RESEARCH ARTICLE

Amanita thiersii and *Amanita foetens* are closely related but genetically and geographically distinct species, leaving the origins of *A. thiersii* and its range expansion enigmatic

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Abstract

Background: The decomposer *Amanita thiersii* was originally described from a Texas lawn. Over time the species appears to have spread its range, but whether *A. thiersii* is an introduced and invading fungus or a native expanding its range remains an open question. A striking morphological similarity between *A. thiersii* and the Argentinian *A. foetens* led us to question whether the two species are the same. We hypothesized *A. thiersii* was simply an *A. foetens* introduced from Argentina.
Methods: We first compared the original species descriptions of both taxa. Next, we used databases associated with iNaturalist and Mushroom Observer to plot the global ranges of *A. thiersii* and *A. foetens*, revealing new reports of *A. thiersii* in Mexico and an expanded range in the United States of America. Next, we sequenced three genomes: an *A. thiersii* specimen from the U.S.A., an isotype of *A. foetens*, and an Argentinian specimen tentatively identified as *A. thiersii*. We reconstructed phylogenies using our own and publicly available data of other *Amanita* species. Because the genetic diversity of *A. thiersii* in the U.S.A appears to be very low, we also searched for mating type loci.
Results: Macroscopic descriptions suggest the two taxa are distinguishable by mushroom stature and the decoration of the stipe. The geographic ranges of the two taxa seem distinct and not overlapping, although the inconsistent names used by database users causes confusion. Phylogenies suggest the genomes of mushrooms

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collected in U.S.A. are different from Argentinian genomes. We discovered an individual which appears to have a mating type locus present in one nucleus of the dikaryon and absent from the second nucleus.
Conclusions: While *A. thiersii* and *A. foetens* appear strikingly similar, each is morphologically, geographically and genetically distinct, leaving the question of whether *A. thiersii* is native or introduced to the U.S.A. unanswered.

Keywords
Biogeography, decomposer fungi, invasive fungi, invasion biology, fungi, geographic distribution, taxonomy, introduced species, integrative species

This article is included in the Ecology and Global Change gateway.

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This article is included in the From genes to genomes: Investigating the population species boundary in non-model Fungi collection.

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Introduction

Humans often move organisms across continents, either deliberately or inadvertently, and by doing so facilitate long distance dispersal. Introductions may lead to invasions, and invasive species are one of the top five threats to Earth's biodiversity (Butchart *et al.*, 2010; Pyšek *et al.*, 2020). We define an invasive species as a species outside its natural range which becomes established in local habitats and threatens native biodiversity (Desprez-Loustau *et al.*, 2007). Invasive species can cause severe, often unpredictable problems. For example, the chestnut blight fungal pathogen, *Cryphonectria parasitica*, caused devastation to Chestnut tree populations in North America, which in turn had cascading, ecosystem-wide effects (Desprez-Loustau & Rizzo, 2011). Invasive nonpathogenic fungi have not received as much attention as invasive vertebrates and plants, but because decomposer and symbiotic fungi shape biodiversity (because they themselves are diverse, and through interactions with animals and plants) and because decomposer fungi drive biogeochemical cycles, their potential impacts on native species and ecosystem services are tremendous (DAISIE, 2012).

Fungi are ubiquitous, but only a fraction of Earth's total species have been described (Blackwell, 2011). Moreover, the past and present ranges of most fungal species remain unmapped, in part because the native habitats of many fungi are not documented: often, species are only known from the one or two specimens used to describe them (Pringle & Vellinga, 2006). In fact many fungi are described from places where they are introduced, for example, botanical gardens (Pringle & Vellinga, 2006). Finding a fungus somewhere does not necessarily mean it is native there (Golan & Pringle, 2017). Often, invasive fungi are identified as invasive only because they are charismatic or dramatically affect humans, for example the invasive Death Cap, which is deadly poisonous (Wang *et al.*, 2023). Unsurprisingly, invasions by plant pathogens like *C. parasitica* are more studied than invasions by decomposers or symbionts (Fisher *et al.*, 2012; Pringle & Vellinga, 2006). For example, while the story of the saprotrophic *Clathrus archeri*'s spread throughout Europe is more than a century old, its potential impacts on native fungal communities remain unknown (Brännhage *et al.*, 2021; Desprez-Loustau *et al.*, 2007). Nonetheless, the scarce data available confirm invasive nonpathogenic fungi matter: for example, the beetle symbiont *Flavodon subulatus*, which was introduced alongside its invasive beetle, suppresses native fungal species in the invasive range (Hulcr *et al.*, 2021; Jusino *et al.*, 2020).

The striking lawn mushroom *Amanita thiersii* is a saprotroph within the asymbiotic clade of the genus *Amanita* (Cui *et al.*, 2018; Tulloss *et al.*, 2016; Wolfe, Tulloss *et al.*, 2012). Originally described from College Station, Texas, U.S.A. in 1957 (Thiers, 1957), herbarium records from the 1960's onward document the dramatic spread of *A. thiersii* out of Texas and across the southern and midwestern United States (Wolfe, Kuo *et al.*, 2012). An expanding range is a hallmark of an invasive species and has been documented for other symbiotic species in the genus, not only for the Death Cap *Amanita phalloides* (Pringle *et al.*, 2009), but also the Fly Agaric *A. muscaria* (Vargas *et al.*, 2019). North American populations of *A. thiersii* appear to be genetically homogeneous (Wolfe, Kuo *et al.*, 2012). The lack of genetic diversity across its entire known distribution suggests an introduction associated with a genetic bottleneck. Although *A. thiersii* possesses characteristics of an invasive species, if it is native to Texas, then by definition it is not invasive. It may still be undergoing a range expansion, perhaps in response to climate change (Hobbie *et al.*, 2017).

But is *A. thiersii* truly native to North America? Morphological similarities between *A. thiersii* and another white decomposer, the Argentinian *A. foetens* (Singer, 1953), raise two questions: are the two species the same species? Was the species introduced to North America from Argentina? If *A. thiersii* was introduced to the U.S.A. from South America, its rapid geographic spread and the dramatic increase in its population size over recent decades would define it as an invasive species. As conservation biology slowly begins to focus on fungi, as well as animals and plants (Gonçalves *et al.*, 2021; May *et al.*, 2018), efforts to record and stop the spread of introduced and invasive nonpathogenic fungi are ramping up (Dickie *et al.*, 2016; Pyšek *et al.*, 2020). An essential prerequisite is the ability to differentiate between native and invasive fungi.

Using an integrative species concept (Barrett & Freudenstein, 2011; Wiens, 2007), we hypothesized the two species are the same; *A. thiersii* is simply an *A. foetens* introduced from Argentina. To test our hypothesis, we first revisited and compared the original species descriptions of *A. thiersii* and *A. foetens*, focusing on morphological similarities and differences. Next, we investigated their current global ranges using the biodiversity databases iNaturalist and Mushroom Observer. Finally, we sequenced three genomes and compared the sequence data of a U.S.A. *A. thiersii*, an isotype of *A. foetens*, and a recently collected Argentinian mushroom initially identified as *A. thiersii.* Our data provide a unique opportunity to document the history of an *Amanita* species currently spreading in North America.

Methods

Comparing the original species' descriptions

The species *Amanita thiersii* was first described by Harry D. Thiers from College Station, Brazos County, Texas, U.S.A. in 1957 (using the invalid name *A. alba* Thiers; Thiers, 1957), and it was later validly named for Thiers (Bas, 1969). The 1969 text is the protologue of *A. thiersii.* The specimens used to describe the species were collected

in September 1952 from a lawn. The species *Amanita foetens* was first described from Pié del Periquillo in Tucumán Province, Argentina by Rolf Singer (Singer, 1953). The specimens used to describe *A. foetens* were collected in December 1951 from a semiarid pasture. *Amanita foetens* was revised again at length by Bas (Bas, 1969). We used all descriptions in our comparisons.

Plotting the current global distributions of *A. thiersii* and *A. foetens*

We used two public databases to establish the current known distributions of *A. thiersii* and *A. foetens*: iNaturalist (iNat) and Mushroom Observer (MO). While MO uses the name *Amanita thiersii,* iNat uses the name "*Saproamanita thiersii.*" The generic name "*Saproamanita*" Redhead, Vizzini, Drehmel & Contu was proposed in 2016 for use with asymbiotic *Amanita* species (Redhead *et al.*, 2016), but it is controversial (Hawksworth, 2016; Tulloss *et al.*, 2016). Confusingly, iNat uses the generic name "*Amanita*" for *A. foetens,* even though it is also asymbiotic. Both iNat and MO are populated with observations of mushrooms submitted by the public. Names for observations are determined by popular vote on iNat and by a different, more complex community voting system on MO. To search in each database, we used the search terms "*Amanita thiersii*" and "*Amanita foetens*". Searching for "*Amanita thiersii*" in iNat leads to the page for "*Saproamanita thiersii,*" and using "*Amanita foetens*" leads to the page for "*Amanita foetens*". Data from iNat were downloaded between June 14 and 15, 2022, and MO data were downloaded on June 7, 2022. We used iNat data with a data quality assessment of "research grade" and additional observations with photos clearly resembling white *Amanita.* Next, we manually checked each individual observation in both datasets to confirm species identifications using the gross morphology visible in pictures, authors' descriptions, and/or DNA sequence data, as available. Observations without latitude and longitude were almost always excluded, as were observations not strongly resembling one of our target species. However, observations from South America were relatively rare (as compared to observations in North America), and in a few instances we estimated exact latitude and longitude coordinates from observer's location descriptions, especially for MO observations from South America. In these cases, coordinates are not exact. Eventually, all observations made outside of North and South America were removed because none matched the descriptions for either *A. thiersii* or *A. foetens.* Data from each of the databases were compiled into a single dataset (dataset on Dryad) and mapped. The locations of specimens used in genome sequencing were manually added to the dataset.

DNA extraction, genome sequencing and genome assembly

We sequenced the genomes of three mushrooms: AmanitaBASE 10801, 10802 and 10175. AmanitaBASE **10801** (Elmore, 2020) is an isotype of *A. foetens* sent from the University of Michigan herbarium (voucher: MICH4948) originally collected in Pié del Periquillo, Tucumán Province, Argentina by R. Singer and H. Helberger in December 1951 (Singer original voucher: T1672). AmanitaBASE **10802** is an *A. thiersii* mushroom collected by S. Kay from a lawn in Baldwin City, Kansas, U.S.A. in 2009 (Kay voucher: SKay4041). A single spore of mushroom SKay4041 was cultured and its haploid genome previously sequenced by Wolfe *et al.* (Wolfe, Kuo *et al.*, 2012; more fully described in Hess & Pringle, 2014). We re-sequenced the same single spore cultivar to take advantage of improved sequencing technologies. AmanitaBASE **10175** was collected in Córdoba, Argentina in 2014 and it was originally identified as *A. thiersii* by G. Robledo (Robledo voucher: G201); from this point forward, we refer to 10175 as an *Amanita* sp. We also refer to the genomes generated from each mushroom specimen by their AmanitaBASE numbers. DNA extraction for genome sequencing and library preparation followed protocols described by Wang (Wang *et al.*, 2023). Genomes were sequenced on the Illumina HiSeq 2500 short reads platform with 251 bp paired-end reads (Wang *et al.*, 2023).

To assemble the genomes of *A. thiersii* 10802*, A. foetens* 10801, and *Amanita* sp. 10175, the raw reads were first trimmed using bbduk from the BBMap suite ver. 38.32 (kmer length 23; Bushnell, 2016). The genomes were then assembled using SPAdes ver. 3.5.0 with default parameters using two libraries (Prjibelski *et al.*, 2020).

Phylogenetic trees for asymbiotic *Amanita*

Saprotrophic *Amanita* species are closely related to each other and basal to ectomycorrhizal *Amanita* (Wolfe, Tulloss *et al.*, 2012). To clarify the phylogenetic relationship among specimens collected as either *A. thiersii* or *A. foetens*, we obtained DNA sequences of the nuclear regions ITS, NucLSU (28S), and NucSSU (18S), and of the mitochondrial regions MitLSU, and MitSSU loci, from all saprotrophic or asymbiotic *Amanita* available from NCBI as of March 11, 2022. We included all sequences meeting the following criteria: 1) the sequence was from a specimen (Collector's ID) associated with at least two of the five loci of interest, and 2) the mushroom corresponding to the sequence was not identical to any represented by our own genomes. We included NCBI data from two *A. thiersii* specimens; one of them (Collector's ID SKay4041_het) is directly related to our sequenced single spore cultivar. It is the dikaryotic parent of our genome *A. thiersii* 10802, in other words, *A. thiersii* 10802 is the monokaryotic offspring of SKay4041_het. Because the diploid SKay4041_het data captures all of the genetic information of the original specimen, we omitted the haploid genome of *A. thiersii* 10802 from the 5-locus analysis. In total, we included data from eight asymbiotic *Amanita* species, each species represented by between one and three specimens, and from two specimens of an outgroup species (*Pluteus cervinus*) which also met our criteria (Table 1). We also identified and extracted the five loci from our remaining genomes

Table 1. Dataset of saprotrophic *Amanita* loci used to construct single-gene and concatenated gene trees. Table includes species name, the identifier given by the mushroom collector "Collector's ID", and all the NCBI accession numbers associated with that mushroom used in this analysis for the five loci.

Species	Collector's ID	NCBI GenBank Accession Numbers				
		NucLSU.28S	NucSSU.18S	ITS	Mit.LSU	Mit.SSU
Amanita inopinata	BW_GK2005-1	HQ539702.1	HQ539806.1	-	HQ540016.1	HQ539913.1
Amanita inopinata	PDD_95848	MT862260.1	-	HQ533044.1	-	-
Amanita praeclara	KUBOT-KRMK-2020-02	MW029933.1	-	MW031170.1	-	-
Amanita praeclara	RET_387-6	MH806864.1	-	MH806862.1	-	-
Amanita praeclara	RET_726-7	MK351833.1	-	MK351812.1	-	-
Amanita prairiicola	RET_266-1	HQ539727.1	HQ539831.1	HQ625015.1	HQ540038.1	HQ539938.1
Amanita pruittii	RET_343-2	KP866160.1	-	HQ625011.1	HQ540040.1	HQ539940.1
Amanita pruittii	RET_522-3	KM096567.1	-	KM096566.1	-	-
Amanita singeri	LIP_88.056	HQ539738.1	HQ539842.1	-	HQ540049.1	HQ539949.1
Amanita singeri	RET_272-4	MT229872.1	-	MK461186.1	-	-
Amanita sp-Ridley-2	PDD_93780	HQ539742.1	HQ539846.1	-	HQ540053.1	HQ539953.1
Amanita thiersii	DPL_7272	HQ539752.1	HQ539856.1	-	-	-
Amanita thiersii	SKay4041_het	HQ593114.1	-	HQ625010.1	-	-
Amanita vittadinii	HKAS101430	MH486950.1	-	MH508651.1	-	-
Amanita vittadinii	RET_277-1	HQ539757.1	-	-	HQ540068.1	HQ539967.1
Pluteus cervinus	Field_88934	HQ539765.1	HQ539868.1	-	HQ540075.1	HQ539975.1
Pluteus cervinus	Ghobad-Nejhad_4271	MT554283.1	-	MT535687.1	-	-

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(10801 and 10175) by querying the genomes with known sequences of closely related species using blastn from the BLAST+ suite (Altschul *et al.*, 1990). Sequences corresponding to the best BLAST hit were obtained using seqinr in R (Charif & Lobry, 2007; Team, 2016).

We aligned each sequence set using MAFFT ver. 7.490 (code and tags can be found on GitHub; Katoh *et al.*, 2002). Resulting alignments were used to construct maximum-likelihood phylogenies with IQtree ver. 1.6.12. Our pipeline first used the ModelFinder tool to find the best nuclear or mitochondrial substitution model for each alignment (Kalyaanamoorthy *et al.*, 2017), and then ran 1000 bootstraps using the ultrafast bootstrap approximation method (Nguyen *et al.*, 2015). To construct a single phylogeny using the data of all five single-locus phylogenies, we concatenated alignments. The concatenated sequence was used to reconstruct a maximum-likelihood phylogeny to create the best tree to fit the data, with informative branch lengths corresponding to genetic distances. The five-locus phylogeny was created using IQtree run with partition models to distinguish the loci based on the ModelFinder tool, and bootstrapped 1000 times using the ultrafast bootstrap approximation (tags found on GitHub; Dunkirk, 2023). We verified the results of this method by also creating a consensus tree using ASTRAL (Zhang *et al.*, 2018). The trees were rooted with *P. cervinus* as outgroup.

As a final analysis and to contextualize the close relationship between *A. thiersii* and *A. foetens*, we downloaded all 2,237 Agaricales ITS sequences available from NCBI on October 1, 2022, and we included these with the ITS sequences we used to generate the five-locus phylogeny. Sequences were aligned with MAFFT using the '-auto' parameter and trimmed with trimAL using the '-automated1' parameter (Capella-Gutiérrez *et al.*, 2009). The resulting trimmed alignment of 279 bp was used in IQtree to construct a maximum likelihood phylogeny using the 'test' parameter to find the best model as constrained within 'raxml' options. All identical sequences were removed by default. All pairwise distances in the resulting tree were obtained from the 'mldist' file and filtered to only include comparisons within the same genus. Only the lowest distance comparison within and between species was kept for a given sequence. We visualized the data as a histogram to compare pairwise distances between intraspecific and interspecific species, as named in the database.

Constructing BUSCO multi-gene trees

To contextualize the genomes of *A. thiersii* 10802, *A. foetens* 10801 and *Amanita* sp. 10175 within the genus *Amanita*, we downloaded all publicly available *Amanita* genomes, both asymbiotic and mycorrhizal, in addition to those of *Volvariella volvacea* and *Pluteus cervinus*, both used as outgroups (genomes downloaded from NCBI between March 29 and 31, 2022; Table 2). We identified a set of fungal Benchmarking Universal Single-Copy Orthologs (BUSCOs) from each of 15 genomes using the program BUSCO (ver 3.0.2 run with *Laccaria bicolor* as reference; Simão *et al.*, 2015).

Table 2. Dataset of *Amanita* genomes used to extract BUSCOs for phylogenetic analysis. Table includes data downloaded from NCBI detailing the species name, genome assembly accession number, and genome ID associated with the genome.

Species	NCBI GenBank Genome Assembly Accession	NCBI GenBank genome ID
10175 *Amanita* sp.	SRR23983940	PRJNA947219
10801 *Amanita foetens*	SRR23983939	PRJNA947219
10802 *Amanita thiersii*	SRR23983941	PRJNA947219
Amanita bisporigera	GCA_001983365.1	ASM198336v1
Amanita brunnescens	GCA_001691785.2	ASM169178v2
Amanita inopinata	GCA_001691775.3	ASM169177v3
Amanita jacksonii	GCA_000497225.1	AmaJack1.0
Amanita muscaria	GCA_001691765.1	ASM169176v1
Amanita phalloides	GCA_001983385.1	ASM198338v1
Amanita polypyramis	GCA_001691755.2	ASM169175v2
Amanita pseudoporphyria	GCA_003316615.1	ASM331661v1
Amanita rubescens	GCA_015039365.1	Amarub1
Amanita subjunquillea	GCA_020011035.1	ASM2001103v1
Pluteus cervinus	GCA_004369065.1	Plucer1
Volvariella volvacea	GCA_001691835.3	ASM169183v3

We subset this dataset to include only those BUSCOs present as single copies in all 15 taxa (n = 55 BUSCOs). We aligned the sequences of each BUSCO with MAFFT and constructed single-BUSCO maximum-likelihood phylogenies for each resulting alignment with IQtree.

We took two approaches to generate subsequent multi-gene species-trees: first, we concatenated the alignments and made a single tree, and second, we used a consensus tree method to generate a consensus tree. First, concatenated sequence data were used to generate a maximum-likelihood phylogeny using IQtree run with a partition model, using methods parallel to the methods used to generate the five-locus tree (described above). Second, ASTRAL ver. 5.7.8 was used to reconstruct a consensus tree (Zhang *et al.*, 2018) based on the phylogenies constructed for each individual BUSCO. Essentially, using ASTRAL, the BUSCO species tree was created by using single-gene-BUSCO trees as inference and by considering discordance among the single-gene trees. As an extra check to verify the topology of the BUSCO species trees, we used additional methods. To determine the number of informative gene-trees which showed topology in concordance with the consensus phylogeny, we re-ran ASTRAL and measured quartet support (Zhang *et al.*, 2020), and we calculated gene concordance with IQtree ver. 2.1.2 (Minh *et al.*, 2020) using the single-gene BUSCO trees and the maximum-likelihood phylogeny previously generated using IQtree. The quartet support option in ASTRAL indicates, at each branch, how much conflict there was between gene-trees in the resulting consensus tree (Zhang *et al.*, 2018). The concordance factor option in IQ-tree indicates the percentage of locus-trees which support that branch (Minh *et al.*, 2020). The tree was rooted with *P. cervinus* and *V. volvacea* as outgroup taxa.

Comparing mating type loci

U.S.A. populations of *A. thiersii* are characterized by a lack of genetic diversity, suggesting sexual reproduction is absent or involves genetically similar pairs (Wolfe, Kuo *et al.*, 2012). In fungi, successful sexual reproduction typically requires the interaction of compatible mating type genes, named as Homeodomains 1 and 2 (*HD1* and *HD2*). To determine if our three genomes include the mating type loci required for sexual reproduction, we used the methods described above to extract the genes *HD1* and *HD2*. To identify *HD2*, we queried genomes 10175, 10801, and 10802 using the tblastn function of BLAST with the amino acid (AA) sequences of the *HD2* gene identified from an earlier annotated genome of *A. thiersii* (Hess *et al.*, 2014), from the genome of another closely related asymbiotic *Amanita, A. inopinata* (Hess *et al.*, 2014), and from *Coprinopsis cinerea* (Stajich *et al.*, 2010). To identify *HD1*, we queried the same genomes using tblastn with the AA sequences of *HD1* from *A. inopinata,* and *C. cinerea.*

The published, annotated genome of *A. thiersii* (Hess *et al.*, 2014) does not appear to include the *HD1* gene region and so we could not use it as a query. To explore this dynamic further, we searched for *HD1* in a publicly available transcriptome (NCBI ID: SRX037158; Wolfe, Kuo *et al.*, 2012) sequenced from a culture of the dikaryotic parent mushroom of the single spore used to generate genome 10802. We first used the *HD1* nucleotide sequence from *Amanita* sp. 10175 as query in an SRA BLAST (Sequence Read Archive Basic Local Alignment Search Tool) of the transcriptome. Next, we downloaded all output sequences and used the program EGassembler to merge sequence fragments into a single consensus sequence (Masoudi-Nejad *et al.*, 2006). We used the resulting consensus sequence as the next query to SRA BLAST, once again searching in the transcriptome, and repeated our searches until no new transcriptomic reads could be incorporated into the consensus.

Because the *HD2* gene includes introns, we used the annotated genome of *A. thiersii* to locate and remove them (Hess *et al.*, 2014). After removing introns, we used the tool Expasy to translate the DNA sequences of both *HD1* and *HD2* into AA sequences (Duvaud *et al.*, 2021). Homeodomain proteins are typically identifiable by three helices (Hull *et al.*, 2002). To confirm the presence of the three helix motif, we used a position-specific iterative predictor, PSIPRED, to predict and confirm secondary structural motifs (McGuffin *et al.*, 2000). We checked the PSIPRED predictions using blastp (Altschul *et al.*, 1990). Next we searched for *HD1* and *HD2* in other *Amanita* and *V. volvacea* (Hess *et al.*, 2018). To compare HD1 and HD2 among species, we aligned only the conserved three-helix homeodomain AA sequences using MAFFT (default settings; Katoh *et al.*, 2002).

To confirm the absence of mating-type genes in assemblies as the result of true deletions and not error related to genome assembly, we aligned raw reads from 10802 to the genome of 10801 that contained both mating type genes using BWA mem (Li, 2013). Resulting alignments were visualized in Integrative Genomics Viewer (Thorvaldsdóttir *et al.*, 2012) to confirm the absence of reads aligning at mating type loci.

Results

Species descriptions of *A. thiersii* and *A. foetens* offer clues

Amanita thiersii and *A. foetens* are morphologically very similar, but key differences are apparent in the original species' descriptions. Features which appear identical include gill characteristics, ring location, and basidiospore shape (Figure 1).

Figure 1. Morphology of *Amanita thiersii* and *A. foetens*. Morphology summarized from the original descriptions by Singer (1953) and Thiers (1957) and secondary descriptions by Bas (1969) for notable characteristics. Photos under creative commons license (Cc-by-sa-3.0) or reproduced with permission from Bas (1969).

Features which appear similar include the general appearance of mushrooms, more specifically their color, height, cap size and wart characteristics; the integrity of the ring on developing mushrooms; and basidiospore size. Conflicting or ambiguous descriptions relate to the structure of the volva, details of stipe and ring morphology, and, notably, mushroom scent.

Both species are described as entirely white in color (*A. foetens* may also be pink or yellowish), with medium to large mushrooms and convex caps (Bas, 1969). Caps possess abundant floccose or fleshy warts with crowded and freely attached gills. The caps of *A. thiersii* appear to be slightly smaller than caps of *A. foetens* but ranges are not disjunct. The mushrooms are described as either "rather thick-fleshed" (*A. thiersii;* Bas, 1969) or with a "rather sturdy fruit body" (*A. foetens;* Bas, 1969). Stipes are reported as textured as opposed to smooth (Bas, 1969). The stipe of *A. thiersii* is described as being equally wide across its height, but with a slight bulb at its base (Thiers, 1957). The stipe of *A. foetens* is described as "white, firm, broad" with a strongly bulbous base (Singer, 1953). Stipe height for both species is reported similarly at between 80-200 (*A. thiersii*) or 80-210 (*A. foetens*) mm (Bas, 1969; Thiers, 1957). Basidiospores appear to have the same shape: globose to subglobose, and mushrooms drop a white spore print (Singer, 1953; Thiers, 1957). Basidiospores are amyloid and less than 10 × 10 µm (Bas, 1969). Basidia lack clamps, a feature typical for stirps *Thiersii*, the subset of the genus *Amanita* housing both *A. thiersii* and *A. foetens* (Bas, 1969).

Other characters in the two species' descriptions are either difficult to compare or are ambiguous. Within the genus *Amanita*, the volva is a key distinguishing feature. If *A. thiersii* and *A. foetens* are the same species, we would expect to find similar or identical volval descriptions. The volva of *A. thiersii* is described as usually evanescent "or present as a series of irregular rows of easily detached, fibrillose warts along the base of the stipe" (Thiers, 1957). The volva of *A. foetens* is described as strongly reduced or absent "or represented by some girdles" (Singer, 1953). Although these descriptions use different words, fibrillose warts *versus* girdles, they both allude to a volva made up of evanescent pieces of mushroom tissue around the stipe; a contrast to the obvious, persistent, and cup-shaped volva of many *Amanita.* Both descriptions point to the lack of a volva, or a volva present as remnants only, but because it is difficult to interpret the original descriptions further, how the volva might compare to each other remains ambiguous.

Less ambiguous are other differences, including differences in the internal morphology of the stipe; the stipe of *A. thiersii* is described as "stuffed to hollow" (Thiers, 1957) while the stipe of *A. foetens* is described as "solid, firm" (Bas, 1969;

Singer, 1953). After revisiting Bas's (1969) species descriptions one of us (Tulloss) a taxonomic expert and specialist of the genus, concluded fresh specimens of the species can be distinguished based on stature. The stature of *A. thiersii* is significantly more "gracile" than the stature of *A. foetens* (Bas, 1969: Fig. 85 *vs.* Fig. 88). The word gracile is used to mean slender, and a comparison of the ratios of stipe length to stipe width can stand in for the qualitative term "gracile." In *A. thiersii* the ratio ranges from 6.9 to 7.3 (based on data of Bas, 1969) while in *A. foetens* the ratio ranges from 10 to 20 (also using data from Bas, 1969).

Descriptions of the ring also emerge as distinct. While both species' rings are described as white, membranous, and located apically on the stipe (Bas, 1969), *A. thiersii*'s ring is described as thin and "easily torn, sometimes disappearing" (Bas, 1969) while *A. foetens*' ring is described as "rather thick", "frequently fragmentary" (Singer, 1953), and as "falling to pieces" (Bas, 1969). While both rings appear fragile, the distinction of thin *versus* thick cannot be ignored.

Finally, the scent of *A. thiersii* is reported as indistinct (and we ourselves have never found a strong-smelling *A. thiersii*), whereas the smell of *A. foetens* is "resolutely stinking" and in mature specimens, like urine (Singer, 1953). In the aggregate, the morphological differences recorded for *A. thiersii* and *A. foetens* suggest they are different species. Later we discuss the usefulness of these diagnostic characters to the field biologist.

A. thiersii continues to expand its range in North America, and collections are clustered around urban centers

The two species were not equally represented across iNat and MO. There were a total of 24 observations of *A. foetens* in iNat, of which we used 15 (we removed nine observations with low quality data grades), and no observations in MO. We manually added the locations of the isotype of *A. foetens* collected from Tucumán, Argentina (AmanitaBASE 10801/MICH4948) and the mushroom collected as *Amanita* sp. from Córdoba, Argentina (AmanitaBASE 10175) to our dataset (total observations included in subsequent analyses: 17). There were a total of 286 observations of *A. thiersii* in iNat, of which we used 155 (we removed 131 observations with aberrant morphologies or low-quality data grades), and 15 observations in MO. We manually added the location of our genome-sequenced sample from Kansas, U.S.A. (AmanitaBASE 10802) to our dataset (total observations included in subsequent analysis: 171). Because the nomenclature within iNat and between iNat and MO is inconsistent (iNat uses the pseudonym *Saproamanita thiersii*, while *A. foetens* remains as *Amanita foetens* (Hawksworth, 2016; Redhead *et al.*, 2016; Tulloss *et al.*, 2016; at the date of download, MO did not use the generic name *Saproamanita*), we use the names *Amanita thiersii* and *Amanita foetens* to describe records from both iNat and MO (except on Figure 2, where for clarity we use the names used in the databases themselves).

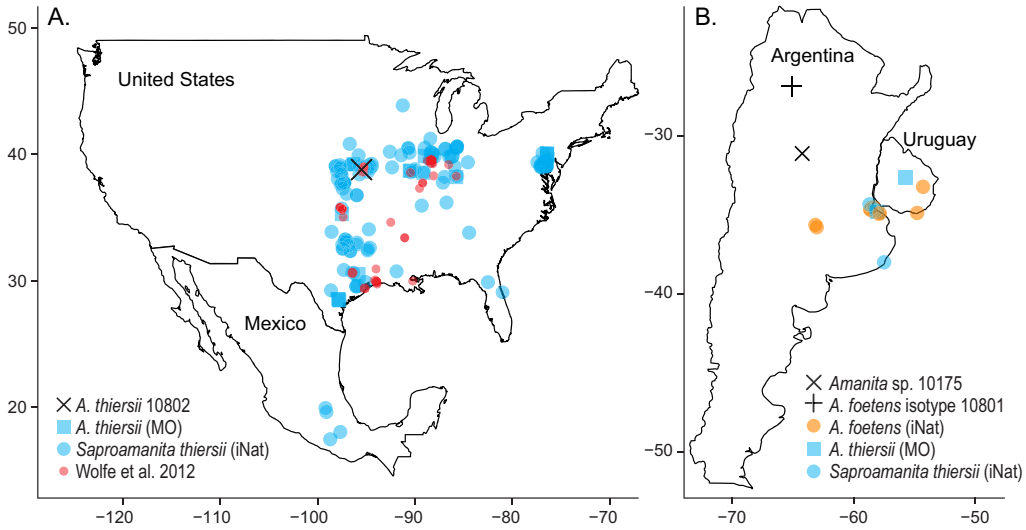

Figure 2. Geographic Distributions of *A. thiersii* and *A. foetens* based on Public Databases. A. Map of U.S.A. and Mexico plotted with observations of *A. thiersii* (as blue squares from MO, and blue circles named as *Saproamanita thiersii* from iNat). Mushroom *A. thiersii* 10802, the source of a sequenced genome, plotted as a black X. Geographic distribution of *A. thiersii* as published in Wolfe, Kuo et al. (2012) shown as small red dots. B. Map of Argentina and Uruguay plotted with observations of *A. foetens* (iNat) as orange circles, *A. thiersii* (MO) as blue squares, and *Saproamanita thiersii* (iNat) as blue circles. Mushrooms *Amanita* sp. 10175 and *A. foetens* 10801, sources of sequenced genomes, plotted as black X and +, respectively. Axes of A. and B. reference latitude and longitude.

Both species are observed within North and South America and not on other continents. While two observations were made from Taiwan and South Africa (iNat observations 118324696 and 117233579, respectively), neither observation matched either species' morphology. *Amanita foetens* was observed predominantly in Argentina (n=15), as far south and east as Buenos Aires and as far north and west as Tucumán. It was also found in Uruguay (n=2). The fungus was most frequently observed in the Argentinian province of Buenos Aires (n=13). *Amanita thiersii* was found overwhelmingly in the U.S.A. (n=160), but its range appears to be expanding. Wolfe *et al.* (2012) reported it as far north as Illinois and east to Kentucky (red dots on Figure 2A), but the fungus now appears as far north as Wisconsin, and as far east as Pennsylvania and Maryland (Figure 2A). It has also been reported from Florida. *Amanita thiersii* appears to be newly common in Illinois, with 16 observations recorded from the state since 2012. But in the U.S.A., *A. thiersii* was most frequently observed in Kansas (n=38), Maryland (n=31), and Texas (n=28). Although MO records observations of *A. thiersii* in Argentina (n=1) and Uruguay (n=1), the records likely reflect a bias towards recording this well-known species. Moreover, within MO, all Argentinian *A. thiersii* observations have comments urging observers to name the observations as *A. thiersii* or "*Amanita* stirps *Thiersii*," and not *A. foetens.* MO users do not appear to use the name *A. foetens.* The iNat records of *A. thiersii* from Mexico (n=4) are among the first observations of the fungus in that country (see below). One of us (Tulloss) keyed out one of the Mexican mushrooms and confirms it is *A. thiersii* (Tulloss, 2020). Mexican *A. thiersii* appear to be collected from lawns; images of the observations show mushrooms growing with lawn grasses and in one case, mulch (see iNat observation 53329070). Lawns are also where U.S.A. *A. thiersii* are found. There are also four iNat records of "*A. thiersii*" in Argentina, one from Mar del Plata, Argentina, and the others from Buenos Aires. The record from Mar del Plata is the southernmost record in South America. We are skeptical the Argentinian *A. thiersii* records are real, and later we discuss the issue.

Most mushrooms in both databases were observed around urban centers. By contrast, the two Argentinian specimens used for genome sequencing were collected far from any city; the isotype of *A. foetens* (AmanitaBASE 10801/MICH4948) was collected in a grass pasture in the Tucumán region (+ on map, Figure 2B) and the specimen originally described as *A. thiersii* (AmanitaBASE 10175), which helped spark our study, was collected from a grassy paddock outside of Córdoba, between Tucumán and Buenos Aires, Argentina (X on map, Figure 2B). The *A. foetens* observations in Argentina and Uruguay are typically pictured in grass lawns, although some are featured in a mulch or heavily wooded environment (see iNat observation 12341687).

A five-locus phylogeny suggests *A. foetens* 10801 and *Amanita* sp. 10175 are the same species, while *A. thiersii* is genetically distinct

We used sequences of the ITS, NucLSU, NucSSU, MitLSU, and MitSSU loci to clarify the phylogenetic relationships of our specimens and other saprotrophic, asymbiotic *Amanita.* We downloaded between two and five gene sequences from a total of 17 mushrooms representing ten different species from NCBI (Table 1), and also used our data from AmanitaBASE specimens 10801 and 10175. (Because publicly available sequences from the dikaryon *A. thiersii* Skay4041_het represent the parent genome of our haploid (monokaryotic) genome *A. thiersii* 10802, we omitted our genome 10802 from this analysis.)

The topology of the concatenated five-locus species tree (Figure 3) is congruent with the topologies of each of the single-locus trees and the consensus tree (not shown). Specimen *Amanita* sp. 10175 is nearly identical to *A. foetens* 10801, evidence it is *A. foetens* and is not *A. thiersii.* Notably, mushrooms of *A. thiersii* and both *A. foetens* 10801 and *Amanita* sp. 10175 are more closely related to each other than they are to any other saprotrophic *Amanita*, a clustering strongly supported by bootstrapping. However, *A. thiersii* specimens from North America form a separate monophyletic clade from *A. foetens* 10801 and *Amanita* sp. 10175, and bootstrap support is moderately strong. While the samples from North and South America appear to be in two distinct monophyletic groups, the genetic distance between *A. thiersii* 10802 and *A. foetens* 10801 in our distance matrix is only 0.000001: the two species appear very closely related to each other (Figure 4). However, in this analysis of the ITS locus, interspecific measures of genetic distance are frequently very low (Figure 4). The overlap between intraspecific and interspecific genetic distances is striking.

A phylogeny reconstructed from BUSCO genes supports the genetic distinction of *A. thiersii* 10802

The BUSCO analysis resulted in a different number of single-copy BUSCOs from each of the genomes, ranging from 197 in *A. pseudoporphyria* to 279 from *A. inopinata.* The BUSCO completeness values for our own genomes were good for genomes 10175 (94.8%) and 10802 (95.1%) and moderate for genome 10801 (81.7%). We used the sequences of 55 BUSCO genes which were found as single-copy genes from each of 15 *Amanita* genomes (including our genomes of AmanitaBASE specimens 10801, 10175 and 10802) to elucidate phylogenetic relationships between the three *Amanita* spp. specimens and other symbiotic and asymbiotic *Amanita* from across the genus (Table 2). Both methods (concatenation and consensus) used to construct a BUSCO species tree resulted in identical topologies for *A. thiersii* and *A. foetens* (Figure 5; concatenated sequence tree not shown).

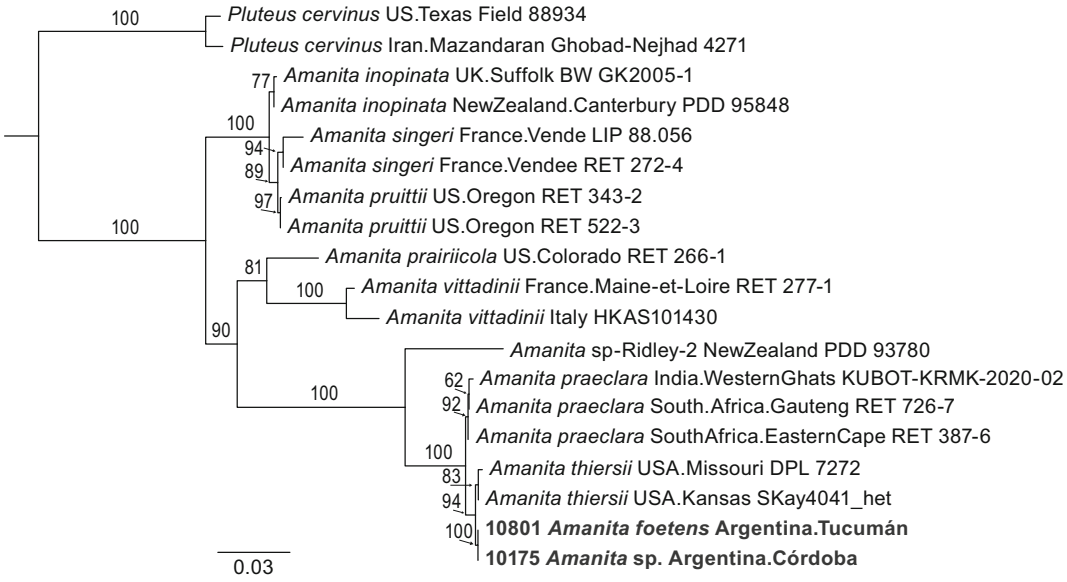

Figure 3. Multi-Gene Tree of Saprotrophic *Amanita*. Single phylogeny generated from five concatenated loci of saprotrophic *Amanita*, with *Pluteus cervinus* used as outgroup. Branch lengths correspond to genetic distances.

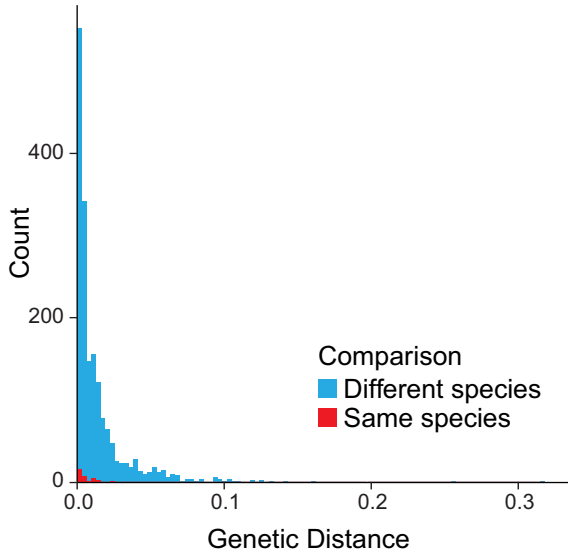

Figure 4. Pairwise comparison between or among species. Histogram of all pairwise genetic distances inferred between all non-identical publicly available Agaricales ITS sequences on NCBI. Comparisons of species from different genera are not reported.

In the BUSCO-species tree, our three specimens form a monophyletic group distinct from all other *Amanita*, and the clustering is supported by high levels of ASTRAL quartet support and IQtree concordance factor support (Figure 5). Consistent with the first analysis (Figure 3), the topology of the BUSCO phylogeny shows the Argentinian samples 10801 and 10175 as clustering together to form a monophyletic clade, additional evidence *Amanita* sp. 10175 is the same species as *A. foetens* 10801. These two are consistently separate from *A. thiersii* 10802, additional evidence *A. foetens* and *A. thiersii* are distinct species.

Mating type loci are found in some nuclei but not others

The presence of the mating type genes *HD1* and *HD2* in a genome would signal the potential for sexual reproduction by an individual. Using AA sequences as queries, we were unable to find the *HD1* gene in the *A. thiersii* 10802 genome

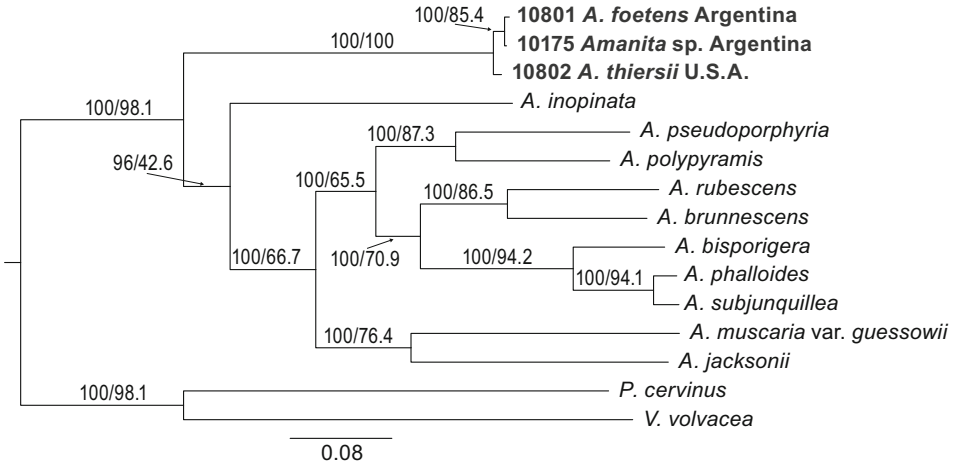

Figure 5. Phylogeny Reconstructed from Available *Amanita* Genomes. A 55-BUSCO-gene phylogeny of *Amanita* based on all available *Amanita* genomes from NCBI with *P. cervinus* and *Volvariella volvacea* used as outgroups. The phylogeny was generated with IQtree (with concatenated sequences), and a phylogeny reconstructed with ASTRAL has the same topology. Branch supports indicate ASTRAL's quartet support test and IQtree's concordance factor (shown before and after slashes, respectively).

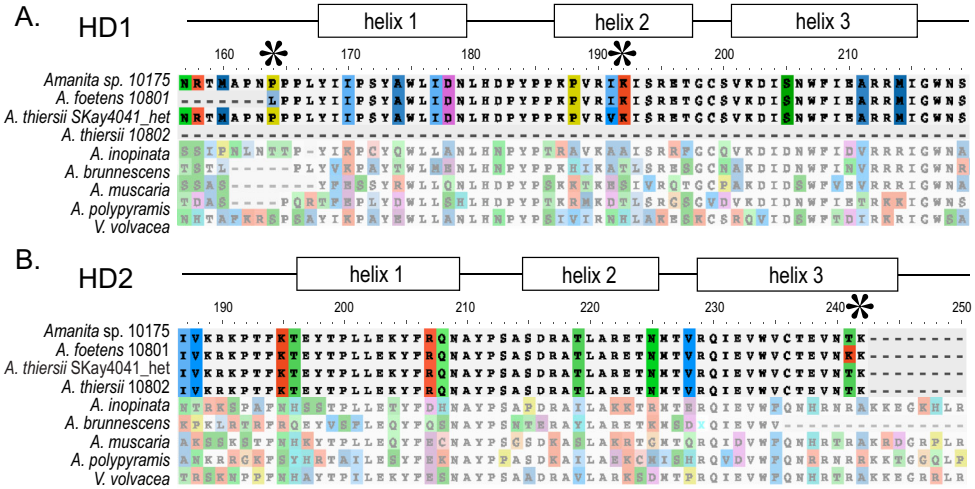

Figure 6. Mating type Genes of *Amanita* species. Amino acid sequences of mating type loci *HD1* (A) and *HD2* (B). *A. thiersii* 10802 is missing the *HD1* gene, but a transcriptome generated from the dikaryotic parent of 10802 (*A. thiersii* Skay4041_het) houses a transcript of *HD1*, suggesting one of the nuclei of the dikaryotic parent houses *HD1* while the other does not. Sequence similarity among *A. thiersii* and *A. foetens* is very high, with only one or fewer amino acid differences in the conserved homeodomain region of both mating type genes. Asterisks are near key amino acid differences.

assembly (a finding we confirmed using genome alignment methods), but we could isolate partial sequences in both *Amanita* sp. 10175 (AA length=222) and *A. foetens* 10801 (AA length=103). We also isolated a sequence of HD1 from the transcriptome of *A. thiersii* SKay4041_het (AA length=533), a transcriptome sequenced from a dikaryotic mushroom, the parent of the germinated basidiospore used to generate the genome sequence *A. thiersii* 10802. In other words, while the transcriptome of the dikaryotic parent does have HD1, the genome of one of its nuclei does not: the second nucleus of the parent must be the source of the transcriptome's HD1. The HD1 sequence found in the genome of *Amanita* sp. 10175 possesses a single AA substitution distinguishing it from the HD1 sequence found in the genome *A. foetens* 10801 and in the transcriptome of *A. thiersii* SKay4041_het across the conserved three-helix homeodomain region (AA length=60; Figure 6A, HD1; AA substitutions shown with asterisks). Using AA sequences as queries we were able to identify the complete sequence of HD2 in each of our three genomes and the transcriptome of *A. thiersii* SKay4041_het (AA lengths=289), and each sequence possessed the typical three-helix structure of the conserved homeodomain motif (AA length=60; Figure 6B, HD2). The HD2 sequences possess only one AA substitution, between *Amanita* sp. 10175 and *A. foetens* 10801. This is the first report of mating type loci for these *Amanita* spp.

Discussion

Species' descriptions, ranges, and phylogenies contextualized by other taxa in the genus *Amanita* each suggest *A. thiersii* and *A. foetens* as two different species. The morphological differences distinguishing them may enable identification in the field. However, identifying fungi based on morphology alone is often difficult (Houbraken *et al.*, 2020; Looney *et al.*, 2020), and if a specimen is old or weathered, key characters may be absent (a full description of the characters used to identify *Amanita* species is provided by Tulloss (Tulloss, 2023). While it may be possible to identify fresh material as *A. thiersii* or *A. foetens* using features of the stipe and smell; by measuring stipe length and width, and deciding if a specimen smells and, if so, what it smells like, in practice many will find these to be difficult field characters (or not know to measure or record them). For example, the smell of *A. thiersii* mushrooms is described as indistinct, while mushrooms of *A. foetens* are supposed to smell like urine (Figure 1). But collectors posting to MO report *A. thiersii* as having a range of scents, from indistinct and "scentless" to "smells like urine", "has a fishy, bad odor", all the way to "the odor was unpleasant, a bit like a sweaty locker room". Collectors posting to iNat do not include details of *A. foetens* smell. While morphology emerges as formally useful, it may not serve as a practical guide for choosing whether a particular mushroom is *A. thiersii* or *A. foetens.* It is also possible the original species descriptions (especially of *A. thiersii*) are missing descriptions of intraspecific variability in scent, or variability between e.g. young and old mushrooms.

The geographic origin of any material is likely to be a more useful diagnostic for most collectors. Both iNat and MO document *A. thiersii* as growing throughout much of North America. The fungus continues to expand its range and is now found throughout the U.S.A. east of the Rocky Mountains, from Texas and Florida up to Wisconsin, Pennsylvania and Maryland (Figure 2, compare to red dots denoting range of *A. thiersii* in Wolfe, Kuo *et al.*, 2012). The continuing spread of *A. thiersii* is remarkable. In the U.S.A., *A. thiersii* is most often recorded from typical lawn environments, e.g., iNat observations 94653088 from Kansas and 60276048 from Indiana. Notably, some of what are now the eastern-most collections, for example collections in Florida, show the mushroom growing from dead leaves, for example iNat observation 74461419, described at "woodroad's edge" and pictured without any grass in sight. The fungus is hypothesized to be moving north and east in response to climate change (Hobbie *et al.*, 2017).

With this hypothesis in mind, the northward and eastward movements of the fungus are perhaps less surprising than the discovery of *A. thiersii* in Mexico. In Mexico the fungus is found directly north of Mexico City and to the south in Oaxaca. However, we do not know if the Mexican observations reflect the ongoing range expansion or the discovery of the native range. We hypothesize the discovery of *A. thiersii* in Mexico represents a new range for the fungus, and not the discovery of a native range, basing our hypothesis on the habitats of the Mexican mushrooms. As is true for *A. thiersii* in the U.S.A., in Mexico *A. thiersii* appear to grow in lawns, for example iNat observations 86939921 and 86236720 from México, Mexico. Lawns of grasses are habitats grown by humans and the current scarcity of collections from natural habitats suggests a link between human activity and the fungus.

Amanita foetens mushrooms have not been reported in North America. Instead, the observations of *A. foetens* from iNat suggest it is a South American mushroom of urban and anthropogenic habitats, including lawns, pastures, and gardens. It grows in both Argentina and Uruguay. The preponderance of urban observations may reflect a simple bias; perhaps people in cities are more frequently using the database, as compared to people outside of cities. The fifteen observations are clustered in Buenos Aires Province, Argentina. By contrast, the two Argentinian mushrooms we used in genome sequencing were found far from urban habitats. The isotype and other specimens used in the original species' description were discovered in a pasture near Tucumán (although the exact coordinates are unknown and the location is given simply as "Pie del Periquillo, Tucumán Province, Argentina"). The specimen which began our debate about *A. thiersii* and *A. foetens*, *Amanita* sp. 10175, was collected similarly in a paddock in the hills near Córdoba, well outside of the city center.

Observations of *A. thiersii* or "*Saproamanita thiersii"* (depending on the database) are also reported from Argentina and Uruguay. Because the name *A. foetens* is not used by MO collectors, it is difficult to know if records of South American "*A. thiersii*" in MO are actually records of *A. foetens*; in fact, because of the general confusion, iNat "*Saproamanita thiersii*" records may also represent misidentifications. The difficulty highlights the complications of using data in public databases. In iNat, Argentinian "*Saproamanita thiersii"* were sometimes observed from locales very close to iNat observations of *A. foetens.* In MO, the question of whether *A. thiersii* and *A. foetens* are the same species has been debated for nearly a decade, and the community has actively encouraged the naming of Argentinian samples as "*A. thiersii"* or "*Amanita* stirps *Thiersii*" (the latter convention encompasses both *A. thiersii* and *A. foetens*). Either *A. thiersii* is also growing in Argentina alongside *A. foetens*, or the Argentinian MO and iNat records are misidentifications (the records are *A. foetens* mislabeled as *A. thiersii* or *S. thiersii*). We favor the second hypothesis. Comments within MO offer support for our hypothesis, for example, MO observation 200425 is annotated with "… Of note to me is that this amanita [sic] was not found in its typical lawn habitat, but with trees and shrubs.".

Phylogenies consistently place mushrooms of *A. thiersii* and *A. foetens* apart from each other, although the two species appear to be very closely related. A discussion of intraspecific diversity of the ITS locus is beyond the scope of our current study, but we were struck by the overlap between intraspecific and interspecific comparisons of genetic distances (Figure 4). We considered an hypothesis of the two species as distinct populations of the same species, but using the morphological and geographic data as context, we consider the phylogenies as better supporting the hypothesis of two distinct species. Although they were collected 570 kms away from each other, specimen *Amanita* sp. 10175 consistently groups with the isotype of *A. foetens* and is clearly an *A. foetens*, additional evidence *A. thiersii* is absent from Argentina.

The species most closely related to *A. thiersii* and *A. foetens* is *A. praeclara* (A. Pearson) Bas, a species originally described from the Cape Province of South Africa as an "aberrant" *Lepiota* (Figure 3; Pearson, 1950). It is another "white to whitish" (Reid & Eicker, 1991) decomposer *Amanita* collected from lawns; the original collections were made in the "grassy ground of paddock" and from "football" [soccer] fields (Pearson, 1950). The fungus was recently reported from India (Kantharaja & Krishnappa, 2022), and the Indian record suggests another potential introduction involving this different asymbiotic *Amanita.* However, the habitat for the Indian *A. praeclara* is described as "on soil under in [sic] dry deciduous forest region". Moreover, while South African *A. praeclara* are described as white staining yellow (sulfur- or lemon-yellow), the Indian *A. praeclara* appears to be somewhat different; the description states mushrooms are "white, covered with pale yellow to orange yellow lanose-floccose covering when young … staining pale yellow afterwards" (Kantharaja & Krishnappa, 2022). One of us (Tulloss) does not believe the Indian *A. praeclara* is the same as the South African *A. praeclara.* Once again, the question of whether or not a newly discovered species is native or introduced is unanswered. Regardless, Bas (Bas, 1969) grouped *A. praeclara* in the same stirps as *A. thiersii* and *A. foetens*, and his grouping is now confirmed by our DNA sequence data (Figure 3).

Invasive species often reproduce asexually, and clonal propagation can facilitate spread (Gao *et al.*, 2018). In North America, *A. thiersii* lacks genetic diversity (Wolfe, Kuo *et al.*, 2012). While we identified the mating type locus *HD1* in the transcriptome of a dikaryotic strain of *A. thiersii*, the locus is missing from its monokaryotic offspring (which was cultured from a single basidiospore of the mushroom used to generate the dikaryotic strain, see also Elmore, 2020). The *HD1* locus appears to be present in some nuclei and absent from others. Mushrooms are sexual structures and *A. thiersii* clearly grows mushrooms. But the lack of genetic diversity and existence of a nucleus missing *HD1* suggests unusual mating dynamics, an analog to the biology of invasive Death Caps in California (Wang *et al.*, 2023), and to other unusual basidiomycete mating systems (Coelho *et al.*, 2017). We found no evidence for missing mating type loci in either of the *A. foetens* genomes, however, neither genome involved a monokaryotic culture.

For the moment, we recommend all mushrooms keyed to *A. thiersii* and *A. foetens* observed in North America be named as *A. thiersii* and all those found in South America be named as *A. foetens.* Using geography to choose names will simplify identification of morphologically ambiguous mushrooms. However, basing identification on geography will also obscure future introductions. If or when either species is introduced to the other continent, the introduction will be difficult to recognize. There is a great need for simple molecular tools enabling straightforward identification of specimens to species, and tools would be strengthened by greater efforts to sequence types and apparent novelties.

Conclusions

In the aggregate, morphological descriptions, data on occurrence and habitat, and phylogenies based on both a few loci and many specimens, as well as many loci of a few specimens, support *A. thiersii* and *A. foetens* as closely related but distinct species. The species appear morphologically and genetically distinct, and geographically isolated. We reject the hypothesis of *A. thiersii* as an *A. foetens* introduced to Texas from Argentina by humans. While our data do not establish *A. thiersii* as an introduced and now invasive species, they also do not establish it as native; the native range of *A. thiersii* remains unknown. It may have been introduced to North America from a country other than Argentina. For the first time, we report *A. thiersii* in Mexico. The question of why *A. thiersii* is spreading rapidly throughout North America remains open. In this instance, baseline data on fungal biodiversity have failed us; there are no baseline data for *A. thiersii.* Thus, we started with an enigma, and end with one as well.

Data availability

Dryad: Mushroom Observations of *Amanita thiersii* and *A. foetens*, https://doi.org/10.5061/dryad.7h44j1008 (Dunkirk *et al.*, 2023a).

This project contains the following underlying data:

- Database_Mushroom_Observations.csv

NCBI: Are Amanita thiersii and Amanita foetens the same species? Accession numbers SRR23983939, SRR23983940, and SRR23983941, https://identifiers.org/NCBI/bioproject:PRJNA947219 (Dunkirk *et al.*, 2023b).

This project contains the following underlying data:

- Genome sequences Athiersii 10802, Afoetens 10175, and Afoetens 10801

Source code available from: https://github.com/noramushrooms/Amanita_thiersii.

Archived source code at time of publication: https://doi.org/10.5281/zenodo.7996518 (Dunkirk, 2023).

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We thank Dr. Timothy James and the University of Michigan herbarium for sending us the voucher specimen of *A. foetens.*

References

Altschul SF, Gish W, Miller W, *et al.*: **Basic local alignment search tool.** *J. Mol. Biol.* 1990; **215**(3): 403–410.
Publisher Full Text

Barrett CF, Freudenstein JV: **An integrative approach to delimiting species in a rare but widespread mycoheterotrophic orchid.** *Mol. Ecol.* 2011; **20**(13): 2771–2786.
PubMed Abstract | Publisher Full Text

Bas C: **Morphology and Subdivision of Amanita and a Monograph of its section Lepidella.** *Persoonia.* 1969; **5**(4): 285–579.

Blackwell M: **The fungi: 1, 2, 3 ... 5.1 million species?** *Am. J. Bot.* 2011; **98**(3): 426–438.
PubMed Abstract | Publisher Full Text

Brännhage J, Beenken L, Gross A: **Champignons introduits en Suisse.** *Notice Pour Le Praticien.* 2021; **69**: 1–12.

Bushnell B: *BBMap short read aligner.* Berkeley, CA: University of California; 2016.
Reference Source

Butchart S, Walpole M, Collen B, *et al.*: **Global biodiversity: indicators of recent declines.** *Science.* 2010; **328**: 1164–1168.
Publisher Full Text

Capella-Gutiérrez S, Silla-Martínez JM, Gabaldón T: **trimAl: a tool for automated alignment trimming in large-scale phylogenetic analyses.** *Bioinformatics.* 2009; **25**(15): 1972–1973.
PubMed Abstract | Publisher Full Text | Free Full Text

Charif D, Lobry JR: **SeqinR 1.0-2: A Contributed Package to the R Project for Statistical Computing Devoted to Biological Sequences Retrieval and Analysis.** *Structural Approaches to Sequence Evolution.* 2007; pp. 207–232.
Publisher Full Text

Coelho MA, Bakkeren G, Sun S, *et al.*: **Fungal Sex: The Basidiomycota.** *Microbiology Spectrum.* 2017; **5**(3).
PubMed Abstract | Publisher Full Text | Free Full Text

Cui YY, Cai Q, Tang LP, *et al.*: **The family Amanitaceae: molecular phylogeny, higher-rank taxonomy and the species in China.** *Fungal Divers.* 2018; **91**(1): 5–230.
Publisher Full Text

DAISIE: **Delivering Alien Invasive Species Inventories for Europe.** *DAISIE (online).* 2012; 134–135.
Reference Source

Desprez-Loustau ML, Rizzo DM: **Fungi.** Simberloff D, Rejmanek M, editors. *Encyclopedia of Biological Invasions.* 1st ed. University of California Press; 2011; pp. 258–263.

Desprez-Loustau ML, Robin C, Buée M, *et al.*: **The fungal dimension of biological invasions.** *Trends Ecol. Evol.* 2007; **22**(9): 472–480.
PubMed Abstract | Publisher Full Text

Dickie IA, Nuñez MA, Pringle A, *et al.*: **Towards management of invasive ectomycorrhizal fungi.** *Biol. Invasions.* 2016; **18**: 3383–3395.
Publisher Full Text

Dunkirk N: noramushrooms/Amanita_thiersii: Data for Amanita thiersii manuscript v1 (v1.0.0). [Dataset]. *Zenodo.* 2023.
Publisher Full Text

Dunkirk N, Wang YW, Drott MT, *et al.*: Mushroom Observations of *Amanita thiersii* and *A. foetens.* [Dataset]. *Dryad.* 2023a.
Publisher Full Text

Dunkirk N, Wang YW, Drott MT, *et al.*: Are Amanita thiersii and Amanita foetens the same species? [Dataset]. *NCBI Bioproject.* 2023b.
Reference Source

Duvaud S, Gabella C, Lisacek F, *et al.*: **Expasy, the Swiss Bioinformatics Resource Portal, as designed by its users.** *Nucleic Acids Res.* 2021; **49**(W1): W216–W227.
Publisher Full Text

Elmore H: *Ecological population genomics in the emerging Amanita system.* Harvard University ProQuest Dissertations Publishing; 2020; pp. 1–144.

Fisher MC, Henk DA, Briggs CJ, *et al.*: **Emerging fungal threats to animal, plant and ecosystem health.** *Nature.* 2012; **484**(7393): 186–194.
PubMed Abstract | Publisher Full Text | Free Full Text

Gao L, Hou B, Cai ML, *et al.*: **General laws of biological invasion based on the sampling of invasive plants in China and the United States.** *Glob. Ecol. Conserv.* 2018; **16**: e00448.
Publisher Full Text

Golan JJ, Pringle A: **Long-distance dispersal of fungi.** *Microbiol. Spectr.* 2017; **5**(4): 309–333.
Publisher Full Text

Gonçalves SC, Haelewaters D, Furci G, *et al.*: **Include all fungi in biodiversity goals.** *Sci. Mag.* 2021; **373**(6553): 403–405.

Hawksworth DL: **Sense and sensitivity in naming.** *IMA Fungus.* 2016; **7**(1): A1–A2.
Publisher Full Text

Hess J, Pringle A: **The natural histories of species and their genomes: Asymbiotic and ectomycorrhizal amanita fungi.** *Advances in Botanical Research.* 1st ed. Vol. 70. Elsevier Ltd.; 2014.
Publisher Full Text

Hess J, Skrede I, De Mares MC, *et al.*: **Rapid divergence of genome architectures following the origin of an ectomycorrhizal symbiosis in the genus Amanita.** *Mol. Biol. Evol.* 2018; **35**(11): 2786–2804.
PubMed Abstract | Publisher Full Text | Free Full Text

Hess J, Skrede I, Wolfe BE, *et al.*: **Transposable element dynamics among asymbiotic and ectomycorrhizal amanita fungi.** *Genome Biol. Evol.* 2014; **6**(7): 1564–1578.
PubMed Abstract | Publisher Full Text | Free Full Text

Hobbie EA, Schubert BA, Craine JM, *et al.*: **Increased C3 productivity in Midwestern lawns since 1982 revealed by carbon isotopes in Amanita thiersii.** *J. Geophys. Res. Biogeo.* 2017; **122**(2): 280–288.
Publisher Full Text

Houbraken J, Kocsubé S, Visagie CM, *et al.*: **Classification of Aspergillus, Penicillium, Talaromyces and related genera (Eurotiales): An overview of families, genera, subgenera, sections, series and species.** *Stud. Mycol.* 2020; **95**: 5–169.
PubMed Abstract | Publisher Full Text | Free Full Text

Hulcr J, Gomez DF, Skelton J, *et al.*: **Invasion of an inconspicuous ambrosia beetle and fungus may affect wood decay in Southeastern North America.** *Biol. Invasions.* 2021; **23**(5): 1339–1347.
Publisher Full Text

Hull CM, Davidson RC, Heitman J: **Cell identity and sexual development in Cryptococcus neoformans are controlled by the mating-type-specific homeodomain protein SXI1α.** *Genes Dev.* 2002; **16**(23): 3046–3060.
PubMed Abstract | Publisher Full Text | Free Full Text

Jusino MA, Skelton J, Chen CC, *et al.*: **Sexual reproduction and saprotrophic dominance by the ambrosial fungus Flavodon subulatus (= Flavodon ambrosius).** *Fungal Ecol.* 2020; **47**: 100979.
Publisher Full Text

Kalyaanamoorthy S, Minh BQ, Wong TKF, *et al.*: **ModelFinder: Fast model selection for accurate phylogenetic estimates.** *Nat. Methods.* 2017; **14**(6): 587–589.
PubMed Abstract | Publisher Full Text | Free Full Text

Kantharaja R, Krishnappa M: **Amanitaceous fungi of central Western Ghats: taxonomy, phylogeny, and six new reports to Indian mycobiota.** *J. Threat. Taxa.* 2022; **14**(4): 20890–20902.
Publisher Full Text

Katoh K, Misawa K, Kuma KI, *et al.*: **MAFFT: A novel method for rapid multiple sequence alignment based on fast Fourier transform.** *Nucleic Acids Res.* 2002; **30**(14): 3059–3066.
PubMed Abstract | Publisher Full Text | Free Full Text

Li H: **Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM.** 2013; 1–3.
Reference Source

Looney BP, Adamčík S, Matheny PB: **Coalescent-based delimitation and species-tree estimations reveal Appalachian origin and Neogene diversification in Russula subsection Roseinae.** *Mol. Phylogenet. Evol.* 2020; **147**(May 2019): 106787.
PubMed Abstract | Publisher Full Text

Masoudi-Nejad A, Tonomura K, Kawashima S, *et al.*: **EGassembler: Online bioinformatics service for large-scale processing, clustering and assembling ESTs and genomic DNA fragments.** *Nucleic Acids Res.* 2006; **34**(WEB. SERV. ISS): W459–W462.
PubMed Abstract | Publisher Full Text | Free Full Text

May TW, Cooper JA, Dahlberg A, *et al.*: **Recognition of the discipline of conservation mycology.** *Conserv. Biol.* 2018; **33**(3): 733–736.
PubMed Abstract | Publisher Full Text

McGuffin LJ, Bryson K, Jones DT: **The PSIPRED protein structure prediction server.** *Bioinformatics.* 2000; **16**(4): 404–405.
Publisher Full Text

Minh BQ, Hahn MW, Lanfear R: **New methods to calculate concordance factors for phylogenomic datasets.** *Mol. Biol. Evol.* 2020; **37**(9): 2727–2733.
PubMed Abstract | Publisher Full Text | Free Full Text

Nguyen LT, Schmidt HA, Von Haeseler A, *et al.*: **IQ-TREE: A fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies.** *Mol. Biol. Evol.* 2015; **32**(1): 268–274.
Publisher Full Text

Pearson AA: **Cape agarics and boleti.** *Trans. Br. Mycol. Soc.* 1950; **33**(3–4): 276–IN8.
Publisher Full Text

Pringle A, Adams RI, Cross HB, *et al.*: **The ectomycorrhizal fungus Amanita phalloides was introduced and is expanding its range on the west coast of North America.** *Mol. Ecol.* 2009; **18**(5): 817–833.
PubMed Abstract | Publisher Full Text

Pringle A, Vellinga EC: **Last chance to know? Using literature to explore the biogeography and invasion biology of the death cap mushroom Amanita phalloides.** *Biol. Invasions.* 2006; **8**(5): 1131–1144.
Publisher Full Text

Prjibelski A, Antipov D, Meleshko D, *et al.*: **Using SPAdes de novo assembler.** *Curr. Protoc. Bioinformatics.* 2020; **70**(e102).
Publisher Full Text

Pyšek P, Hulme PE, Simberloff D, *et al.*: **Scientists' warning on invasive alien species.** *Biol. Rev.* 2020; **95**(6): 1511–1534.
PubMed Abstract | Publisher Full Text | Free Full Text

Redhead SA, Vizzini A, Drehmel DC, *et al.*: **Saproamanita, a new name for both Lepidella E.-J. Gilbert and Aspidella E.-J. Gilbert (Amaniteae, Amanitaceae).** *IMA Fungus.* 2016; **7**(1): 119–129.
PubMed Abstract | Publisher Full Text | Free Full Text

Reid DA, Eicker A: **South African fungi: the genus Amanita.** *Mycol. Res.* 1991; **95**(1): 80–95.
Publisher Full Text

Simão FA, Waterhouse RM, Ioannidis P, *et al.*: **BUSCO: Assessing genome assembly and annotation completeness with single-copy orthologs.** *Bioinformatics.* 2015; **31**(19): 3210–3212.
Publisher Full Text

Singer R: **Quelques Agarics nouveaux de l'Argentine.** *Revue de Mycologie.* 1953; **18**(1): 1–17.

Stajich JE, Wilke SK, Ahrén D, *et al.*: **Insights into evolution of multicellular fungi from the assembled chromosomes of the mushroom Coprinopsis cinerea (Coprinus cinereus).** *Proc. Natl. Acad. Sci. U. S. A.* 2010; **107**(26): 11889–11894.
PubMed Abstract | Publisher Full Text | Free Full Text

Team, R: *R: A language and environment for statistical computing [Computer software manual].* 2016.

Thiers HD: **The Agaric Flora of Texas. I. New Species of Agarics and Boletes.** *Mycologia.* 1957; **49**(5): 707–722.
Publisher Full Text

Thorvaldsdóttir H, Robinson JT, Mesirov JP: **Integrative Genomics Viewer (IGV): high-performance genomics data visualization and exploration.** *Brief. Bioinform.* 2012; **14**(2): 178–192.
PubMed Abstract | Publisher Full Text | Free Full Text

Tulloss RE: *Amanitaceae: Amanita thiersii.* Amanitaceae Studies; 2020.
Reference Source

Tulloss RE: *Studying Morphology.* Amanitaceae Studies; 2023.
Reference Source

Tulloss RE, Kuyper TW, Vellinga EC, *et al.*: **The genus Amanita should not be split.** *Amanitaceae.* 2016; **1**(July): 1–16.
Reference Source

Vargas N, Gonçalves SC, Franco-Molano AE, *et al.*: **In Colombia the Eurasian fungus Amanita muscaria is expanding its range into native, tropical Quercus humboldtii forests.** *Mycologia.* 2019; **111**(5): 758–771.
PubMed Abstract | Publisher Full Text

Wang Y-W, McKeon MC, Elmore H, *et al.*: **Invasive Californian death caps develop mushrooms and sporulate unisexually and bisexually.** *BioRxiv.* 2023.
Publisher Full Text

Wiens JJ: **Species delimitation: New approaches for discovering diversity.** *Syst. Biol.* 2007; **56**(6): 875–878.
PubMed Abstract | Publisher Full Text

Wolfe BE, Kuo M, Pringle A: **Amanita thiersii is a saprotrophic fungus expanding its range in the United States.** *Mycologia.* 2012; **104**(1): 22–33.
PubMed Abstract | Publisher Full Text

Wolfe BE, Tulloss RE, Pringle A: **The irreversible loss of a decomposition pathway marks the single origin of an ectomycorrhizal symbiosis.** *PLoS One.* 2012; **7**(7): e39597.
PubMed Abstract | Publisher Full Text | Free Full Text

Zhang C, Rabiee M, Sayyari E, *et al.*: **ASTRAL-III: Polynomial time species tree reconstruction from partially resolved gene trees.** *BMC Bioinformatics.* 2018; **19**(Suppl 6): 15–30.
PubMed Abstract | Publisher Full Text | Free Full Text

Zhang C, Scornavacca C, Molloy EK, *et al.*: **ASTRAL-pro: Quartet-based species-tree inference despite paralogy.** *Mol. Biol. Evol.* 2020; **37**(11): 3292–3307.
PubMed Abstract | Publisher Full Text | Free Full Text

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Current Peer Review Status: ? ✗ ?

Version 1

Reviewer Report 30 August 2024

https://doi.org/10.5256/f1000research.147897.r246580

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Martin P A Coetzee
Departments of Biochemistry, Genetics and Microbiology, Forestry and Agricultural Biotechnology Institute (FABI), Faculty of Natural and Agricultural Sciences, University of Pretoria, Pretoria, South Africa

In their study, Dunkirk and colleagues investigated the taxonomy and biogeography of *Amanita thiersii* and *A. foetus*. *Amanita thiersii* was described from Texas and is present in the eastern United States and Mexico, while *Amantita foetense* was described from Argentina. The basidiocarps of both species are morphologically very similar. The current question is whether *A. theirsii* is an introduced and invading fungus, or native and expanding its range. The working hypothesis of this study was that *A. thiersii* is an *A. foetens* and that it was introduced into North America. The authors, therefore, investigated whether the two species are conspecific and, if *A. thiersii* is a different species from *A. foetens*, whether it is native and spreading. To test this hypothesis, the authors used distribution data from iNaturalists and Mushroom Observer, conducted phylogenetic studies, and compared the phenotypic characteristics of the two species. They also investigated the mating locus of the two species. Based on their results, the authors concluded that *A. thiersii* and *A. foetens* are two different species. However, it is still unclear if *A. thiersii* is native or introduced into the U.S.A. The HD1 and HD2 mating genes are present, but one dikaryotic individual had the HD1 present in one nucleus but not the second nucleus.

I enjoyed reading the paper and found the results interesting. The manuscript is well written, in my opinion. However, I do have some recommendations for the authors and some questions, which are outlined below.

I am concerned about the small sampling size to determine if there are one or two species. However, this is not unusual in fungal taxonomy. Firm conclusions can, therefore, not be drawn, but the results set a working hypothesis for future studies. The authors might want to address this in their discussion section.

The authors should provide some background information regarding the mating system of Amanita in the Introduction section. The HD1 and HD2 genes are typically associated with the one

mating type locus in Basidiomycota, while the second locus has the pheromones and pheromone receptors. Since the authors only searched for the HD1 and HD2 genes, the question is, what about the second locus, or are the pheromone and pheromone receptors linked to the HD1 and HD2 genes at the same mating type locus? Is it tetrapolar or bipolar, is heterothallic or homothallic?

The authors could have used the term phylogenomic trees for the BUSCO-gene trees. Or at least add the word phylogenetic. For example, BUSCO-gene phylogenetic tree. Still, I recommend using the term phylogenomic tree as the analysis was based on a phylogenomic approach.

I do not think trees generated with ASTRAL are consensus trees. These should be referred to as species coalescent trees. A consensus tree is generated from a set of trees, but it only shows the agreement among the trees. This is different from what ASTRAL does.

Page 5: "Saprotrophic *Amanita* species are closely related to each other and basal to ectomycorrhizal Amanita (Wolfe et al. 2012)". This is wrong; in the paper, they are shown as two monophyletic sister groups.

Page 7: Change "... used the ModelFinder tool to find the best nuclear or mitochondrial substitution model ..." You meant the best nucleotide substitution model; there is no model for substituting the nucleus or mitochondria. Similarly, you use an amino acid substation model if amino acid sequences were used.

Page 7: To get statistical support, 1000 bootstraps using the ultrafast bootstrap approximation method were used. When using an ultrafast bootstrap, a Shimodaira-Hasegawa-like (SHL) approximate likelihood ratio test replicates is recommended in addition to the bootstrap analysis. It may not affect the conclusions, but it is something to consider in the future.

Page 7: The authors stated that they verified the results of their phylogenetic method using concatenated data by also creating a consensus tree using ASTRAL. I do not think that it would really verify the results, as the supertree is generated from individual five locus trees. I am unsure how they obtained the gene trees, as the data available were very patchy for most of the genes (Table 1). There are 17 strains in Table 1, data only from the nulLSU.28S is complete, while the rest have less than 50% sequences available. What was the effect of this on the Astral analysis? I recommend following a Bayesian approach, which often performs better with missing data, and is an alternative to verify the results. Please state what version of ASTRAL was used.

Page 7: It is not clear what the authors meant with "To contextualize the genomes of *A. thiersii* 10802, *A. foetens* 10801 and Amanita sp. 10175 within the genus *Amanita*, ..." The data was used to construct phylogenomic trees and to determine concordance among the gene trees, which are then related to genealogical concordance (i.e. genealogical concordance phylogenetic species recognition). How did this contextualize the genome?

On page 10 the heading reads "*A. thiersii* continues to expand its range in North America, and collections are clustered around urban centers" My issue here is with the word "continues". How does one know that it is continuing to expand its range? Also, the fact that it was not reported on iNaturalists or Mushroom Observer does not mean it is not already in an area outside its current range. Perhaps this can be addressed and better discussed in relation to the methods used by

Wolf et al. (2012) in which historical collections were used to follow the presence/absence of the fungus over time.

Page 11: The authors stated, "However, *A. thiersii* specimens from North America form a separate monophyletic clade from *A. foetens* 10801 and Amanita sp. 10175." A clade is, by definition, monophyletic; remove the word "monophyletic" before clade at all instances.

Page 12: The authors stated that the HD1 locus was not found in the nucleus of some monokaryotic offspring of a dikaryotic strain. While there is precedent for this finding (Wang et al. 2023), do the methods used to find the HD1 gene enable proving that it is not present? How would it have been removed if it was indeed absent, and what would the effect be?

The link to Dryad: Mushroom Observations of Amanita thiersii and A foetens, https://doi.org/10.5061/dryad.7h44j1008, gives an error and is not accessible.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Not applicable

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Fungal systematics, fungal genomics

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 27 February 2024

https://doi.org/10.5256/f1000research.147897.r246576

Julieta Alvarez Manjarrez
Universidad Nacional Autonoma de Mexico, Mexico City, Mexico City, Mexico

The manuscript compares two saprotrophic species *Amanita thiersii* and *A. foetens*. The authors hypothesized that they could be the same species because they present morphological similarities and nutrition mode. *Amanita thiersii* was described from Texas and now its consider an invasive species, while *A. foetens* inhabits in Argentina. After exhaustive methods including public observation databases and fresh specimens, they conclude they are different species but sister clades.
The information is well written however for me it is uncomprehensive the justification of using genomics for a simple taxonomic question. A more easy way was to amplify the barcode and/or other DNA regions would be enough. Additionally, subtle but observable features help to distinguish between the two species.
With the obtained data they confirmed they were two different species closely related. And because the data did not help to elucidate the potential introduction of *A. thiersii*, I think the manuscript should be written just about the differences between the two species rather than left an unanswered question about *A. thiersii* distribution.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
No

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Not applicable

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Mycology, taxonomy, fungal ecology, ectomycorrhiza, tropical ecology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for

reasons outlined above.

Reviewer Report 28 September 2023

https://doi.org/10.5256/f1000research.147897.r201477

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Duur K Aanen
Wageningen University, Wageningen, The Netherlands

This is an interesting and generally well-written manuscript. It describes the results of a study to answer the question whether a recently described mushroom species from north America, Amanita thiersii, is a recently introduced invasive species, or a native species that recently has expanded its range. It studies its relationship with a resembling species from Argentina, Amanita foetens, using morphological, geographical and genetic data. The paper finds a close relationship between the two species, although both seem separate species (but see my comments). Overall, the topic is interesting and relevant, and interesting data are presented. I think the paper can be accepted for indexing if my comments are addressed.

My main comment is about the interpretation of your results. I think your data point to a very close relationship between the two taxa. None of the data you present provide conclusive evidence to conclude that they are two different species. The only convincing data, which in principle could show reproductive isolation would be genetic data of a larger sample of strains. The four specimens for which sequence data are provided are not enough for any form conclusions. Just randomly rearranging branches will give a large fraction where the two will form monophyletic groups. And even if this is confirmed for a larger sample, you might still argue that genetic differentiation has occurred after migration of an invasive population. I would suggest to discuss the implications of your findings in a more open way, without firm conclusions on species identity. To me your data seem consistent with an introduction of this taxon from south America, or from a related taxon not included in your sample (a possibility you also discuss).

I have the following more detailed suggestions to improve the manuscript:

- On page 4, the term “integrative species concept” is used with a reference to the literature. Since the species concept used in this paper seems highly relevant for the conclusions drawn, it is necessary to explain this species concept and perhaps to discuss it in the context of competing species concepts.

- On page 4: “As conservation biology slowly begins to focus on fungi, as well as animals and plants”. This can be misunderstood as that animals and plants slowly begin to focus on fungi, which I guess is not what you mean. Please change to: “As conservation biology slowly begins to focus on fungi, as well as on animals and plants”.

- Page 5: “basal to”, please change to “are the sister group of”.

- Why don't you present all taxa used for the 2-5 gene analysis in Table 1? Now, I was struggling to find the origin of taxa of Figure 3, because two were not included in table 1.

- You searched for the presence of mating-type loci and were able to find HD2 for all three genomes, but for one of the strains HD1 only was present in the (dikaryotic) transcriptome, but not in the genome of one of its nuclei. From this you conclude that HD1 is missing in one of the nuclei of the dikaryon. Since 'absence of proof is not the proof of absence" you need to be more careful in drawing any conclusions from this. So, for example, in the abstract you should rephrase "We discovered an individual which appears to have a mating type locus present in one nucleus of the dikaryon and absent from the second nucleus." to "We discovered mating type sequences in some strains, but in one of the dikaryotic strains, we were able to find a mating-type locus in one of its nuclei, but not in the other".

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Not applicable

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: mycology, phylogenetics, population genetics, social evolution, kin selection, symbiosis

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Comments on this article

Version 1

Reader Comment 08 Sep 2023

Chanel Thomas, University of Pretoria, Pretoria, South Africa

Dear authors

We read your paper as part of our Genomes Journal Club at the Forestry and Agricultural Biotechnology Institute (FABI) at the University of Pretoria. We'd like to share a few of our thoughts and comments with you.

Overall, we thought the article was really well written. The use of citizen science data was particularly interesting and we feel this is something that biologists should strive to do more often. We also felt that the way transcriptome data was used to search for the HD1 gene was an elegant and thorough identification method.

The only "negative" comment was related to the flow of the results. We felt it would have made more sense to put the phylogenies after the species description section, and only after that to bring in the location data results.

Another comment that came up was over the necessity of the recommendation to name all the observations in North America as *A. thiersii* and those in South America as *A. foetens*. Most of us felt that we would have drawn no conclusions about the naming, but it should be noted that none of us are experts in taxonomy. However, this could be considered a fair recommendation, especially since (1) you mention the pros and cons of this choice, (2) you say it is only for the time being and (3) you emphasise the need for molecular identification tools. The only question that remains for us is if this conclusion is necessary, i.e. is there a need to standardise the naming by location.

Otherwise, it was a very interesting article which we all enjoyed reading!

Competing Interests: No competing interests were disclosed.