UNIVERSITY OF WISCONSIN-LA CROSSE

Graduate Studies

POPULATION GENOMIC INSIGHTS INTO THE ESTABLISHMENT OF NON-NATIVE GOLDEN OYSTER MUSHROOMS (*PLEUROTUS CITRINOPILEATUS*) IN THE UNITED STATES

A Manuscript Style Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biology

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December, 2018

POPULATION GENOMIC INSIGHTS INTO THE ESTABLISHMENT OF NON-NATIVE GOLDEN OYSTER MUSHROOMS (*PLEUROTUS CITRINOPILEATUS*) IN THE UNITED STATES

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We recommend acceptance of this thesis in partial fulfillment of the candidate's requirements for the degree of Master of Science in Biology.

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ABSTRACT

Bruce, A.L. Population genomic insights into the establishment of non-native golden oyster mushrooms (*Pleurotus citrinopileatus*) in the United States. MS in Biology, December 2018, 47pp. (T. Osmundson)

The naturalization of non-native golden oyster mushrooms (*Pleurotus citrinopileatus*) represents the first known case of a cultivated mushroom spreading quickly and widely outside of its native range, exhibiting characteristics of invasiveness in the U.S. The first observations of wild fruitings in American woodlands occurred approximately 6 years ago. User-generated biogeographical databases indicate observation frequency has increased significantly over the last two years, with sightings recorded from 9 states so far. To gain insights into the mechanisms behind this species' introduction and spread, I used population genomic data to test the hypothesis that naturalized golden oyster populations are the result of multiple introductions from cultivation operations. I analyzed genome-wide single-nucleotide polymorphisms (SNPs) from 29 wild mushroom specimens collected in six states, plus 6 commercially cultivated isolates. Clustering patterns revealed by the SNP data are consistent with a larger gene pool of commercial strains from which a limited number of strains differentiated via recombination or mutation. High genetic similarity was found between all wild samples plus two of the commercial isolates examined, presenting possible source strains of wild populations. Genotypic subdivision of the wild samples does not closely correlate with geographic location, suggesting multiple introductions and human-mediated spread.

ACKNOWLEDGEMENTS

I thank my research advisor, Todd Osmundson, for his guidance, encouragement, generosity, and support of all my mycological endeavors. I thank my thesis committee members, Tom Volk, Kris Rolfhus, and Bonnie Bratina, for their help and support throughout my thesis work. I thank Tom Volk further as an excellent professor of mycology and a generous mentor. I am very grateful for the support of my parents, Joel and Paula Bruce, my cousins, Eric Remais and Joyce Gordon, my best friend, Conner Reisdorf, and the rest of my loving family and friends. This research was funded by the University of Wisconsin-La Crosse's Research, Service, and Educational Leadership Grant, as well as the Mycological Society of America's Translational Mycology Award. I thank the citizen scientists who collected specimens for this study: Dave Layton, Roger Heidt, Kyle Martin, Tusha Yakovleva, Leah Wesselmann, Marty Gearhart, David Work, Seth Chrisman, Carol Withers, Seana Krohn, Liza Wallner, Claire Sykes, and Jacob Zuelke. Shana McDevitt provided assistance with genomic library quality control and Illumina sequencing at the Vincent J. Coates Genomics Sequencing Laboratory, University of California, Berkeley. Fungal cultures were provided by the Reference Culture Collection at the USDA Center for Forest Mycology Research in Madison, WI.

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INTRODUCTION

Pleurotus citrinopileatus is a saprotrophic fungus commonly referred to as the golden oyster mushroom. Golden oysters share features common among mushrooms in the genus *Pleurotus* (Pleurotaceae, Agaricales, Basidiomycota), including subcentral stipes and decurrent gills, but possess caps with a central depression on top and a bright yellow color that fades to nearly white with age (Fig. 1). They grow in clusters, often quite gregariously, depositing copious numbers of whitish-pink spores. Golden oysters are commonly found growing on oak, elm, beech, and other hardwoods (Stamets, 1993) in the U.S. as well as their native range, which includes eastern Russia, China, Korea, and Japan (Miyazawa, Dejima, Takahashi, & Matsuda, 2011).

Golden oysters have been cultivated for nearly 40 years in Asia for culinary use (S.-T. Chang & Miles, 2004) and have become a popular delicacy in China, Japan, and Taiwan in recent years (Liu et al., 2012). Several studies published over the last 10 years have demonstrated a number of potential health benefits from compounds found in golden oyster extract. These include antioxidant, antihyperglycemic, mitogenic, immunomodulatory, and HIV-1 reverse transcriptase activity (Chen, Ma, Tsai, Wang, & Wu, 2010; Y. R. Li, Liu, Wang, & Ng, 2008). The purported health benefits of these mushrooms, along with their flavor and striking color, has led to the rise in demand for golden oyster mushrooms.

Golden oysters are relatively new arrivals in the American market, cultivated primarily by specialty mushroom growers, often outdoors in close proximity to wooded areas. An examination of evidence found in the literature and on the internet, has

revealed a rough timeline of the species' introduction. Some commercial isolates examined in this study were deposited into American culture banks in the mid-1980s, although there is little evidence to suggest many cultivators grew the species at that time. In his mushroom cultivation guide, Paul Stamets (1993) noted that it was difficult to

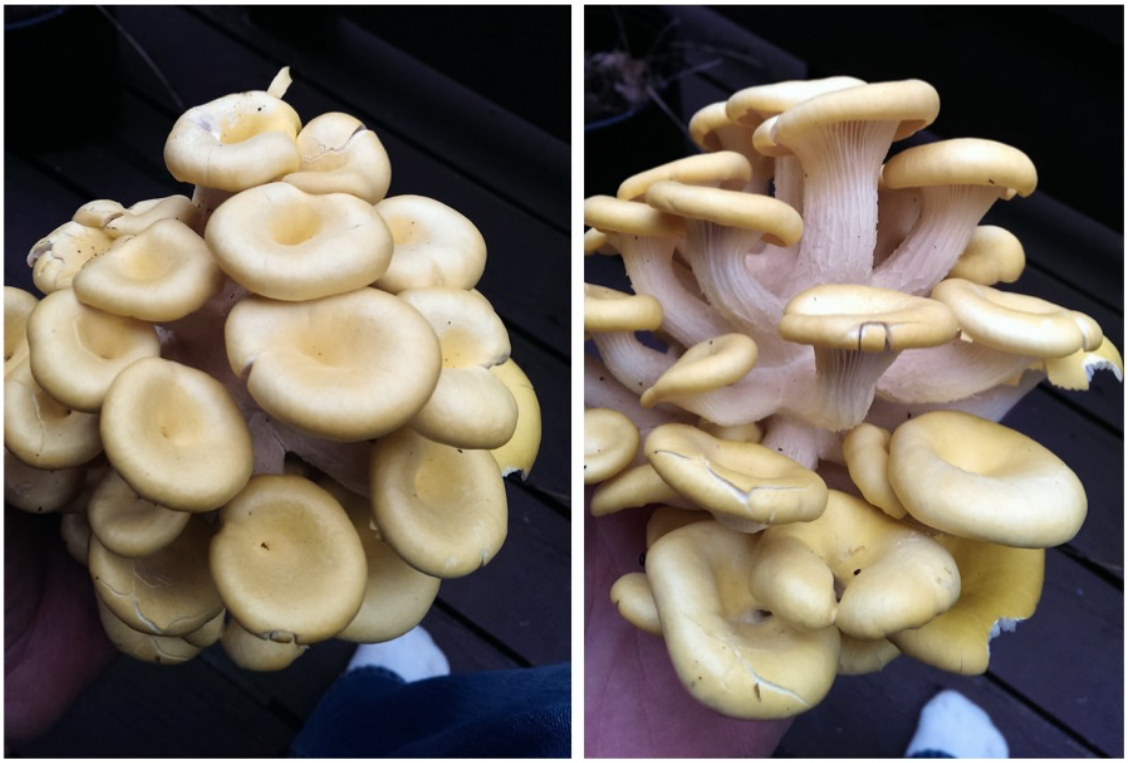

Figure 1. Naturalized golden oyster specimens found growing wild in Ohio (https://mushroomobserver.org/204314).

acquire strains of golden oysters in North America at the time of writing, and that no specimens had been found growing wild. In 2000, the Mycological Society of San Francisco reported a recently introduced, daffodil-yellow cultivated *Pleurotus* mushroom on the market (Freedman, 2000), and in a study submitted in 2001, growth experiments were conducted on a yellow strain of *P. cornucopiae* (previously used synonymously with *P. citrinopileatus*) that was noted to be used commercially in the United States (Royse, 2002).

In 2014, the first recorded wild sighting of golden oysters in the U.S. was entered on Mushroom Observer (Wilson & Hollinger, 2006). Mushroom Observer is a web-based database of user-submitted mushroom observations launched in 2006 that currently hosts over 308,000 fungal observations worldwide, providing open-access biogeographical data for fungi. The first observation of wild golden oysters in the U.S. was entered in June of 2014 in Madison, Wisconsin (Fig. 2), followed by a single 2015 entry observed in an unspecified city in Ohio. Two sightings were recorded in 2016, followed by 9 in 2017, and 11 in 2018 as of October 1st (Fig. 3). Mushroom Observer is one of two lines of evidence available to estimate the frequency of recent wild golden oyster fruitings in the U.S. based on observations reported by the public.

Figure 2. The first known recorded observation of wild golden oyster mushrooms in the U.S., collected by Mushroom Observer user travisalbertbc in Madison, WI in 2014 (https://mushroomobserver.org/167130).

iNaturalist is another web-based reservoir of user-submitted biogeographical and biodiversity data launched as an LLC in 2011, which also suggests an increasing frequency of golden oyster fruitings in American woodlands (Fig. 4; Agrin *et al.* 2018). The first wild golden oyster observation submitted to iNaturalist occurred in 2016 and was the single observation reported that year (Fig. 3). In contrast, 15 observations were submitted in 2017, followed by 93 as of October 1, 2018. The popularity of iNaturalist has increased over these years, which likely accounts for some of the increase in observation records. It remains notable, however, that out of over 220,000 combined observations of mushrooms from 2005 through 2016, the golden oyster mushroom was only reported once, followed by a sharp increase in observation reports in 2017 and 2018, suggesting an extremely low frequency prior to 2016 for such a noticeable, brightly colored mushroom. Furthermore, it is unlikely that these highly recognizable, brightly-colored mushrooms would have gone overlooked by mushroom observers. These observation data indicate that the species is a recent arrival in the U.S., and that fruiting events are being observed more frequently over time.

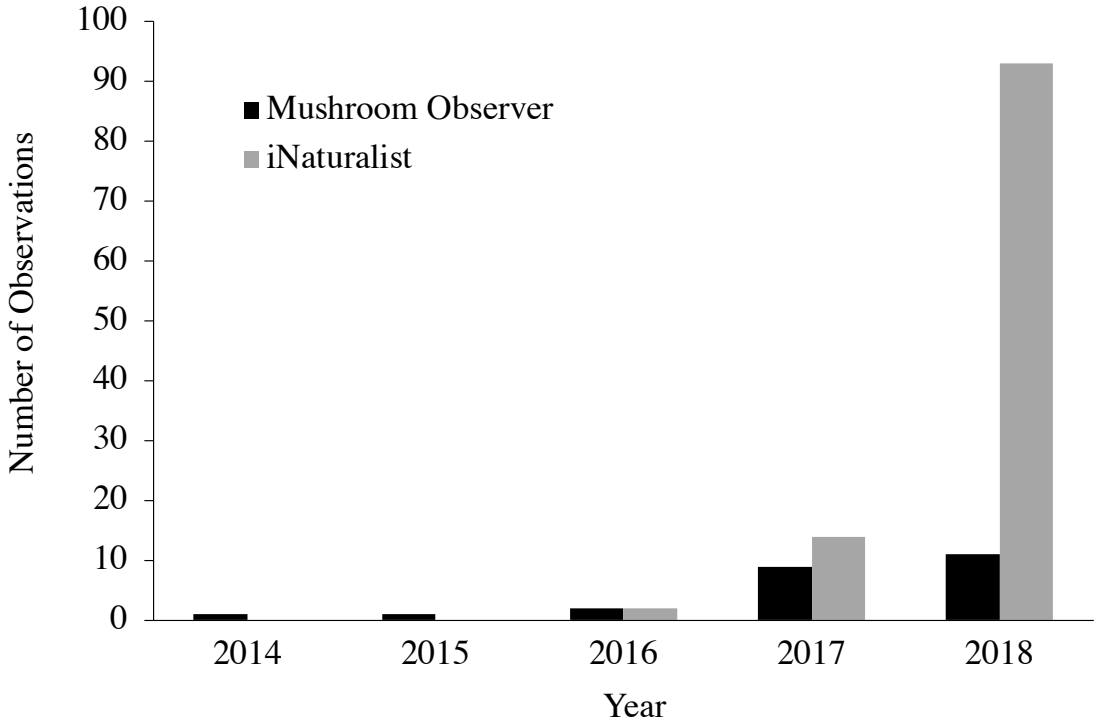

Figure 3. Observations of golden oyster mushrooms in the U.S. over time, as submitted to two user-generated online biogeographical databases. The 2018 observations were submitted between Jan. 1 and Oct. 1, 2018.

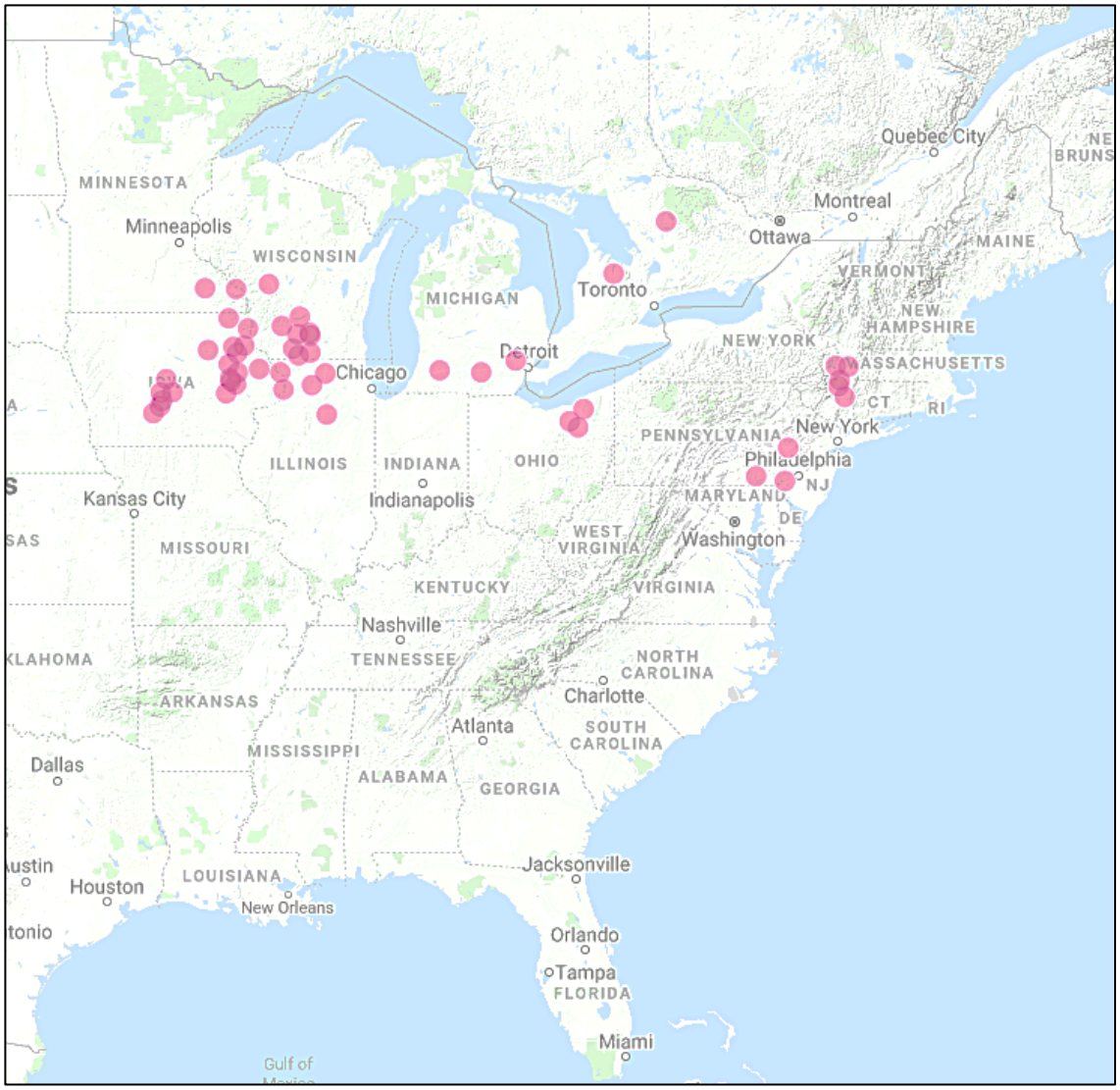

Figure 4. The known range of wild golden oysters in the U.S., as represented by user-submitted observations on iNaturalist from the first record in 2016 through October 1, 2018 (https://www.inaturalist.org/observations?place_id=any&taxon_id=504060).

The naturalization of golden oysters represents the first known case of a cultivated mushroom species spreading quickly and widely outside of its native range. European strains of *Agaricus bisporus*, the white button mushroom, have been cultivated in the U.S. for over 150 years (S. T. Chang & Hayes, 1978), and although Kerrigan and others confirmed that these genotypes have naturalized in some areas of North America, they appear fairly restricted in distribution and no significant spread has been observed

(Kerrigan, Carvalho, Horgen, & Anderson, 1995; Kerrigan & Ross, 1989). Clearly then, non-nativeness does not necessarily implicate invasiveness. Elena Litchman (2010) defines invasive microbes broadly as those that proliferate in a new range and have an impact on local communities or ecosystems. If these impacts on native ecosystems are found to be caused by golden oysters, this definition would place golden oysters alongside better-known invasive fungi, including *Cryphonectria parasitica*, the cause of chestnut blight, and *Ophiostoma novo-ulmi*, the cause of Dutch elm disease of American elms.

The majority of research examining invasive fungi thus far has dealt with pathogenic fungi, due to their more apparent effect on native species and the significant economic impact of crop devastation. There has also been some research investigating the distribution and origin of non-native mycorrhizal fungi, in particular *Amanita* species in North America (Pringle, Adams, Cross, & Bruns, 2009; Vellinga, Wolfe, & Pringle, 2009; Wolfe, Richard, Cross, & Pringle, 2010) and New Zealand (Dickie & Johnston, 2008). Only two species of saprotrophic fungi (*Favolaschia calocera* and *Clathrus archeri*) seem to have been acknowledged as potentially invasive in the literature, primarily based on a combination of anecdotal evidence, historical records, and one single-locus genetic study examining the ribosomal ITS region of less than 20 isolates with global distributions (Arora & Burk, 1982; Johnston et al., 2006; Vizzini, Zotti, & Mello, 2009). There currently exist no data in the scientific literature on the population genetics of invasion for saprotrophic fungi, the absence of which has drastically hindered our understanding of the mechanisms behind their introduction, establishment, and spread. Non-native species are considered one of the most significant threats to intact ecosystems and biodiversity (Pimentel, Lach, Zuniga, & Morrison, 2000), so we have no

grounds by which to assume that alien saprotrophs would have negligible effects on the ecosystems they invade. Possible alterations to native communities include the decline or loss of less competitive native species, shifts in resource availability, and the creation of novel hybrid species.

Examining a species' invasion biology requires an understanding of its native range, but this can be difficult to determine for many microorganisms, including fungi. The range and distribution of most mushroom-forming fungi are not well known, predominantly due to two factors. One of these factors is our heavy reliance on their brief fruiting cycles to offer a glimpse of a species' presence (Vilgalys & Sun, 1994). The other factor is the historically-held notion that microorganisms have global distributions, popularized by Baas Becking's idea that "everything is everywhere, but the environment selects" (1934). The application of the morphological species concept, compounded by a paucity of morphological characters, has traditionally resulted in fungi with near-identical characteristics across continents being considered to represent the same species. However, in many cases, transoceanic distributions have been refuted by DNA sequence data (Taylor, Turner, Townsend, Dettman, & Jacobson, 2006).

Golden oysters have been found to be closely related to *Pleurotus cornucopiae*, an oyster mushroom species found primarily in Europe with a pale, ochre-colored cap. The two species share similar phenotypic characters: a central depression on the top of mature caps; branching, subcentral stems; and a dimitic hyphal system (Bao, Kinugasa, & Kitamoto, 2004). In nature, golden oysters can be differentiated from *P. cornucopiae* by habitat, a brighter yellow cap, smaller spore size, and a lighter spore color (Hilber 1982, as cited in Zervakis and Balis 1996). Although *P. citrinopileatus* was originally described as a distinct species (Singer, 1986), Ohira proposed reducing golden oysters to

varietal status under *P. cornucopiae* due to their sexual compatibility and morphological similarities, suggesting the name *P. cornucopiae var. citrinopileatus* (Ohira 1990, as cited in Bao *et al.* 2004). However, Vilgalys and Sun (1994) point to the controversy surrounding the biological species concept and recommend relying instead on a phylogenetic species concept where monophyletic geographic populations represent species groups. As such, I will defer to the original name for golden oysters, *P. citrinopileatus*, due to its genetic distinction and the fact that allopatric sibling species commonly have incomplete reproductive isolation (Le Gac & Giraud, 2008).

Given their recent arrival and rapid spread following a significant increase in cultivation, there is a high likelihood that naturalized golden oysters escaped from outdoor cultivation, although the mechanisms of invasion remain unknown. The so-called genetic paradox of invasion, *i.e.,* a reduction in adaptive potential caused by the low genetic diversity of a small founder population, is a common hurdle for non-native species in novel ecosystems. However, fungi appear to overcome this hurdle fairly commonly by mating with evolutionary relatives (Gladieux et al., 2015). It is therefore possible that golden oysters may have hybridized with native *Pleurotus* species in the invaded range. A recent review of 47 studies that examined the population genetics of fungal invasions found that 17 contained direct evidence that non-native fungi had either hybridized with other species or admixed with other populations of distinct lineages (Gladieux et al., 2015). Only 2 of the 47 studies concluded that this did not occur, and 28 did not examine this phenomenon. Strains of golden oysters have been shown to be sexually compatible with *P. pulmonarius* (Rosnina, Tan, Abdullah, & Vikineswary, 2016), an oyster species that also fruits in warm-weather conditions and is native to the regions known to be invaded by golden oysters. Of those same 47 studies, 21 found

evidence that the non-native fungi had spread from multiple introduction events, only 6 suggested a single introduction, and 20 did not examine this possibility (Gladieux et al., 2015). Multiple introductions are more likely to introduce multiple genotypes into the invaded area, creating a larger founder population, potentially with higher adaptive potential. The number of golden oyster introductions, the number of genotypes present, and the presence or absence of admixture are fundamental aspects of the invasion biology of *P. citrinopileatus*, which I examined in the present study using population genomics.

Comparing genomic sequence data among naturalized isolates collected throughout a non-native species' introduced range allows inferences to be made regarding the genotypic diversity present, number and location of introductions, naturalized populations' origins, and method of spread (Branco et al., 2017, 2015). While it is possible to track the migration history of well-established naturalized populations using population genetics, i.e., analyses using targeted loci, the number of polymorphic sites is likely too few in cases of recent introductions to glean significant findings. Given the likelihood for very high similarity among wild golden oysters in the U.S., I opted to employ whole-genome sequencing data to examine a much larger number of potential markers, providing greater resolution to track invasion history. For example, Broders *et al.* (2012) compared genomic sequence data of 101 isolates of the non-native *Ophiognomonia clavigignenti-juglandacearum*, the butternut canker fungus, collected throughout its known range in North America. They identified only 17 single-nucleotide polymorphisms (SNPs), which they used to examine the genotypic diversity and spatial distribution of genotypes present using a combination of Bayesian and principal component clustering analyses. They found three distinct populations in North America, each with a strong clonal structure, suggesting multiple introduction events and

a spread by asexually produced propagules. Non-genomic scale data would have been insufficient for generating enough SNPs to infer population structure and invasion history of this species.

In the present study, similar analyses utilizing genome-wide variant data were employed to answer 3 primary questions regarding the invasion of golden oyster mushrooms in the U.S.: (a) Are wild strains genetically similar to commercial strains commercially cultivated in the United States? (b) Was there likely one introduction event, or multiple? (c) Do wild golden oysters represent clones of a single strain? Given the frequency with which mushroom cultivators buy, sell, and trade strains, I hypothesize that naturalized golden oysters in the U.S. are the progeny of one or very few commercial strains, that they were introduced into the wild several times in different geographic regions, and that they are spreading sexually. My sampling strategy involved the recruitment of citizen scientist volunteers to collect golden oyster specimens from different geographic regions within the species' known range, combined with the purchase of commercial isolates from spawn retailers and culture banks for comparison. I employed a combination of clustering, ordination, and model-based population structure analyses on genomic SNP data to make the first inferences of demographic history and method of spread of golden oysters in the United States. To my knowledge, this work represents the first genome-level analysis of non-native saprotrophic fungi anywhere in the world, providing critical insights into the invasion biology of the rarely observed non-native decomposer fungi. As a particularly distinctive macrofungus, the golden oyster is an excellent model species for studying the impacts that invasive saprotrophic fungi may have on intact ecosystems, and furthermore is an excellent candidate for the incorporation of citizen science to assist in that effort.

METHODS

Specimen collection and processing

Thirty-four wild golden oyster isolates were collected and donated by citizen scientists in the U.S. for the characterization of naturalized population structure, representing 6 of the 8 states where species sightings had been reported at the time (Table 1, Fig. 5). Collectors were given instructions to first dry mushrooms either in a food dehydrator on its lowest temperature setting or in an oven on its lowest temperature setting with the door propped open a few inches and a fan pointed at the open door. Six additional isolates sourced from commercial mushroom kits (labeled as "Commercially sourced" in Table 1) were obtained to assess similarity between wild isolates and cultivated isolates that may have escaped. Of the commercial isolates, sample 2 (strain CBS-276.33), sample 3 (strain FP-102361-Sp), and sample 4 (strain FP-140077-Sp) were obtained from the Reference Culture Collection at the USDA Center for Forest Mycology Research in Madison, WI. The others, samples 5, 6, and 7, were purchased from spawn retailers and isolated from grain spawn.

Each live commercial isolate was cultured on potato dextrose agar (PDA) growth medium supplemented with gentamycin. To avoid confounding factors that might be caused by the agar in downstream processes, nitrocellulose filter paper was cut into 80-mm discs, autoclaved, and placed on top of new PDA plates. Each disc was wetted with autoclaved deionized water to help it adhere flat to the agar medium. An agar plug of each isolate was transferred onto a separate plate, on top of the nitrocellulose disc, and

incubated at room temperature for 29-36 days until plates were amply colonized.

Mycelium was then scraped off the top of the discs for DNA extraction.

Table 1. Collection information for golden oyster specimens used in the final analyses.

Sample	Collection Location	Collection Date
1	Tivoli, New York	9/29/2017
2	Commercially sourced	--
3	Commercially sourced	--
4	Commercially sourced	--
5	Commercially sourced	--
6	Commercially sourced	--
7	Commercially sourced	--
8	La Crosse, Wisconsin	6/14/2017
9	La Crosse, Wisconsin	10/1/2017
10	Clinton, Iowa	7/25/2017
11	Clinton, Iowa	8/19/2017*
12	Clinton, Iowa	8/18/2017
13	Muscatine, Iowa	9/12/2017*
14	Morrison, Illinois	9/12/2017*
15	Clinton, Iowa	9/12/2017*
16	Clinton, Iowa	9/12/2017*
17	Hudson, New York	7/10/2017
18	Saugerties, New York	7/8/2017
19	Red Hook, New York	7/11/2017
20	Kingston, New York	7/11/2017
21	Kingston, New York	8/31/2017
22	Kingston, New York	8/31/2017
23	Saugerties, New York	8/31/2017
24	Hurley, New York	8/30/2017
25	Montgomery County, Pennsylvania	6/17/2017
26	Williamston, Michigan	6/23/2017
27	Elkader, Iowa	8/11/2017
28	Toddville, Iowa	10/15/2017
29	Oregon, Wisconsin	9/17/2017
30	Edgerton, Wisconsin	9/30/2017
31	Rhinebeck, New York	10/6/2017*
32	Rosendale, New York	10/4/2017*
33	Rhinebeck, New York	10/4/2017*
34	Muskego, Wisconsin	6/25/2017

*In cases where the collection date was not provided by specimen contributors, the postmark date on the mailed specimen was used.

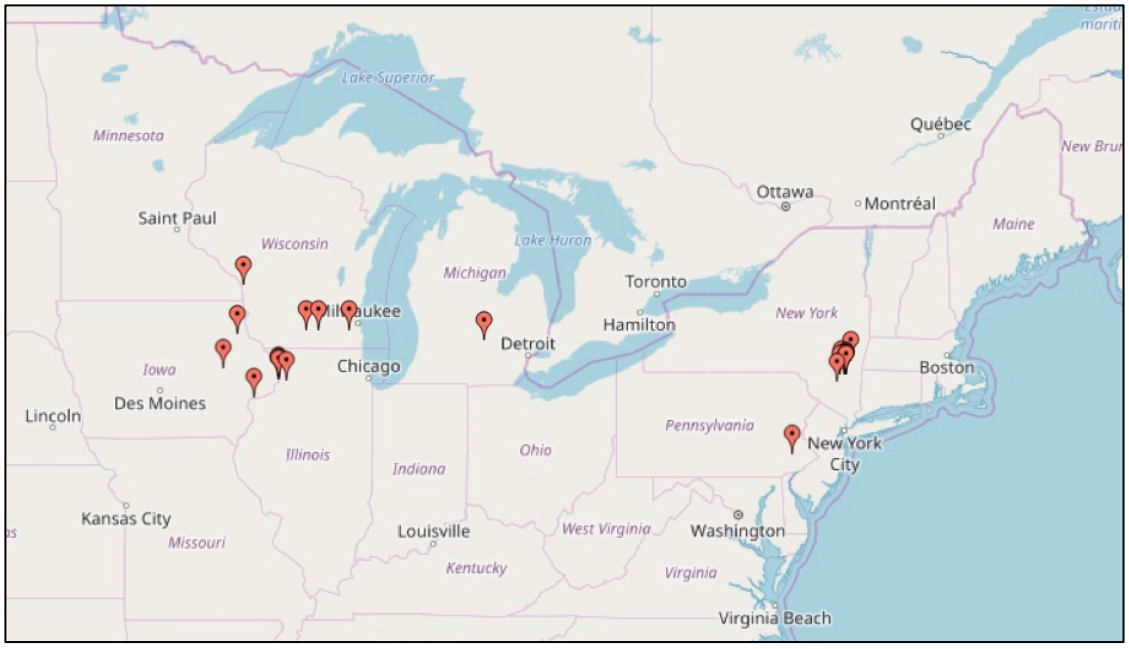

Figure 5. Collection locations of wild golden oyster specimens obtained from citizen scientist contributors.

For DNA extraction from dried mushrooms, clean tissue was taken from the interior of the basidiome whenever possible, i.e., from the pileus or stipe trama, to reduce the inclusion of microbial or spore contaminants. If clean tissue was not obtainable due to excessive damage to the fruit body or due to evidence of insect larvae inside the mushroom, gills were preferentially used over other basidiome tissue.

DNA extraction and genomic sequencing

Tissue samples of both fresh and dried isolates were prepared for DNA extraction by pulverization with liquid nitrogen and a 6-mm glass bead in a mini beadbeater for 1 minute. Genomic DNA was extracted using a CTAB / phenol chloroform extraction followed by column purification using GeneClean GNomic Turbo Salt Solution® (MP Biomedicals Inc.) following Ivors *et al.* (2004). The NEBNext® Ultra™ II DNA Library Prep kit was used for dual indexed library construction for Illumina sequencing following the manufacturer's instructions. Library preparation involves the fragmentation of DNA

molecules to an optimal length range and the addition of sequencing adapters, while indexing attaches a unique short sequence identifier to each sample for downstream analyses after samples have been pooled together (multiplexed). DNA concentrations were measured using a Qubit fluorometer, then pooled with equimolar concentration per sample, and sequenced on the Illumina HiSeq 4000 platform (2 x 250 bp paired-end) at the Vincent J. Coates Genomics Sequencing Laboratory, University of California, Berkeley.

Sequence data processing

Raw whole-genome sequence data were first quality checked using FastQC 0.11.7 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc), and read trimming, filtering, and mapping were carried out using the Galaxy platform 18.09.rc1 (Giardine et al., 2005). Sequence reads were trimmed using Trim Galore! 0.4.3.1 to remove sequencing adapters, (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/), and further quality-filtered using a Phred score cut-off value of 20. Forward and reverse read files were then processed to remove orphan reads (sequences remaining in only one direction after quality filtering) and to sort the remaining reads in matching order between files using a python script (Cock, 2011). The forward and reverse reads were then mapped to a reference genome using Bowtie 2.3.4.1 (Langmead & Salzberg, 2012). The *P. citrinopileatus* reference genome assembly contained 10,689 scaffolds with an N50 of 7,719 bp and a BUSCO result of 82.10%, indicating a fairly high level of completeness (Li *et al.* 2018; https://www.ncbi.nlm.nih.gov/genome/?term= txid98342).

Sequence data analysis

Genotype likelihoods were then calculated using ANGSD 0.921-29-g2753d4c (Korneliussen, Albrechtsen, & Nielsen, 2014). Genotype likelihoods represent the probability of the sequencing data given a genotype in a particular individual. Some of the samples mapped to the reference genome with low or medium coverage, in which case downstream analyses have been shown to improve when working with the raw genotype likelihood data in place of genotype calls (Korneliussen et al., 2014). Genotype likelihoods incorporate statistical uncertainty stemming from the random sampling of reads from dikaryotic isolates, as well as mapping and sequencing errors.

The genotype likelihood data was filtered to remove single nucleotide polymorphisms (SNPs) with minor allele frequencies (MAFs) less than 5% using Plink 1.9 (https://www.cog-genomics.org/plink2/). Plink 2.0 was then used to prune linked genomic variants (linkage disequilibrium pruning) from the dataset, in order to meet the assumption of independently assorting variants required by downstream analyses. The pruned variant data were then used to generate a covariance matrix using PCAngsd 0.95 (https://github.com/Rosemeis/pcangsd/). The resulting covariance matrix was used to construct a neighbor-joining tree, and a principal component analysis (PCA) was conducted to produce a 2-dimensional plot of the first two eigenvectors. The neighbor-joining tree and PCA plot were constructed using R 3.2.2 (https://www.r-project.org/).

ADMIXTURE 1.3.0 (Alexander, Novembre, & Lange, 2009) was used to estimate ancestry proportions of the isolates using the pruned variant data. Simulations were run at parameter values for *K* (the number of inferred ancestral populations) of 1 through 10. Cross-validation errors were generated for each estimate, the lowest of

which represents the best-fit K value (Appendix A). Admixture proportions for each sample were plotted using R for K values 1-4 with low cross-validation errors.

Reciprocal straight-line distances between collection sites of select samples were measured using www.distance.to (Table 2).

RESULTS

We obtained over 691 million raw Illumina reads from 34 golden oyster genomes, of which over 640 million high-quality reads passed quality filtering (Table 2). Filtered reads mapped to the reference genome with a median alignment rate of 80.50% per sample. Filtering of genotype likelihood data generated 1,014,818 SNPs with minor allele frequencies greater than 5%, of which 842,837 were found to be in linkage disequilibrium and pruned. The remaining 171,981 high-quality SNPs across the 36.84 Mb genome were analyzed.

Wild golden oyster isolates are genetically similar to two commercial isolates

Three distinct genetic clusters of samples emerged from the data: two comprised exclusively of commercial strains (clusters 1 and 2) and one comprised of two commercial strains plus all wild samples (cluster 3). Cluster 3 represents the largest cluster outlined in the principal component analysis of the SNP data (Fig. 6), and a single clade in the neighbor-joining tree (Fig. 7), indicating high similarity between samples. Within cluster 3, commercial isolate sample 5 is among a tightly grouped subset of wild isolates (samples 12, 13, 16) collected in Iowa, indicating higher similarity than the rest of the samples. These samples are indistinguishable in the PCA plot at coordinates (0.06, 0.075); in the neighbor-joining tree, they cluster in a nested clade within cluster 3, separated by a long internal branch. The ADMIXTURE analysis depicts these isolates as sharing the same, single ancestor, supporting clonality among samples 5, 13, and 16; sample 12 likely has very few genotypic differences when considering the short branch separating it from the others on the neighbor-joining tree (Figs. 7, 8). It is interesting to

note that, although these samples are not unusual in terms of depth of sequence coverage, they all show a very low (< 2.5%) rate of read alignment to the reference genome. Sample 5 was obtained from a commercial spawn supplier, while the other commercial isolate within cluster 3 (sample 2) was obtained from one of the USDA reference cultures

Table 2. Sequence read and alignment metrics for 34 golden oyster mushroom (*Pleurotus citrinopileatus*) samples. Sample numbers correspond to Table 1.

Sample	Raw reads	High quality reads	Alignment rate (%)
1	14273540	13096309	86.65
2	11933486	11145613	36.70
3	15891090	14776735	84.17
4	14009384	13150160	78.23
5	68424730	63130256	1.32
6	42612734	39873448	81.31
7	62145008	58526317	71.20
8	33065254	31128179	79.73
9	7020208	6446414	71.25
10	13449028	12343846	22.86
11	18024512	16530443	85.30
12	20714000	18912663	0.29
13	15670050	14413517	1.09
14	9961140	9241758	62.76
15	16441562	15306293	85.15
16	16001790	14917185	2.31
17	15442754	14127370	80.19
18	12070674	11136001	86.61
19	14120806	12910556	80.20
20	15401922	14193944	86.20
21	12840914	11953746	82.18
22	14245030	13203394	80.79
23	17253902	16070357	85.84
24	10952514	10221956	79.56
25	72738400	66898664	82.97
26	17056944	15644897	78.49
27	18511782	17175072	70.00
28	17087162	15748983	86.70
29	12010076	11130549	85.88
30	12573010	11470661	85.91
31	11947918	10992595	83.48
32	9018862	8239440	86.24
33	12986280	11876523	86.40
34	15612626	14334967	62.64

under the name *Pleurotus cornucopiae*; no other collection information is available.

The distance between clusters 1 and 2 from cluster 3 suggests that the genotypes represented in clusters 1 and 2 are not direct clonal progenitors of the naturalized populations. The neighbor-joining analysis places clusters 1 and 2 on distinct branches from cluster 3. The samples in clusters 1 and 2 are also consistently differentiated from cluster 3 in the admixture plots across different values of K, and the pattern is further supported by clear isolation of the clusters in the PCA plot. Although all wild isolates grouped in cluster 3, commercial isolates were present in all genotypic clusters, indicating that there is more overall variability across commercial isolates than among wild isolates. Cluster 1 is comprised of samples 3 and 4, both obtained from the Reference Culture Collection. Sample 3 was deposited into the culture bank in 1988, and both samples were reported to be sourced from commercial mushroom kits, but no additional information is available. Based on our estimate that golden oysters were not commonly cultivated in the U.S. during the 1980s, these samples may more closely represent a wild or early commercial Asian genotype. Cluster 2 is comprised of samples 6 and 7, which were sourced from two American spawn suppliers located roughly 1,500 km apart.

Wild golden oyster isolates are not clonal

The high degree of similarity observed between samples 5, 13, 16, and 12 across analyses represents a degree of overlap that we would expect to see between all samples if spread were occurring clonally. Instead, there is measurable genotypic variability among the closely related wild samples, suggesting that spread via sexual propagules (basidiospores) is an important aspect of the invasion of this species.

Landscape genetic patterns suggest multiple introductions

Genotypic subdivisions within cluster 3 do not closely correspond to geographic location. Both the neighbor-joining analysis and the PCA plot indicate the lack of a clear geographic structure, often grouping samples collected multiple states away with those collected within 5 km (Figs. 6, 7). For example, a subset of samples tightly grouped at PCA coordinates (0.1, 0.0), is dominated by samples collected in New York (Fig. 6, inset). The straight-line distances between New York samples in this group range from 3.1 to 11.1 km (Table 2), a proximity within which it is possible that the samples may be siblings within a sexually reproducing population. However, this tight grouping of samples also includes isolates from Illinois and Iowa, over 1,300 km away. It is unlikely that the Illinois and Iowa samples share the same gene pool as the New York samples as a result of sexual reproduction, i.e., spore dispersal, at such a long range, suggesting multiple introductions of the same source strain as a more reasonable explanation of the observed geographic pattern.

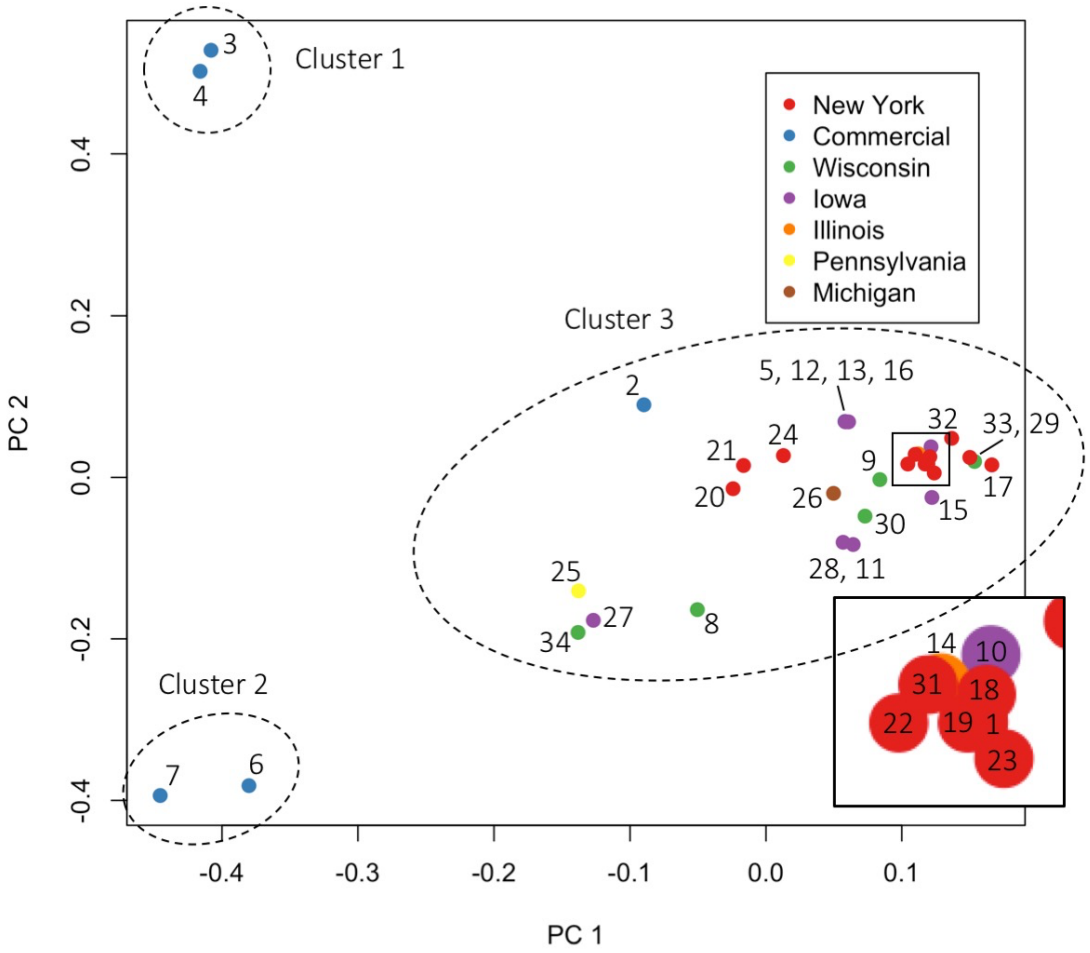

Figure 6. Principal component analysis (PCA) of 34 golden oyster samples based on analysis of 171,981 single nucleotide polymorphisms. Sample numbers correspond to Table 1. The cluster outlined at coordinates (0.1, 0.0) is shown in the inset to better visualize this closely-spaced group of samples. Labeled clusters 1, 2, and 3 represent three genotypically distinct groups of samples also supported by the neighbor-joining tree (Fig. 7).

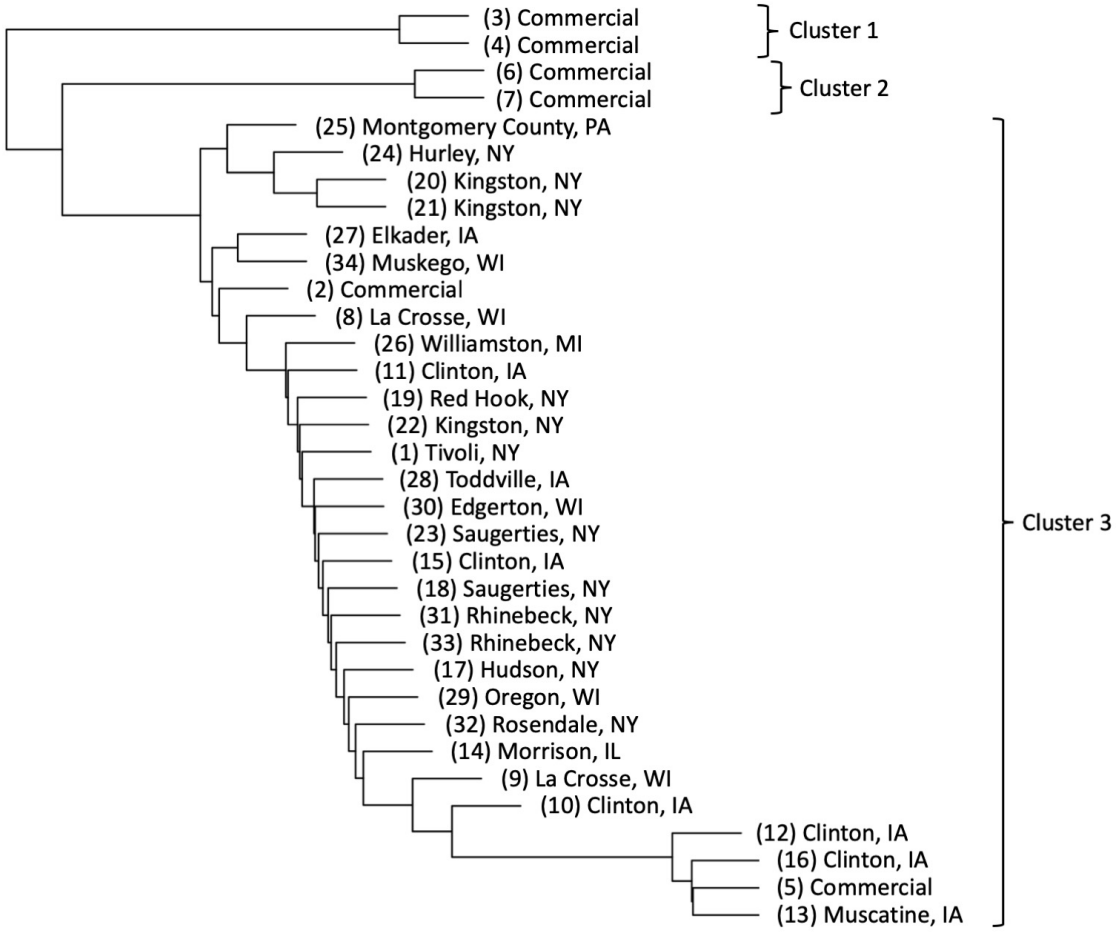

Figure 7. Neighbor-joining tree constructed from genetic distances calculated from 171,981 SNPs among 34 samples of golden oyster mushrooms (*Pleurotus citrinopileatus*). The three clusters identified on the right side of the figure correspond to the three genotypic clusters determined in the PCA (Fig. 6).

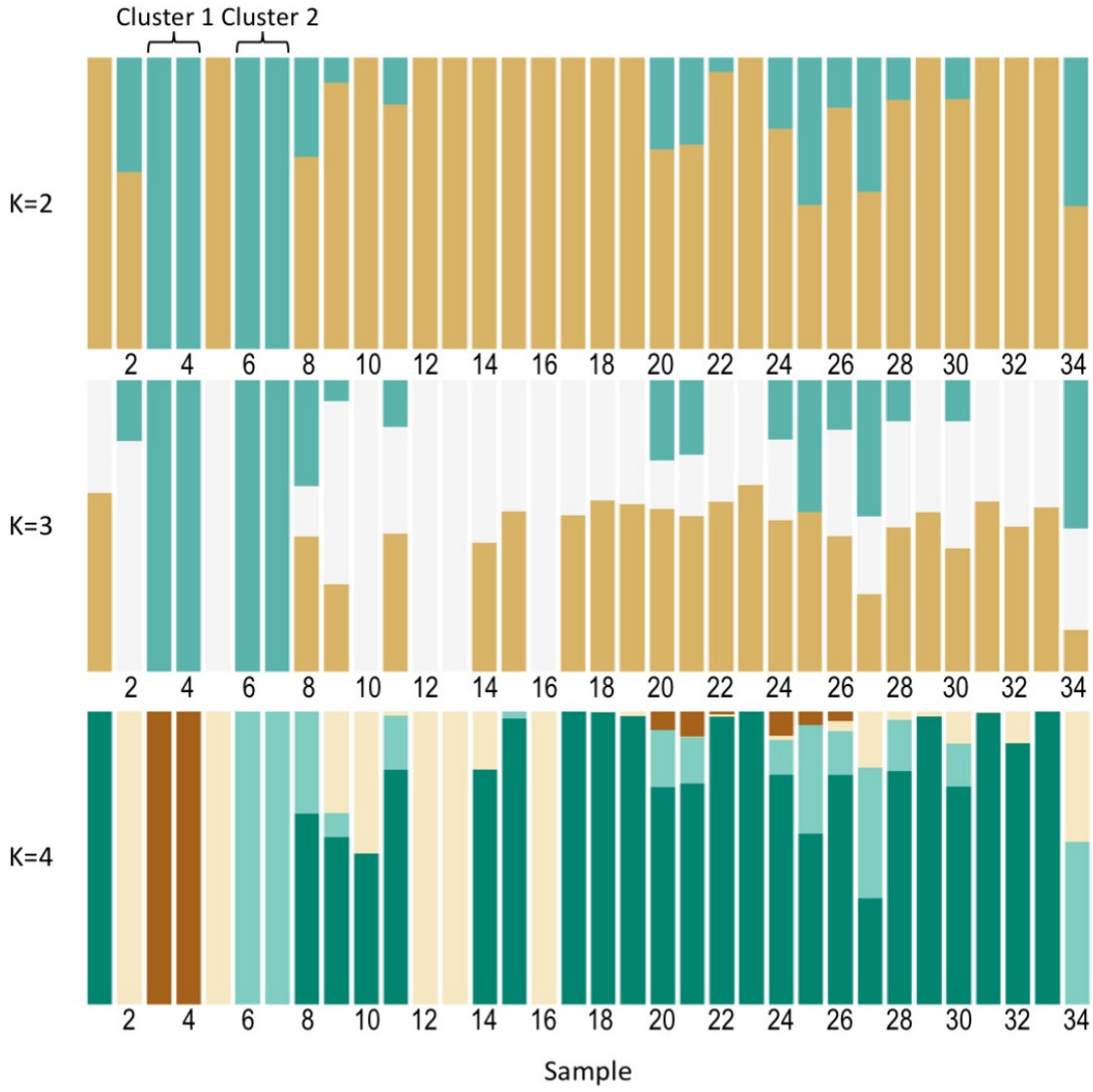

Figure 8. Admixture proportions for 34 sampled golden oyster mushrooms (*Pleurotus citrinopileatus*), as inferred by ADMIXTURE. Sample numbers correspond to Table 1, with each sample represented by a vertical bar. K represents the number of inferred populations, represented by different colors.

Table 3. Reciprocal straight-line distances (km) between collection locations of samples outlined at PCA coordinates (0.1, 0.0) (Fig. 6).

Sample	1	10	14	18	19	22	23	31
1	-							
10	1343.1	-						
14	1329.8	18.5	-					
18	11.1	1332.2	1318.9	-				
19	4.5	1344.3	1331.0	13.7	-			
22	7.7	1340.3	1327.0	12.3	4.9	-		
23	4.7	1340.8	1327.4	10.5	3.6	3.1	-	
31	10.8	1344.2	1330.8	10.9	6.4	5.3	7.6	-

DISCUSSION

The naturalization of non-native golden oyster mushrooms (*Pleurotus-citrinopileatus*) represents the first documented case of a cultivated mushroom spreading quickly and widely outside of its native range in the United States. The first observations of wild fruitings in American woodlands occurred approximately 6 years ago, and user-generated biogeographical databases indicate that observation frequency has increased significantly over the last two years. To gain insights into the mechanisms behind this species' introduction and spread, I examined genomic data from naturalized specimens collected across golden oysters' known invaded range in addition to strains cultivated commercially in the United States. To account for the probability that samples of the same, recently introduced species may not contain a high number of polymorphic sites, genome-wide single-nucleotide polymorphisms (SNPs) were analyzed to track migration history.

This work, to my knowledge, represents the first genome-level analysis of non-native saprotrophic fungi anywhere in the world, providing critical insights into the invasion biology of a now commonly cultivated mushroom. To test the hypothesis that naturalized golden oyster populations are the result of multiple introductions from outdoor cultivation operations, and that spread is occurring via sexual basidiospores, I employed a combination of clustering, ordination, and model-based population structure analyses on the genomic SNP data. I examined similarity clustering results, ancestral admixture proportion estimates, and landscape genetic patterns to infer the history and method of spread of the naturalized populations.

The sudden emergence and rapid spread of golden oyster mushrooms in the U.S. are consistent with the characteristics of a recently introduced invasive species, as are the results of our genomic analyses. These results provide insights into possible mechanisms underlying this invasion, while also identifying several important unknowns that require further research.

In a simple invasion scenario, a human-mediated introduction event of golden oysters by basidiospores could induce the establishment of a wild population comprised of founder siblings and their descendants. This event would create a regional pocket of related individuals with small amounts of genetic variation due to independent assortment and recombination. In our analyses, this scenario would be evidenced by a clustering of one commercial isolate with a group of wild samples collected within the same geographic region. In the event that this simple invasion scenario occurred over multiple introductions with different cultivated strains, our results would show similar clustering patterns where different commercial isolates cluster with corresponding geographically proximate wild isolates. However, the actual pattern revealed by our analyses differs significantly from this simple invasion scenario. Most of the commercial strains cluster separately from the wild isolates captured in our sampling, and wild isolates do not form discrete clusters according to geographic region.

Our cluster and ordination analyses reveal that commercial isolates comprise a greater level of genetic variability than the wild isolates, suggesting that a limited subset of this larger gene pool of cultivated strains became the founders of the naturalized populations (Figs. 6, 7). In several cases, genetically similar samples were collected from sites separated by significant geographical distances. For example, samples 15 and 23 appear to originate from the same gene pool, but they were collected in Clinton, IA and

Saugerties, NY respectively. Although they appear to be related, at over 1,300 km away, they are unlikely to be siblings within a single sexually reproducing population, as this scenario would require two sexually compatible spores to travel across several states and germinate on the same log to form the mushroom-producing dikaryon. A more plausible explanation is the occurrence of multiple introductions of the same source strain in different parts of the country. Variation among closely related wild isolates may have been driven in part by selection practices by independent cultivators, perhaps coupled with subsequent differentiation of individuals via recombination or mutation.

One group of samples appears to more closely align with the model of establishment and spread described in the simple invasion scenario above. A high level of similarity was found between commercial sample 5 and wild samples 12, 13, and 16, all of which were collected in eastern Iowa. This group was clustered tightly in the neighbor-joining and ordination analyses, and the admixture proportion analysis indicates shared ancestry among them. However, our results show that there is variation among several other samples collected in the same region, indicating that clonal spread is not a significant mechanism of this invasion. It is possible that the formation of this grouping in our analyses was in some way an artifact of these samples' low alignment proportions to the reference genome; further study is required to determine whether this is the case, or whether this group's differentiation truly reflects strong genetic divergence from the other wild isolates. Additional examination of the unaligned reads may help clarify why so few aligned. Overall, both the lack of geographic structure in the dataset and the high degree of similarity among wild isolates compared to the commercial cultivars supports our hypothesis that naturalized golden oysters in the U.S. are likely the progeny of one or

very few founder strains, introduced into the wild several times in different geographic regions.

Although our results confirm that wild golden oysters in the U.S. have escaped from cultivation, as shown through the high similarity between some commercial isolates and the wild isolates examined, details regarding the wild populations' invasion history remain unclear. The rather high number of high-quality SNPs represent more variability than might have been expected among a recently introduced population where sexual reproduction is only likely to occur in patches of its widely scattered geographic range; in other words, it is unlikely that 171,981 independently assorting variants would have arisen from mutation or recombination alone. These results indicate the presence of additional source populations that were not captured in our sampling. For example, the clustering of commercial samples 2 and 5 with all wild isolates might point toward a source gene pool for the naturalized populations, but the admixture proportions of these samples indicate that these commercial isolates only comprise a fraction of wild samples' genotypic make-up. It is unclear whether the source population not sampled in this analysis is among the modern American golden oyster population and presently admixing, whether we detected historic admixture of an ancestral population in Asia, or whether the genotype actually comes from a native *Pleurotus* species with which golden oysters are hybridizing. A closer examination of the SNPs omitted by linkage disequilibrium pruning could determine whether large numbers of SNPs are concentrated in regions of high difference compared to the reference, which could identify genome regions characteristic of the naturalized population for further examination. In other cases where admixture between heterogenous gene pools has been detected within an invasive fungal population, the source populations have rarely been identified (Gladieux

et al., 2015). As many invasive fungi have been introduced via hitchhiking on globally transported plant materials, any number of the wide diversity of native genotypes could have been introduced, making the discovery of source populations near impossible. However, as golden oysters are commonly cultivated mushrooms with a comparatively limited number of strains, we are more likely to discover golden oyster source populations with continued sampling, which will help resolve the origins of naturalized populations.

Examination into the alleles present among samples may help resolve population structure analyses further. If variation among samples is strictly a result of recombination of a single source genome, there should be at most two alleles present for all loci in the population, in various combinations. If a more thorough sampling of commercial strains failed to account for alleles present in the wild isolates, the commercial strains could not be the sole genetic source, which might point toward hybridization as a potential reservoir of novel genotypes. Moreover, the presence of private alleles – alleles present in only one population – would further differentiate populations and offer insights into their admixture history.

Even though golden oysters are commonly cultivated across the U.S., the current naturalized range of oysters appears to be restricted to northern hardwood forests. It is interesting that the species has not been observed in the more temperate southern regions of the country, where forest conditions might resemble its subtropical native habitat more closely. Rather, naturalized golden oysters tolerate the freezing winters of the introduced range, and in fact appear not to have invaded regions with average winter temperatures warmer than 30° F (-1.1° C) (Fig. 9). This preference for a seemingly ill-suited climate may be explained by considering the window for establishment created by an

ecosystem emerging from winter dormancy in the spring. Observation records from iNaturalist indicate that golden oysters are among the earliest spring mushrooms to appear in its invaded range, fruiting in large numbers within the first two weeks of May (Fig. 10). Few other species in these regions revive from winter dormancy quickly enough to form mushrooms this early in the season. The ability to fruit so early in the spring following a freezing winter may provide golden oysters an opportunity for establishment while potential competitor fungi are still coming out of dormancy; this is an opportunity that does not exist in regions south of their current range. While these factors may restrict golden oysters from migrating southward, westward migration is likely restricted in the United States due to the lack of hardwood forests west of Minnesota (Fig. 11).

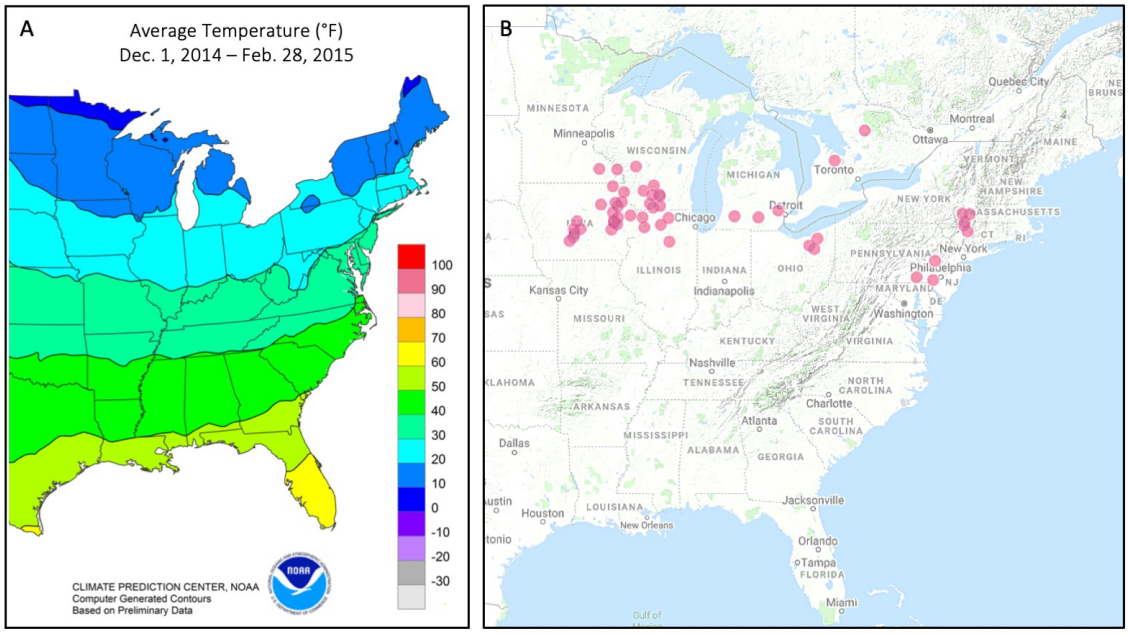

Figure 9. An examination of winter temperatures throughout the known invaded range of golden oyster mushrooms. (A) The most recent available data of average winter temperatures (°F) in the U.S. from Dec. 1, 2014 – Feb. 28, 2015 (http://www.cpc.noaa.gov/products/analysis_ monitoring/regional_monitoring). (B) The known invaded range of golden oysters based on observation records.

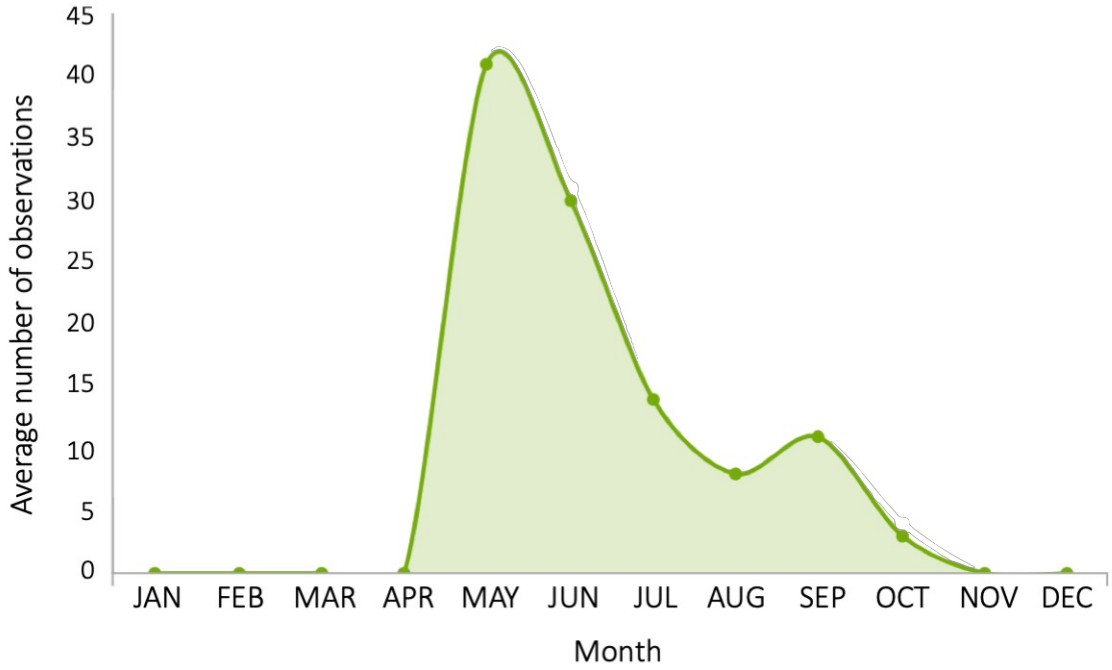

Figure 10. Average number of golden oyster observations in North America per month, based on user-submitted observations to iNaturalist (https://www.inaturalist.org/taxa/504060-Pleurotus-citrinopileatus).

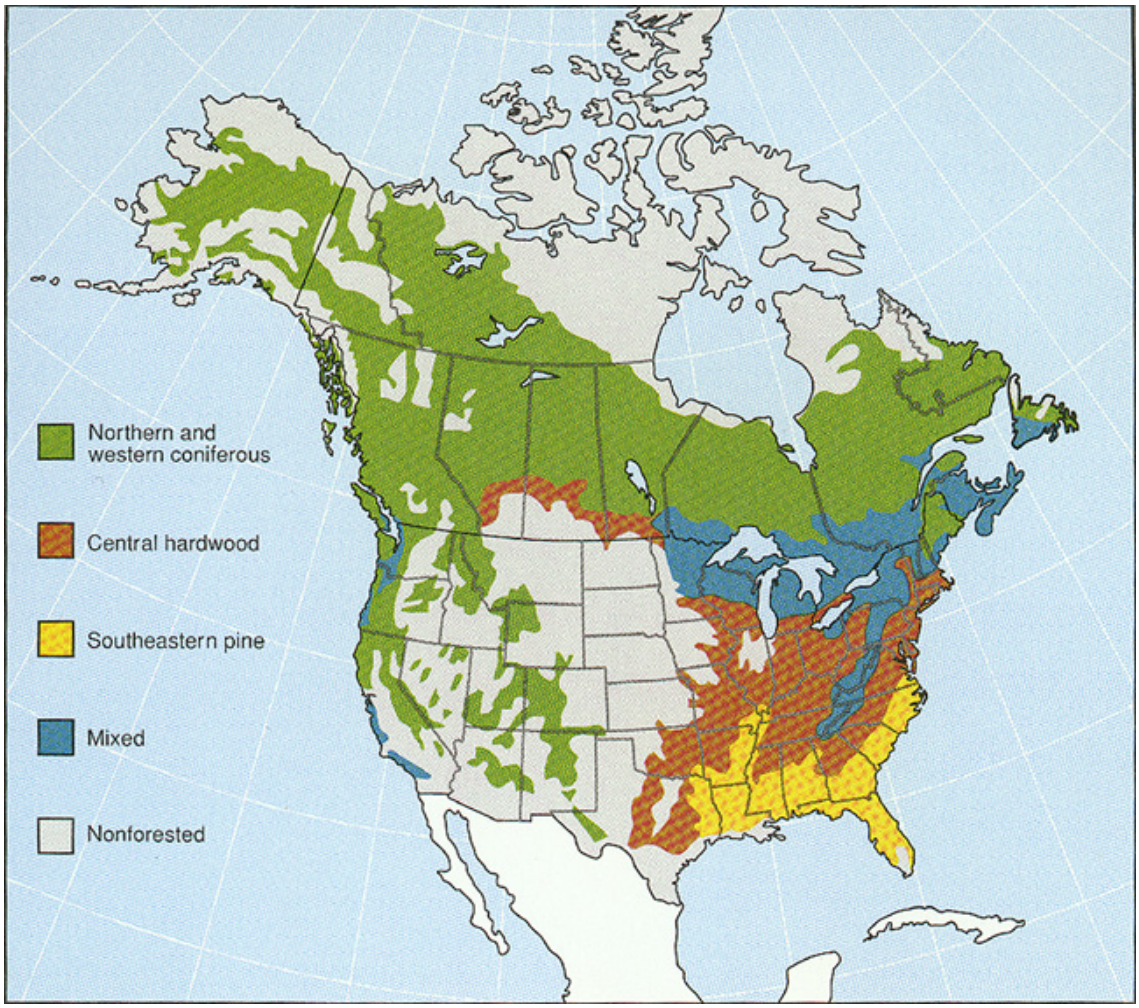

Figure 11. Forest type and coverage in North America (http://geo.msu.edu/extra/geogmich/images/us-foresttypes.jpg).

For these escapees from mushroom cultivation, it is interesting to note that traits golden oysters were likely to have been selected for as cultivated strains are likely to also be beneficial to non-native invaders. Examples of such traits include rapid colonization, an ability to adapt to novel substrates, prolific fruiting, large mushrooms, and a high frequency of fruitings. Cold shocking is a common method used among *Pleurotus* mushroom growers to trigger fruiting for scheduled, heavy flushes of mushrooms. This technique involves lowering the temperature in the fruiting environment by 5° - 10° C (41 - 50° F), often accompanied by lowering CO_2 levels (conditions that effectively

mimic winter conditions in golden oysters' invaded range). A strong response to cold shocking could be an additional trait naturalized isolates were selected for while cultivated. It may be that the cultivation of golden oysters has inadvertently groomed a highly aggressive invader and that outdoor cultivation techniques introduced them directly into suitable habitat.

This study has generated the first inferences into the origins and spread of non-native golden oyster mushrooms in the United States. The continued study of samples collected in forthcoming years will help to refine these findings with greater resolution. The recruitment of citizen scientists as specimen contributors was invaluable to this study, given the wide geographic distribution of the species. Population-level studies offer prime opportunities to employ the assistance of citizen scientists, many of whom are aware of these population shifts well before they appear in the scientific literature. Moreover, golden oysters are excellent candidates for data collection by citizen scientists because their brightly colored sporocarps and often very large fruitings allow them to be easily recognized even among hobbyist mushroom hunters. Online platforms like Mushroom Observer and iNaturalist provided critical windows into the invasion timeline and spread, while also creating more opportunities for the public to engage with the science by generating these data. These online platforms make biogeographical data increasingly easy both to enter and to access through smart phones, offering a bridge to connect citizen naturalists and academic researchers. This partnership importantly offers new, constantly expanding avenues by which we can further our understanding of this rapid invasion and provides a model that other efforts to track species migrations may follow.

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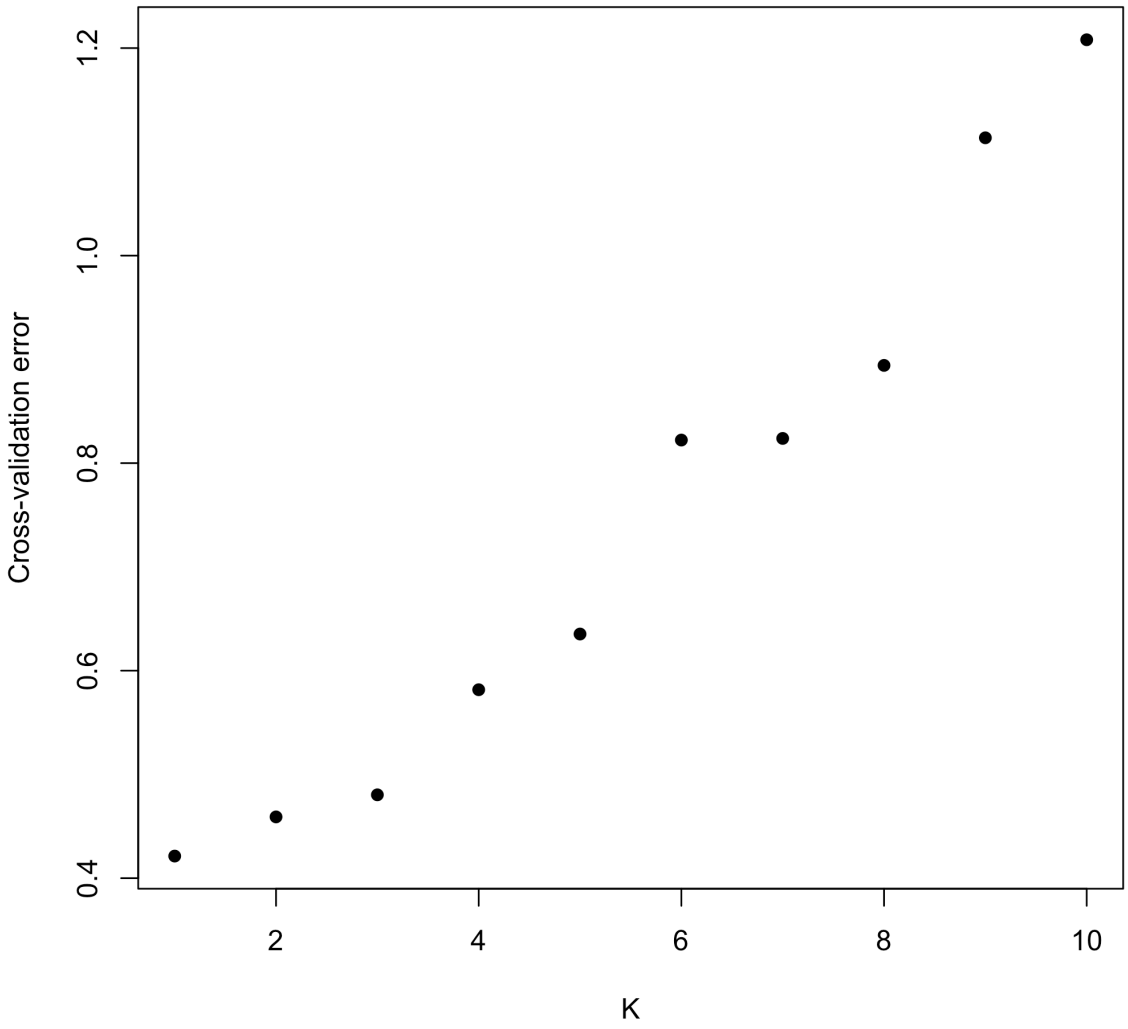
APPENDIX A

ADMIXTURE-GENERATED CROSS-VALIDATION ERRORS FOR EACH VALUE OF *K*, THE NUMBER OF SPECIFIED ANCESTRAL POPULATIONS FOR ALL SAMPLES



APPENDIX A. ADMIXTURE-generated cross-validation errors for each value of *K*, the number of ancestral populations for all samples, suggesting an optimal value of 1, 2, or 3. Plots of admixture proportions for these values of *K* as well as *K*=4, the latter shown to examine genomic proportions at a finer scale, are shown in Fig. 8.