specimen
 233 originally classified as and submitted to GenBank as one of the widely recognized and currently
 234 accepted infraspecific taxa of *A. muscaria* was given the working designation *A. muscaria* s.l.
 235 and later identified using Geml *et al.*'s (2008) clade labels. For our purposes, *A. muscaria* s.l.
 236 includes *A. muscaria* var. *guessowii*, *A. muscaria* var. *muscaria*, and *A. muscaria* var.
 237 *flavivolvata*. However, if a specimen had a GenBank accession for ITS locus sequence data and
 238 was submitted as *Amanita regalis* (or *A. muscaria* var. *regalis*) or *Amanita persicina* (or *A.*
 239 *muscaria* var. *persicina*) we used the modern nomenclature (Tulloss & Yang, 2016), in other
 240 words the names *A. regalis* or *A. persicina*. These two taxa are easily differentiated from *A.*
 241 *muscaria* s.l. on the basis of gross morphology. Specimens classified as *A. pantherina* were kept
 242 as such. For the two specimens lacking an ITS accession, the metadata associated with the LSU
 243 accession was used instead. All collection location data were extracted from the publication
 244 corresponding to each given collection.

245 ***Species tree construction using multi-loci barcodes***

246 We aligned ITS (n = 189), β-tubulin (n = 102), LSU (n = 98), and TEF-1α (n = 52) sequences
 247 with MAFFT v7.511 (Katoh & Standley, 2013) with an automated model selection (L-INS-i,
 248 FFT-NS-i and FFT-NS-2) and adjustment of reverse complements. Alignments were trimmed
 249 with Trimal v1.4.rev15 (Capella-Gutierrez *et al.*, 2009) with the gappyout parameter. Once
 250 trimmed, alignments for all four regions were used to construct a maximum likelihood
 251 phylogeny using an edge-linked partition model with IQ-TREE multicore version 2.2.0 (Minh *et*
 252 *al.*, 2020), with the optimal substitution model determined by ModelFinder (Kalyaanamoorthy *et*
 253 *al.*, 2017) and 1,000 rapid bootstraps.
 254

255 **Specialized Metabolite Biosynthetic Gene Cluster Prediction and Characterization**

256 ***Genome mining for specialized metabolite BGCs***

257 To identify canonical specialized metabolite BGCs (i.e., those with experimentally vetted class-
 258 defining core biosynthetic genes [termed backbone genes], e.g., polyketide BGCs, which are a

259 canonical class of SM defined by polyketide synthase backbone genes), Fungal antiSMASH (v5)
260 (Blin *et al.*, 2019) was run on all of the genomes using the default settings. (However, specimen
261 11662 was erroneously omitted from genome mining analyses.) Closely related terpene or PKS
262 BGCs were networked into gene cluster families (GCFs) using BiG-SCAPE v1.0.1 (Navarro-
263 Munoz *et al.*, 2020). An optimal networking cutoff of 0.3 was determined across a testing range
264 of 0.1 to 0.6 (Fig. S2 and S3).

265
266 We ran cblaster (v1.3.18) (Gilchrist *et al.*, 2021) using a previously characterized ibotenic acid
267 BGC sequence as the query to identify the non-canonical ibotenic acid BGC (Obermaier &
268 Müller, 2020), which is made up of the genes (from left to right) *iboA*, *iboF*, *iboD*, *iboC*, *iboG1*,
269 *iboH*, and *iboG2*. We further investigated the distribution of genes in the ibotenic acid BGC
270 using reciprocal best-hit blast analysis. Briefly: characterized protein sequences were used to
271 replace corresponding protein predictions of IboA, IboC, IboD, IboF, IboG1, IboG2, and IboH
272 (Kohler *et al.*, 2015) in the file containing the proteome of mushroom Gril_1. The resulting
273 proteome was queried against a dataset of all annotated publicly available protein sequences
274 downloaded from NCBI in the spring of 2023 with methods similar to those described previously
275 (Drott *et al.*, 2020). Any two hits that were within 30 kb of each other were considered
276 physically clustered. The presence of a putative ortholog was mapped to a modified version of
277 the whole-kingdom phylogeny from Nickles *et al.* (2023) using ggtree (Yu *et al.*, 2017).
278

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

282 ***Phylogeny of Agaricomycete polyketide synthase (PKS) proteins***

283 A representative query protein was selected from the two conserved PKSs found within the *A.*
284 *muscaria* genomes. Each genome’s protein was BLASTp (Altschul *et al.*, 1990; Schaffer *et al.*,
285 2001) searched against every annotated Agaricomycetes genome publicly available on NCBI as
286 of December 1, 2022 (*n* = 292 genomes) with an e-value cutoff of 1e⁻⁵. Only the top hit for each
287 BLAST search was retained for further analysis. A gene tree of resulting sequences was
288 constructed with the same methodology used to construct the species tree. To confirm if the
289 observed PKS duplication was unique to the *A. muscaria* species complex, we also conducted a
290 targeted analysis of the *Amanita* genus. Genes encoding all PKS enzymes were identified from
291 the six available non-*muscaria* *Amanita* genomes. The resulting 18 PKS protein sequences were
292 used to construct a gene tree with the same methodology described to construct the species tree.
293 Domain analysis revealed a single hybrid NRPS-PKS protein in *A. inopinata* (KAF8636077.1),
294 which was subsequently used to root the tree.
295

296 **Metabolomics for Chemical Identification of Metabolites**

297 ***Methanolic extraction of metabolites***

298 We extracted metabolites from nearly every dried mushroom that had its genome sequenced (*n* =
299 22); the two Australian specimens (Aus332 and NzAUS95) were not included in metabolomics
300 because there were insufficient tissues left. Two additional North American (20031-a and 20045-
301 b) and one additional South African (11671) mushrooms whose genomes were not sequenced
302 were assayed, bringing the total number of metabolite extracts to 26 (*A. muscaria*: 25 and *A.*
303 *pantherina*: 1; Table S1).

304 All samples were imaged prior to extraction (see Fig. S1). Mushroom caps were pulverized and
305 extracted overnight in 20-100 mL (depending on the volume of the mushroom tissue) of HPLC-
306 grade methanol. Extracts were filtered with a 0.2 μ m syringe filter and evaporated to dryness on
307 a rotary evaporator. Compounds were resuspended at 1 mg/mL in methanol or dimethylsulfoxide
308 (DMSO) for chemical analysis or biological assays, respectively.
309

310 ***UHPLC–MS/MS analysis***

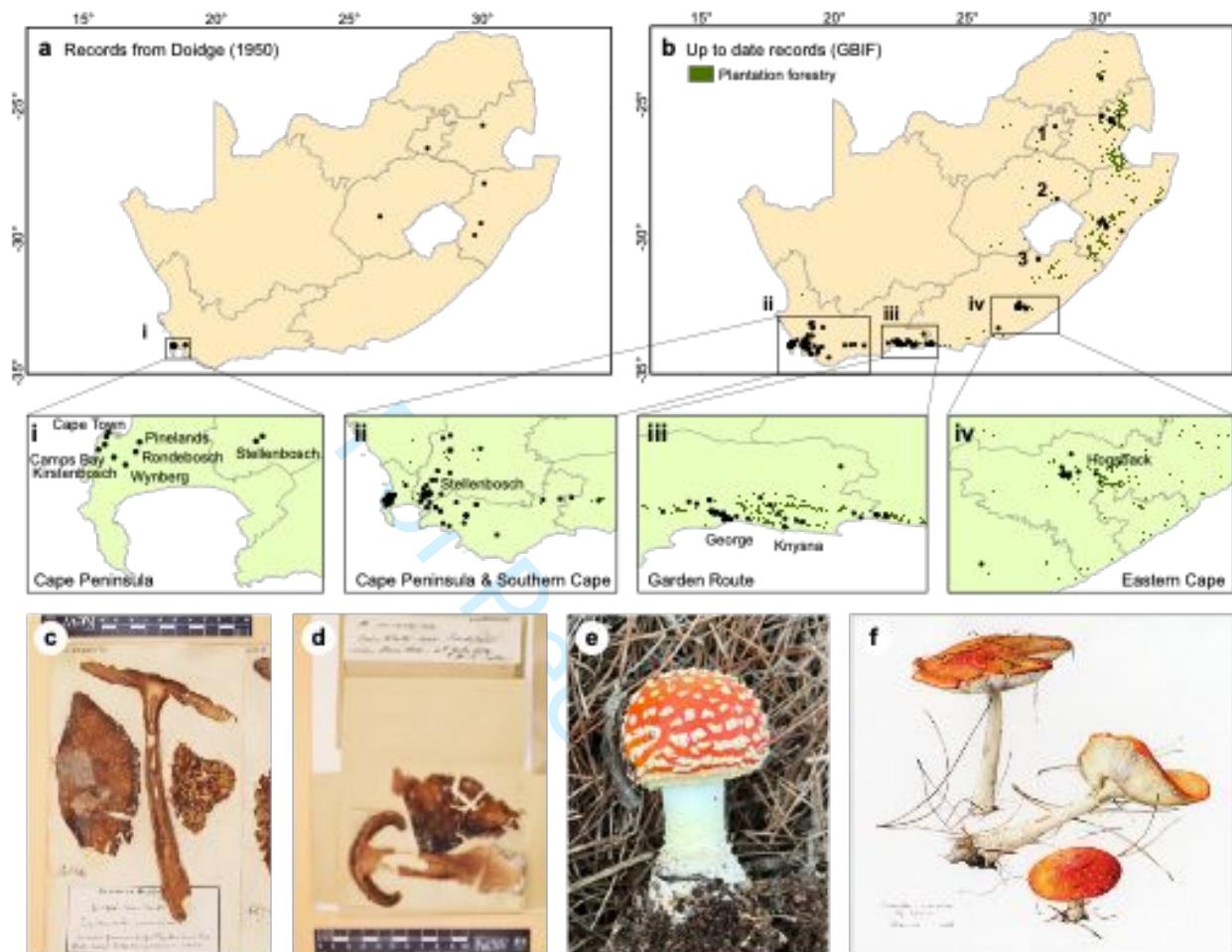
311 Mushroom cap extracts were characterized on Ultra-high-performance liquid chromatography
312 coupled with tandem mass spectrometry (UHPLC–MS/MS) using only spectroscopic grade
313 solvents. Data was acquired using a Thermo Fisher Scientific Q Exactive Orbitrap mass
314 spectrometer (Waltham, MA, USA) coupled to a Vanquish UHPLC (Waltham, MA, USA)
315 operated in positive ionization mode. For all runs, we used a Waters XBridge BEH-C18 column
316 (2.1 mm \times 100 mm, 1.7 μ m) and added 0.05% formic acid to our spectroscopic grade acetonitrile
317 and water (flow rate 0.2 mL/min). The 37 min screening gradient method for the samples is as
318 follows: Starting at 10% organic for 5 min, followed by a linear increase to 90% organic over 20
319 min, another linear increase to 98% organic for 2 min, holding at 98% organic for 5 min,
320 decreasing back to 10% organic for 3 min, and holding at 10% organic for the final 2 min.
321

322 ***Global Natural Product Social Molecular Networking (GNPS) analysis***

323 To construct a molecular network, the raw mass spectra were converted to the mzXML format
324 using RawConverter software (ver.1.2.01., The Scripps Research Institute) (He *et al.*, 2015). We
325 utilized GNPS analysis, the web-based server, with the default parameters provided by the
326 platform: Min pairs cos = 0.7, network TopK = 10, max connected components size = 100, min
327 matched fragment ions = 6, min cluster size = 2. A molecular network was created with a
328 precursor ion mass tolerance of 2.0 Da and fragment ion mass tolerance of 0.5 Da. The resulting
329 molecular network was visualized in Cytoscape (ver.3.10.2.) (Shannon *et al.*, 2003).
330

331 ***Preparing metabolites for bioactivity screening***

332 Every crude extract was tested against a range of the laboratory model organisms available to us
333 at the University of Wisconsin-Madison. Organisms included the pathogenic bacterium
334 Methicillin-resistant *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa*, and the
335 pathogenic fungus *Candida auris*. Because experiments involving invertebrates are more
336 complex, crude extracts from only a subset of the samples were used in tests against the
337 nematodes *Caenorhabditis elegans* and *Brugia spp.*, and the dipterans *Aedes aegypti* and *Musca*
338 *domestica*. For nematode screening, we used three extracts from each region (USA, South
339 Africa, and Europe) in addition to extracts from the outgroup, *A. pantherina*. For insect
340 screening, we used one sample from each of the three regions in addition to the outgroup (Table
341 S1). Crude extracts were redissolved in methanol (MeOH) to test for antimicrobial activity or
342 DMSO for invertebrate assays. Details of bioactivity assays are provided in the Supplemental
343 Methods section. Our focus on model organisms was a practical choice and enables basic
344 insights into bioactivity; future tests with species and populations of South African antagonists
345 are needed.
346

347 **RESULTS**
348

349
350 **Figure 1: Records of *Amanita muscaria* in South Africa.** Occurrence data illustrate the spread
351 of *A. muscaria* in South Africa over the last century. Panel (a) plots historical records up until
352 1945 as compiled by Doidge (1950). Insert i) is an enlargement of the Cape region. Panel (b)
353 plots contemporary records as of 24 July 2023, also illustrating plantation forestry land use.
354 Inserts are enlargements of ii) Cape Town and the southern Cape, iii) the Garden Route, and iv)
355 the Eastern Cape. Numbered occurrences (1-3) are not associated with plantation forestry and
356 instead record *A. muscaria* associated with pine trees in urban areas as in 1) Pretoria or 2)
357 Clarens, or in rural areas as in 3), where pines are often used as windbreaks or to adorn the edges
358 of farm roads. Panels (c) and (d) are images of *A. muscaria* specimens from Wynberg and
359 Rondebosch in Cape Town collected in 1883 and 1874, respectively. Both records provide
360 valuable information about associations with plants, e.g. “under Pine trees” in panel (d). Panel
361 (e) is a photograph of *A. muscaria* collected near pine trees on a golf course (Simola Golf and
362 Country Estate) in 2023 in the Western Cape province. Panel (f) is an image from a card bought
363 at Kirstenbosch Botanical Gardens in Cape Town in 2023, reflecting widespread awareness of
364 the fungus in South Africa.

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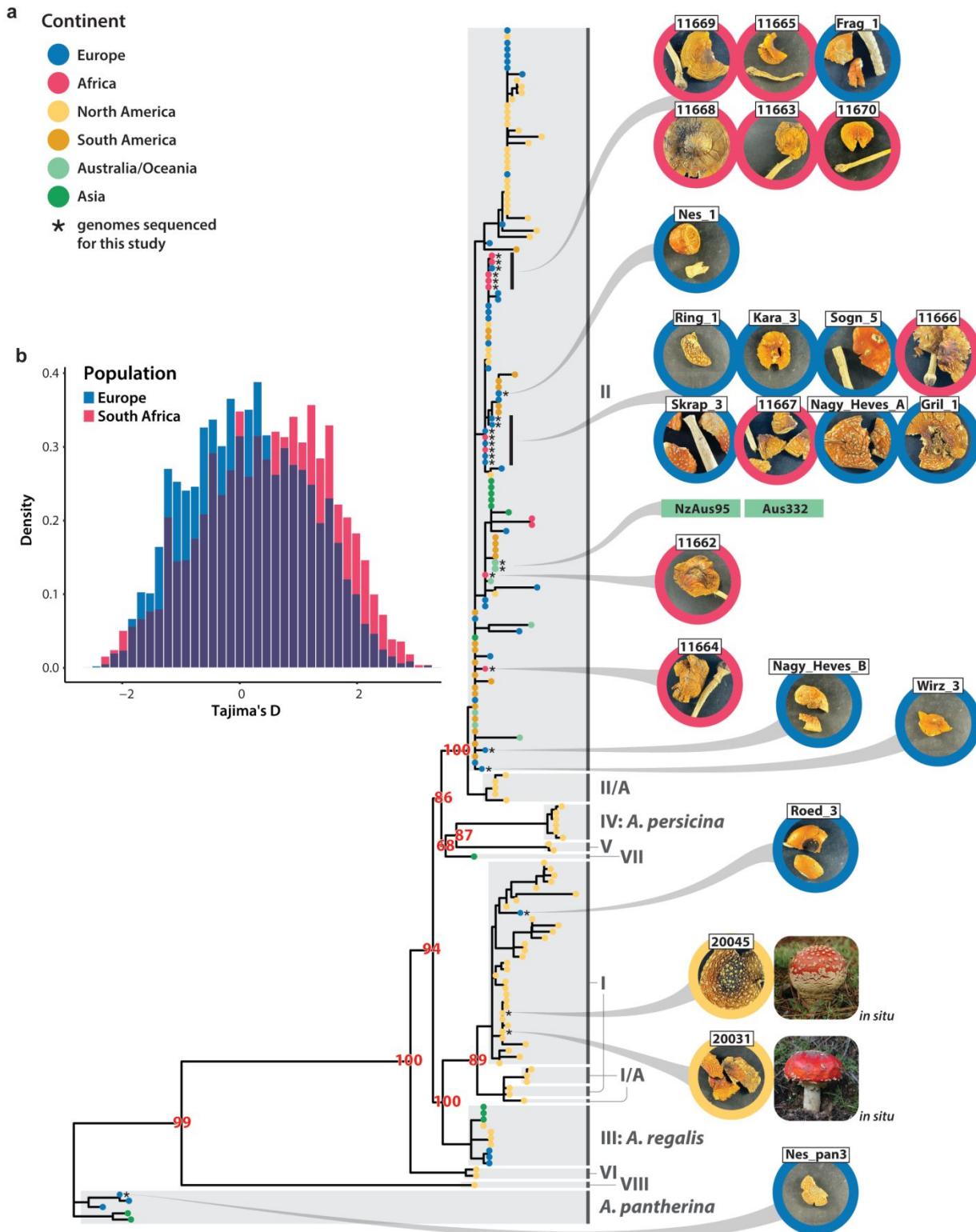
367 **South African *A. muscaria* Derive from a Single European Species**

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

372 To investigate the origin of South African *A. muscaria*, we reconstructed phylogenies of *A.*
373 *muscaria* using data from our own genomes and public repositories (Dataset S1); the topologies
374 of created trees were largely in agreement with minor differences (Sup. Methods; Fig. S4, S5).
375 The nine South African individuals we sequenced belong to Clade II of the *A. muscaria* complex
376 (Fig. 2a), a lineage also including specimens from Australia and South America, where *A.*
377 *muscaria* is also introduced and spreading (Vargas *et al.*, 2019). Clade II broadly corresponds to
378 *A. muscaria var. muscaria*, widespread across Eurasia and into Alaska.
379

380 Site frequency spectrum analyses support a historical introduction of *A. muscaria* into South
381 Africa. European Clade II individuals show a near-zero value (Fig. 2b), while South African
382 individuals display a positive shift, consistent with the loss of rare alleles during a founder event.
383 The European mushrooms consistently have higher nucleotide diversity (0.00166) and mean
384 heterozygosity (0.0799 ± 0.0132 [Table S2]) as compared to the South African population
385 (0.00118 and 0.0684 ± 0.0045 , respectively). Although sampling differences might influence
386 results, the results align with historical records and phylogenetic inferences. Additionally, though
387 the South African population shows signs of a bottleneck, the population remains diverse and is
388 sexual: no clones were detected in either population and kinships are typically below 0.2
389 (Dataset S3). However, one pair of mushrooms from a single *P. elliottii* plantation in KwaZulu-
390 Natal has a higher kinship (~0.33), suggesting some level of inbreeding, perhaps between
391 multiple monokaryotic mycelia of a shared parent.
392

393 Roed_3 was the only European individual we sequenced falling into Clades I-I/A. Clades I-I/A
394 represent a complex of two cryptic taxa widely distributed throughout North America.
395 Interestingly, mitochondrial DNA sequences placed Roed_3 closer to Clade II than Clades I-I/A,
396 a pattern we speculate as reflecting a hybridization event (Figs. S4 and S5).
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Figure 2: A phylogenetic species reconstruction of 184 *A. muscaria* and 5 *A. pantherina* mushrooms. (a) Phylogeny constructed from an edge-linked partition model of ITS, LSU, β -tub, and TEF-1 α gene sequences. Clades are labeled with either the numbers used in Geml *et al.* (2008) or as *A.*

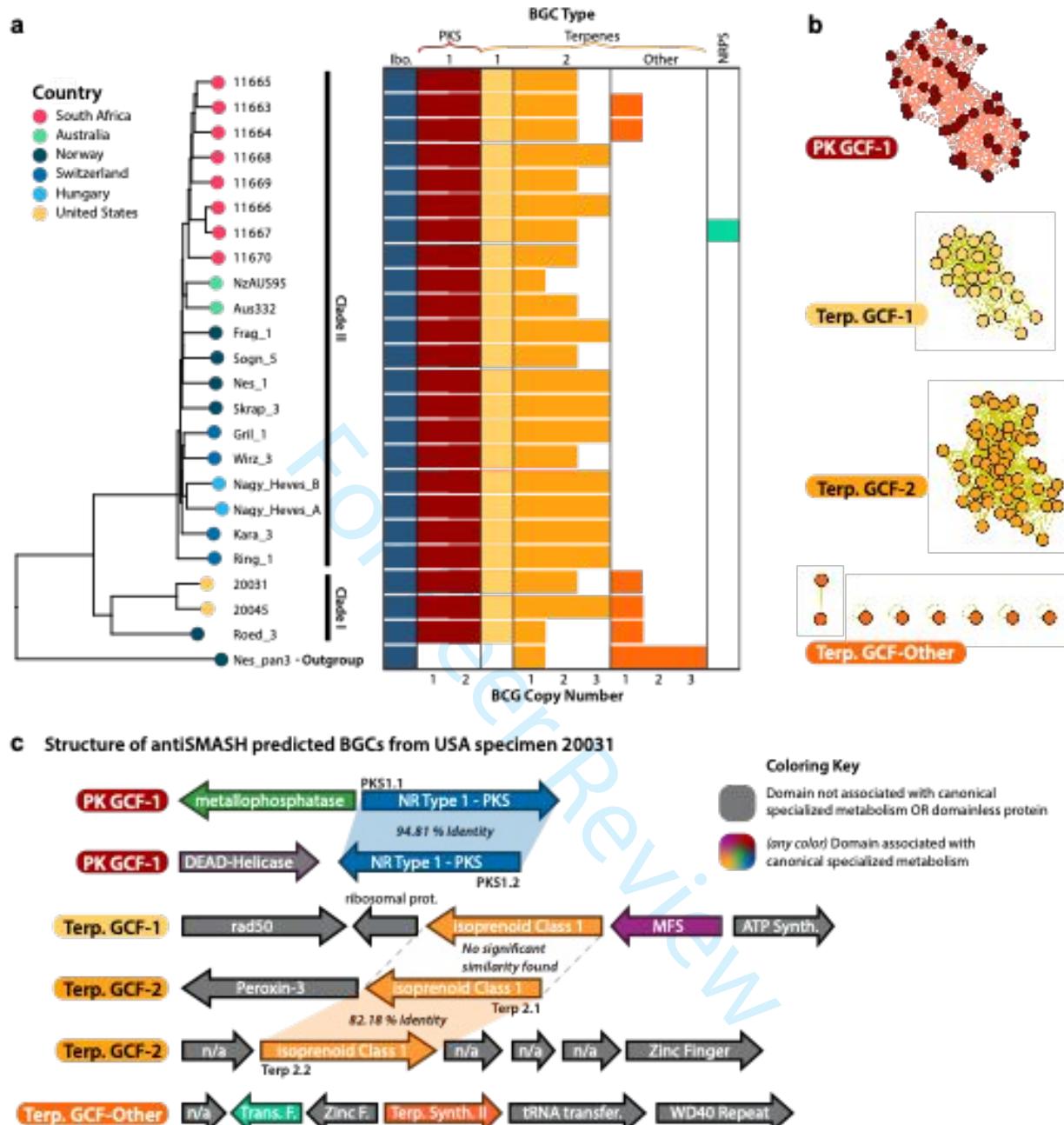
403 *persicina* or *A. regalis*; many of the labels from Geml *et al.* (2008) (e.g. clade VIII) represent undescribed
 404 taxa. Our phylogeny groups two specimens from Clade I in Geml *et al.* (2008) with Clade I/A. The tree is
 405 rooted with *A. pantherina*. Bootstrap support values are shown in red. Each mushroom's continent of
 406 origin is indicated by tip color, and available images of our sequenced mushrooms are displayed to the
 407 right of the phylogeny. Colors of the circles surrounding individual images mark the mushroom's
 408 continent of origin. (b) The distribution of Tajima's D was estimated from 5,000 bp windows across the
 409 reference genome using biallelic SNPs from the European (blue) and South African (red) Clade II
 410 genomes sequenced by us.

411 **Specialized metabolite gene clusters are conserved across geographic regions and**
 412 **phylogenetic clades.**

413 AntiSMASH predicted between 6–8 BGCs per genome, all of which were Type-1 polyketide
 414 synthases (PKSs) or isoprenoid(s) biosynthesis (terpene) backbone enzymes, with the exception
 415 of a single nonribosomal peptide synthetase (NRPS) found in the South African specimen 11667.
 416 All genomes, including the *A. pantherina* outgroup, contained the full ibotenic acid BGC (Fig.
 417 3a, S6, S7) as defined previously (Obermaier & Müller, 2020). While the products of most of
 418 these *A. muscaria* BGCs are unknown, we confirmed the ibotenic acid BGC produced muscimol
 419 in each of the *A. muscaria* mushrooms we sequenced, regardless of phylogenetic grouping or
 420 geographic origin. Muscimol was also made by the *A. pantherina* outgroup (Nes_pan3 in Fig.
 421 S8).

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

438
 439 Although *A. muscaria* has been reported to produce lethal amatoxins (Faulstich & Cochet-
 440 Meilhac, 1976), we found no genomic or chemical evidence—using bioinformatic searches,
 441 relaxed BLAST queries, and direct metabolite analysis—to support these claims (see
 442 Supplemental Methods).



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Figure 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 SNP data and rooted with *A. pantherina* (Nes_pan3). (b) Gene cluster family networks of the two major SM classes within the genomes: polyketides (PK) and terpenes. (c) Domain and genetic architecture of the antiSMASH-predicted BGC loci using Californian sample 20031-a (Clade I) as an example. Genes are depicted approximately to scale.

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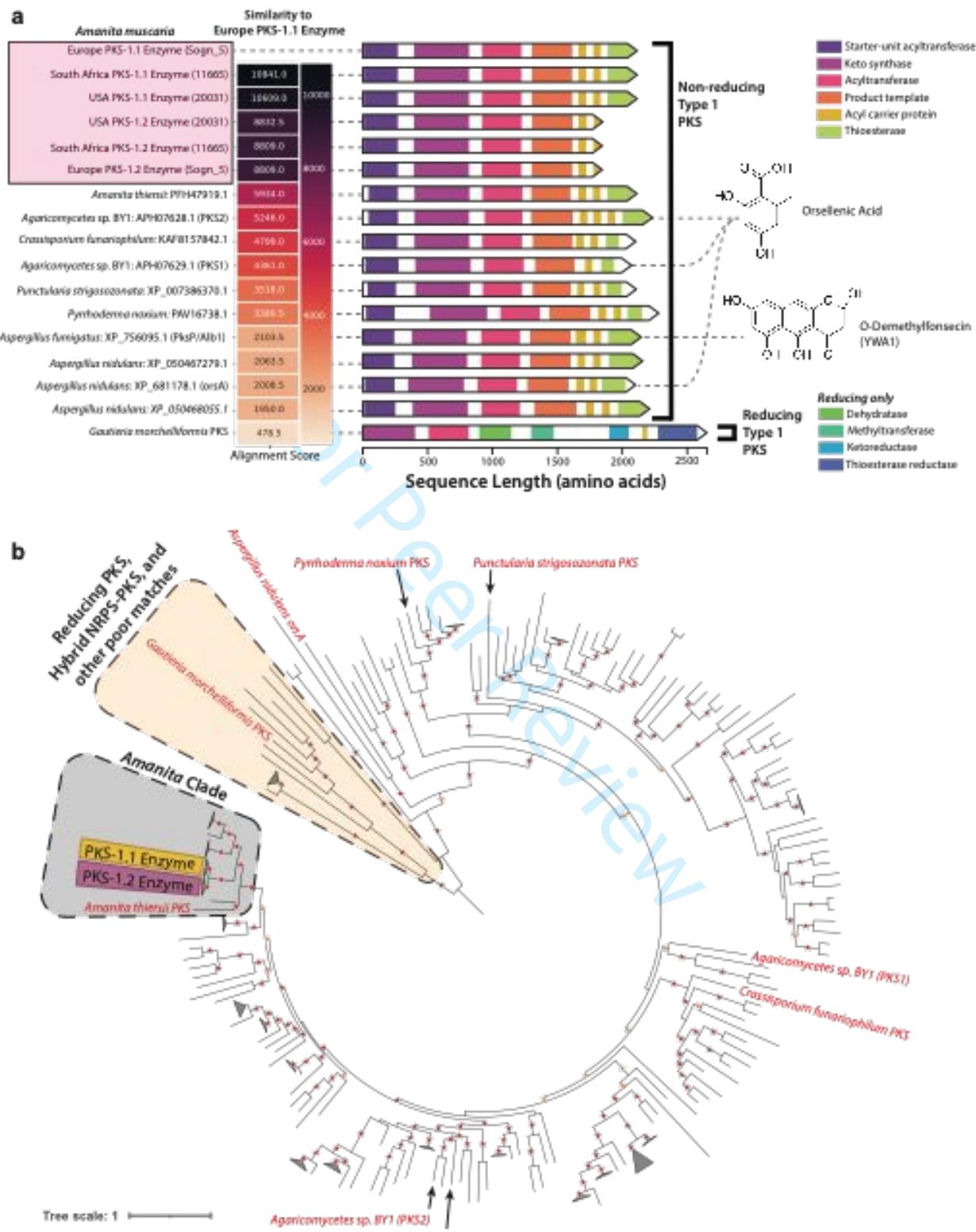
455 **Conserved PKS enzymes in *A. muscaria* originated from duplication in a common ancestor**
 456 **of the species complex**

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

467
 468 To test whether the duplication of PKS-1.1 and PKS1.2 enabled functional divergence, we
 469 compared domain variation between the two copies and a sampling of highly similar PKS
 470 enzymes across diverse fungi. The PKS1.2 backbone in all *A. muscaria* genomes, regardless of
 471 geographic region or clade, has lost its C-terminal thioesterase (TE) domain (Fig. 4a). This result
 472 is not an annotation error; we used tBLASTn to verify that the coding sequence for this domain
 473 was only present in the genomic DNA associated with PKS1.1. The C-terminal domain is
 474 typically involved in the chain release and cyclization of polyketide products (Tang *et al.*, 2019).
 475 While most PKS hits from closely related *Amanita* species were found in single copies within
 476 their genomes and retained the C-terminal thioesterase, a distinct PKS clade - from *Amanita*
 477 *brunnescens* - showed a similar pattern of duplication and loss, suggesting the pattern has
 478 evolved multiple times in the genus (Fig. S9).

479
 480 The domain structure of *A. muscaria*'s PKS enzymes closely mirrors the structure of known
 481 orsellinic acid-producing PKSs in other Agaricomycetes (Lackner *et al.*, 2013; Braesel *et al.*,
 482 2017) and in the Ascomycete *Aspergillus nidulans* (Schroeckh *et al.*, 2009) (Fig. 4a). While
 483 these findings may suggest *A. muscaria* is producing an orsellinic-acid-like compound, a
 484 similarly strong hit to a PKS in the *Aspergillus fumigatus* genome is associated with production
 485 of a melanin intermediate (Fujii *et al.*, 2000; Brakhage & Liebmann, 2005), raising some doubt
 486 about the true end-product of the *A. muscaria* PKS enzymes.
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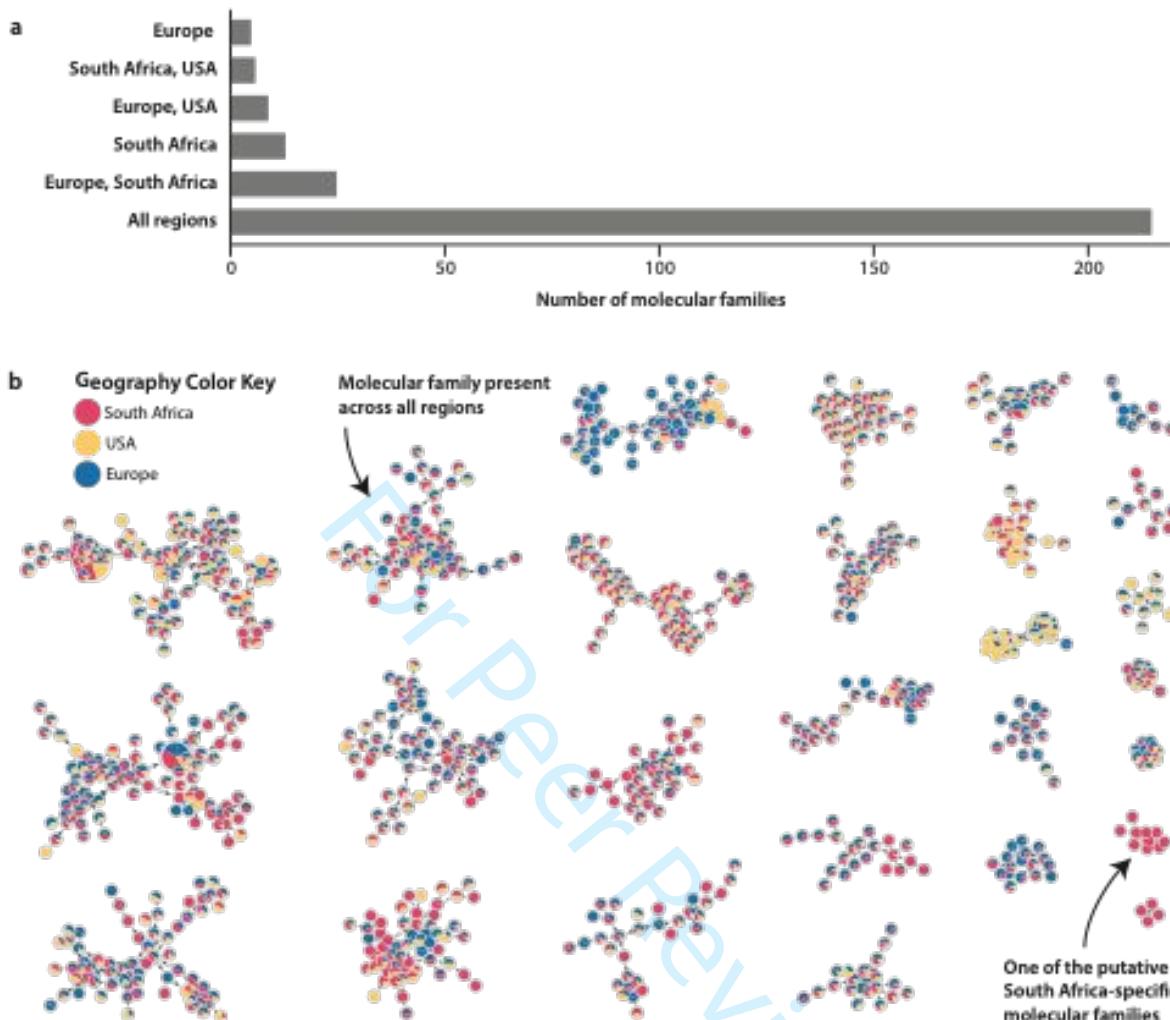
Figure 4: Analysis of the Type 1 polyketide synthase (PKS) gene duplication found in *A. muscaria*. (a) Comparison of the two Type I PKS enzymes found in the PK GCF-1 gene cluster family (termed PKS-1.1 and PKS-1.2), including copies from European, South African and Californian *A.*

494 *muscaria* genomes (highlighted in pink box). Left to right: Similarity scores were calculated by aligning
495 each PKS sequence to the Sogn_5 PKS-1.1 backbone enzyme. The domain architecture of each PKS is
496 color-coded. Known metabolites associated with specific PKSs are linked by dashed lines to the
497 corresponding structures on the right. (b) Midpoint-rooted phylogeny of 244 Agaricomycete PKS proteins
498 with additional hand-selected PKS proteins (e.g., reducing and non-reducing PKSs, *orsA* from *Aspergillus*
499 *nidulans*). The tree was midpoint-rooted to facilitate clade visualization. Clades with an average branch
500 length of less than 0.3 to their leaves were collapsed and displayed as triangles sized proportionally to the
501 number of collapsed leaves. The monophyletic clade containing all *Amanita* PKS proteins is shaded in
502 gray. A detailed analysis of proteins from the genus is provided in Fig. S9. The clades for *A. muscaria*
503 PKS-1.1 and PKS-1.2 are highlighted in yellow and purple, respectively. Nodes with > 80% and > 90%
504 support are indicated with orange and red stars, respectively. A subset of the proteins used to build the
505 phylogeny are labeled for reference.

506
507 **GNPS-based untargeted mass spectrometry analysis suggests the *A. muscaria* metabolome**
508 **is highly conserved across native and novel ranges**

509 GNPS analysis identified 273 unique molecular families (MFs) across all spectra (Fig. S10)
510 including both primary and specialized metabolic features (Fig. 5a; see Methods). Of the 273
511 MFs, 215, including all MFs with at least 11 nodes, were detected in every geographic region.
512 The largest MF (MF-21), comprising 100 unique nodes, was predicted to belong to a primary
513 metabolism phospholipid family. We identified 25 MFs unique to *A. muscaria* Clade II samples
514 (South Africa and Europe) and 13 MFs specific to South African *A. muscaria*. The largest South
515 Africa-specific MF (MF-9) was made up of 10 unique nodes (Fig. 5b). These nodes had
516 precursor m/z values between 900 and 1,200, with no matches to known library entries or
517 previously characterized metabolites.

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519



520
521 **Figure 5: The metabolome of *A. muscaria* contextualized by geography.** (a) Count of
522 molecular families (MF) grouped by geographic region. (b) Network visualization of a subset of
523 MFs. Node size reflects the total precursor intensity. Each node includes a pie chart marking the
524 geographic regions where samples containing that MF were located. The full network of all
525 detected MFs is in Fig. S10.

526
527 ***A. muscaria* extracts reveal biological activity/ inactivity, with no population-specific**
528 **patterns**

529
530 To understand the potential of *A. muscaria* metabolites to have broad-spectrum activity against
531 bacteria, filamentous fungi, and yeasts, with potential relevance for ecological inferences and
532 drug discovery, we performed disk-diffusion assays against Methicillin-resistant *Staphylococcus*
533 *aureus* (MRSA; Gram-positive; Fig. S11), *Pseudomonas aeruginosa* (Gram-negative; Fig. S12),
534 and *Candida auris* (a *Saccharomyces* yeast; Fig. S13). None of these organisms were inhibited
535 by extracts (Table 2). In contrast to the microbial assays, extracts from nine geographically
536 representative *A. muscaria* samples and one *A. pantherina* sample all strongly inhibited the

537 growth of the clade V nematode *Caenorhabditis elegans* (Table 2; Fig. S14). Extracts also
538 immobilized the clade III parasitic nematodes *Brugia pahangi* and *Brugia malayi*, and assays
539 with *B. malayi* reveal the impact of extracts on *B. malayi* was similar to heat-killed controls (Fig.
540 S14). Insect assays using model organisms revealed a more selective pattern: while adult *Musca*
541 *domestica* and *Aedes aegypti* were unaffected by the extracts, first-instar *A. aegypti* larvae were
542 highly susceptible. In general, bioactivity declined as animals reached later developmental stages
543 (Fig. S15). While the model organisms available to us might be unlikely to interact with *A.*
544 *muscaria* in nature, our findings suggest there is variation in dipteran susceptibility, even despite
545 the presence of GABA_A receptors in flies. Our data also suggest potential bioactivity may be
546 limited to early developmental stages in certain species. We note we commonly find dipteran
547 eggs laid among the gills of *A. muscaria* (Drott, Pringle and Stokes, pers. obs.). None of the
548 bioactivities observed were different among the mushrooms collected from different continents.
549 Our experiments targeted model systems, but important differences may exist between the
550 models we used and the populations of species interacting with *A. muscaria* in South Africa and
551 elsewhere.

552

553 **Table 2: Summary of the bioactivity of *A. muscaria* extracts against Methicillin-resistant**
554 ***Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa*, *Candida auris*, *Caenorhabditis***
555 ***elegans*, *Brugia* spp., *Musca domestica*, and *Aedes aegypti*.** The table displays (from left to right)
556 the number of extracts (mushrooms) tested in each experiment, the viability of the tested organism when
557 confronted with extracts, the concentrations used, the method of inoculation, and a reference to the Sup.
558 Figure(s) with full results.

Organism	Number of extracts tested (n)			Bioactivity	Lowest conc. with bioactivity	Highest conc. tested	Bioactivity assay ran	Sup. Figures with all results
	Europe	SA	USA					
 MRSA (Gram positive pathogen)	12	10	4		n/a	50 mg/ml	Disc diffusion assay	S10
 Pseudomonas aeruginosa (Gram negative pathogen)	12	10	4		n/a	50 mg/ml	Disc diffusion assay	S11
 Candida auris (Yeast pathogen)	12	10	4		n/a	50 mg/ml	Disc diffusion assay	S12
 Caenorhabditis elegans (nematode)	4	3	3		50 µg/ml	1 mg/ml	96-well liquid assays	S13
 Brugia spp. (nematode parasite)	4	3	3		10 µg/ml	1 mg/ml	96-well liquid assays	S13
 Adult Musca domestica (Housefly)	1	1	1		n/a	1 mg/ml	Administered by cotton wick	S14
 First/Late Instar Aedes aegypti (yellow fever mosquito)	1	1	1	  First Late	1 mg/ml	1 mg/ml	24-well liquid assays	S14
 Adult Aedes aegypti (yellow fever mosquito)	1	1	1		n/a	1 mg/ml	Administered by cotton wick	S14

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560

561

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 years 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

572 genetically diverse, with multiple distinct individuals present within single forest stands. Despite
573 its extended history in South Africa, the specialized metabolome of *A. muscaria* remains highly
574 conserved, with gene clusters encoding muscimol, terpene, and polyketide products present in all
575 genomes. Bioactivity assays with laboratory model organisms reveal the potent nematicidal
576 effects of the fungus's metabolites, suggesting their potential (as yet untested) role in mediating
577 ecological interactions.

578
579 The European Clade II lineage found in South Africa is also the lineage introduced to Colombia
580 (Vargas *et al.*, 2019) and Australia (Lebel *et al.*, 2024) (Fig. 2a). Remarkably, the global
581 dispersal of *A. muscaria* appears to involve just one of its many cryptic species. Europe has long
582 been suspected as the source of Australian *A. muscaria*, but we are the first to identify Clade II as
583 the specific lineage introduced there. More intensive sampling will be needed to pinpoint the
584 exact European source(s) of South African *A. muscaria*, and additional sequencing may reveal
585 whether other clades within the complex have been introduced elsewhere in the country.
586 However, based on available data, Clade II appears to be the primary lineage driving invasions,
587 suggesting underlying genetic, ecological, or historical factors contributing to its spread.
588 The fungus may also have been introduced to South Africa from another invasive range, for
589 example, Colombia or Australia. But historical records suggest the most parsimonious
590 explanation for the *A. muscaria* in South Africa is a direct introduction(s) from Europe (Box 1,
591 Fig. 1). Interestingly, several of our phylogenies (but not all) suggest South African *A. muscaria*
592 share their most recent common ancestry with Australian populations, a pattern which may
593 reflect a shared European source population (Fig. S4, S5).

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

600 The introduction of *A. muscaria* into South Africa is also reflected in site-frequency spectra,
601 where a positive shift in Tajima's *D* estimates among Clade II mushrooms (Fig. 2b) is consistent
602 with a founder event followed by insufficient time and/or evolution to regenerate rare alleles. A
603 similar pattern has been observed in *A. phalloides* populations introduced to California from
604 Europe (Drott *et al.*, 2023). Other population genetic statistics, including Fst estimates, also
605 suggest South African populations are differentiated from their European counterparts (Fig. S16-
606 a). Loci with the highest Fst scores encode genes associated with oxidoreductase activity, metal
607 binding, and cell membranes—functions previously linked to host and environmental stress
608 responses in fungi (Fig. S16-b) (Staerck *et al.*, 2018; Feng *et al.*, 2023). Further research is
609 needed to clarify the significance of the differences.

610 Despite the different ecological contexts of Europe, South Africa, and Australia, all sequenced *A.*
611 *muscaria* genomes housed a similar complement of BGCs, including the ibotenic acid BGC (Fig.
612 3, S6, S7), an expanded set of terpenes, and two PKS gene clusters (Fig. 4). Research on the
613 ecology of *A. muscaria* has focused primarily on its ecological niche; the ECM symbiosis has
614 one of its origins in the *Amanita* genus (Wolfe *et al.*, 2012). Although little is known about the
615 role of specialized metabolites in the ECM symbiosis, we identified an expansion of an orsellinic

acid-like PKS ortholog maintained across populations (Fig. 3, 4; how the duplicate genes are expressed remains a target for future research). One of these orthologs has lost a TE domain, suggesting 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 specialized metabolites 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 biosynthetic gene clusters (BGCs) are highly conserved, we also identified 13 molecular families 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 molecular families may represent metabolic innovations influencing fungal interactions in the novel range. The differentiation of metabolic space between fungal populations is often small 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 and 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 claims of its efficacy. As early as 1779, French botanist Jean Bulliard challenged claims of insecticidal activity, noting that 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, bacteria, or yeast (Table 2). The strong nematicidal properties of *A. muscaria*, which our study has reproduced, have previously been attributed to its specialized metabolites, ibotenic acid and muscimol, which are known agonists to the GABA_A receptor complex (Johnston, 2014). Muscimol resistance has been documented among mycophagous dipterans but

660 not their frugivorous counterparts (Tuno *et al.*, 2007), emphasizing questions about the
661 susceptibility of relevant antagonists in the introduced range.
662
663 In the aggregate, our data highlight the genetic and metabolic versatility of *A. muscaria*, a
664 globally invasive ectomycorrhizal fungus. While our results offer insights into the origin of
665 introduced populations, the evolution of the *A. muscaria* lineage, and the genomic signatures of
666 these introductions (or their lack), these findings also emphasize many unanswered questions:
667 how does *A. muscaria* interact with local antagonists in its novel ranges, and does enemy release
668 or do novel weapons play a role in the spread of the fungus? Do population-specific molecular
669 families contribute to its success? Our results offer insights into the global distribution of *A.*
670 *muscaria*, creating a strong foundation for future research exploring how fungal invasions and
671 co-invasions shape ecosystems across the globe.

For Peer Review

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685 legacy of service to mycology.

686 COMPETING INTERESTS

687 The authors declare no competing interests.

688

689 AUTHOR CONTRIBUTIONS

690 Our study stems from a unique collaboration between scientists working in South Africa and the
691 United States, and together we span diverse countries and career stages. Our collaboration began
692 at a U.S. National Science Foundation-funded workshop (Grant No. 1953299 awarded to JDH
693 and JSB) held at Future Africa (www.futureafrica.science) in November 2022. The workshop
694 initially focused on introducing students to invasion biology and providing bioinformatics
695 training, using *Amanita muscaria* s.l. genomes as a teaching tool. AP and JDH conceived the
696 study, and it was developed fully over the following 14 months. GRN designed and drafted the
697 manuscript, leading the bioinformatic and natural product experiments and analyses. SRF,
698 KMTL, DLN, and CKS (listed in reverse alphabetical order as equal co-second authors)
699 contributed substantially to analyses and writing. CB also contributed significantly to analyses
700 and writing. GRN, NPK, JWB, and AP sequenced genomes. Historical research was led by
701 MAH, AN, and AP. Y-WW led kinship analyses. Metabolic extractions and mass spectrometry
702 analyses were conducted by GRN, SCP, and NPK. Bioactivity work was designed, executed, and
703 analyzed as follows: microbial assays by GRN, CKS, and NPK; insect assays by GRN, TKW,
704 HLN, MEMM, and KLC; and nematode assays by GRN, KTR, and MZ. JDH, MTD, and AP
705 contributed to data analysis, interpretation, and writing, with MTD and AP leading revisions to
706 interpretations and the manuscript. All authors participated in workshop discussions, contributed
707 to initial analyses, and helped edit the final manuscript.

708

709 DATA AVAILABILITY

710 All genomic data analyzed in this study were generated by the authors. Raw reads and
711 assemblies are publicly available through NCBI BioProject [###]. Other data and methods
712 enabling replication are provided with this manuscript and in the Supporting Information (which
713 contains Supporting Methods, Tables, and Figures).

714

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1076 **SUPPORTING INFORMATION**

1077 Supporting Information may be found online in the Supporting Information section at the end of
1078 the article.

1079 **Table 1** Collection metadata for the specimens collected and analyzed.

1080 **Table 2** Summary of the bioactivity of *Amanita muscaria* extracts against Methicillin-resistant
1081 *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa*, *Candida auris*, *C. elegans*, *Brugia*
1082 spp., *Musca domestica*, and *Aedes aegypti*.

1083 **Fig. 1** Records of *Amanita muscaria* in South Africa.

1084 **Fig. 2** A species reconstruction of 184 *Amanita muscaria* and 5 *Amanita pantherina* isolates.

1085 **Fig. 3** Specialized metabolism (SM) in *Amanita muscaria* genomes.

1086 **Fig. 4** Analysis of Type 1 polyketide synthase (PKS) gene duplication found in all *Amanita*
1087 *muscaria* samples.

1088 **Fig. 5** The metabolome of *Amanita muscaria* grouped by the geographic region of the sample.

1089 **Dataset S1:** Full metadata for *A. muscaria* data retrieved from prior publications.

1090 **Dataset S2:** Genome summary statistics

1091 **Dataset S3:** Kinship analyses of the sequenced genomes

1092 **Table S1** Summary of the isolates used in this study.

1093 **Table S2** Summary of heterozygosity in sequenced genomes.

1094 **Fig. S1:** Dried tissue of each sample prior to chemical analyses.

1095 **Fig. S2:** Determination of optimal cutoff to generate the PKS GCF predictions.

1096 **Fig. S3:** Determination of optimal cutoff to generate the Terpene GCF predictions.

1097 **Fig. S4:** Rooted phylogenies from the fully sequenced *Amanita muscaria* genomes.

1098 **Fig. S5:** Tree compatibility comparisons between different species reconstruction methods.

1099 **Fig. S6:** A cblaster analysis showing the presence and absence of genes in the ibotenic acid
1100 cluster in the *A. muscaria* and outgroup genomes.

1101 **Fig. S7:** Whole fungal kingdom phylogeny depicting the number of Reciprocal best-hit BLAST
1102 hits to genes in the ibotenic acid gene cluster.

1103 **Fig. S8:** Quantity of putative muscimol in every sample.

- 1104 **Fig. S9:** Comparison of polyketide synthases found in *Amanita* genomes.
- 1105 **Fig. S10:** Full GNPS output of every molecular family, including singletons
- 1106 **Fig. S11:** Bioassays of metabolite extracts against Methicillin-resistant *Staphylococcus aureus*.
- 1107 **Fig. S12:** Bioassays of metabolite extracts against *Pseudomonas aeruginosa*.
- 1108 **Fig. S13:** Bioassays of metabolite extracts against *Candida auris*.
- 1109 **Fig. S14:** Nematode bioassay results after treatment with several concentrations of extracts.
- 1110 **Fig. S15:** Viability assays in *Ae. aegypti* and *M. domestica*.
- 1111 **Fig. S16:** GO-Term map corresponding to genes that overlapped with 5 kb sliding windows
1112 containing at least 100 SNPs and where estimates of Fst corresponded to the right 5% tail of
1113 estimates.
- 1114 **Fig. S17:** Phylogenetic trees constructed using a codon-aware alignment of the iboF and iboH
1115 genes.
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