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Population Genomics of *Macrophomina* spp. Reveals Cryptic Host Specialization and Evidence for Meiotic Recombination

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Knowledge of the factors structuring populations of pathogenic fungi is fundamental to disease management efforts and basic biology. However, this crucial information is missing for many important pathogens, including broad-host-range and drought-associated pathogens from the globally distributed *Macrophomina* genus. The objectives of this work were to evaluate the evidence for host specialization, geographic adaptation, and recombination using a global survey of *Macrophomina* isolates from diverse geographic, temporal, and host sources. We obtained high-quality short-read sequence data for 463 *Macrophomina* spp. isolates, representing four putative species, collected from 91 host plant species and soil in 23 countries. Analysis of bi-allelic, single-nucleotide polymorphisms revealed high diversity, admixture, and equal mating type ratios, suggesting ongoing recombination. Although most tested isolates asymptomatically colonized strawberry, only strawberry-derived isolates caused disease on this host. These isolates were all in a single lineage, suggesting that the ability to cause disease on strawberry is not widespread among *M. phaseolina*. Significant associations were also found between isolations from soybean plants and specific population clusters, suggesting that specialization for virulence or reproduction has also occurred for soybean. Geography × isolate genotype associations were weak, suggesting that *Macrophomina* spp. were frequently trafficked between regions. Reference-free whole-genome comparisons support current boundaries among four *Macrophomina* species, and new molecular markers were designed to specifically identify each species. Contrary to expectations, *M. phaseolina* should be considered a single species with both specialist and generalist populations in which meiosis can maintain genetic diversity.

Keywords: meiotic recombination, molecular diagnostics, population genomics

Effective management of plant disease requires knowledge of the factors structuring pathogen genomic diversity, such as host and geographic specialization, and the mechanisms driving the evolution of new pathogenic strains. For example, host specialization, which refers to a pathogen's adaptation to cause more severe disease on a specific host, can occur at the subspecific level (Edel-Hermann and Lecomte 2019; Gladieux et al. 2018), and it must be understood to develop accurate diagnostic methods and evaluate host resistance. Associations between geographic regions and pathogen genotype can illuminate routes of dispersal that could be disrupted by phytosanitary regulations (Ali et al. 2014; Feurtey et al. 2023; Sotiropoulos et al. 2022). Additionally, the frequency of meiotic or parasexual recombination can indicate the likelihood of new, virulent strains emerging (McDonald and Linde 2002). A durable understanding of these factors can only be achieved with globally representative sampling, as international trade continues to move pests and pathogens to new areas (Schertler et al. 2024; Sikes et al. 2018). These factors are only characterized for a few well-studied fungi, and expanding research to novel systems is necessary to broaden knowledge of fungal evolution.

The genus *Macrophomina* contains economically important plant pathogens, such as the cosmopolitan type species *M. phaseolina*. This species was first identified in the early 1900s, has been reported worldwide (Pennerman et al. 2024), and can cause total crop losses in soybean and strawberry (Bradley et al. 2021; Steele et al. 2023). As a species, *M. phaseolina* has a broad host range of at least 97 plant species, in addition to humans with compromised immune systems (Pennerman et al. 2024). It is widely speculated that abiotic stresses induced by climate change will lead to increased damage by *M. phaseolina* (Cohen et al. 2022),

as this fungus causes greater disease severity in warmer and drier conditions (Baggio et al. 2021; Chilakala et al. 2022; de Sousa Linhares et al. 2020; Diourte et al. 1995). Four other *Macrophomina* species were recently described: *M. pseudophaseolina* (Sarr et al. 2014), *M. euphorbiicola* (Machado et al. 2019), *M. tecta* (Poudel et al. 2021), and *M. vaccinii* (Zhao et al. 2019). These species have only subtle differences in morphology and were probably mis-identified as *M. phaseolina* before the widespread adoption of molecular phylogenetics (Poudel and Vaghefi 2023). Thus, their global distribution and host range are unknown and could be much wider than is reported.

Pathogens are typically classified along a continuum ranging from host specific, infecting a single host, to generalists that infect many. Between these extremes are host specialists that predominantly infect a single host but may retain virulence on others (Gladieux et al. 2018; Mercier et al. 2019; Resende et al. 1994). Efforts to describe host specialization in *M. phaseolina* have usually relied on comparing isolates from different hosts without knowledge of their genomic differences, and this approach has not yielded clear results (Gupta et al. 2012; Manici et al. 1995; Reznikov et al. 2019; Su et al. 2001). An exception to this trend is for strawberry-associated strains of *M. phaseolina*, which were found to cause severe strawberry disease in Spain, Argentina, and California, United States (Burkhardt et al. 2019a; Koike et al. 2016; Martin et al. 2015; Viejobueno et al. 2022). However, the broad applicability of these findings is unknown, as no association between host isolation source and virulence in strawberry was found in Israel (Zveibil et al. 2012), and the populations of *M. phaseolina* from strawberry in Chile and Spain appear to be genetically distinct (Sánchez et al. 2017). High-resolution genotypic information is needed to resolve these discrepancies and illuminate the status of host specialization in *M. phaseolina*.

Although isolate genotype × geography associations have been reported for specific populations of *M. phaseolina* (Ortiz et al. 2023; Sánchez et al. 2017), it remains unknown if the global population of *Macrophomina* spp. is structured by geography or if specific genotypes are found in disparate locations. A geographic influence on population structure seems plausible, given the apparent absence of aerial dispersal and the fact that specific environmental conditions play a large role in disease development. However, there is also some evidence for the ability of *Macrophomina* spp. to asymptomatically infect plants (Fuhlbohm et al. 2012; González and Tello 2011; Jamal et al. 2022; Koike et al. 2016; Moslemi et al. 2024) and be seedborne (Dell'Olmo et al. 2023; Fuhlbohm et al. 2013; Mengitsu et al. 2012; Reuveni et al. 1983; Singh and Singh 1982), so they could have a high potential for dissemination between geographic regions.

Continuous adaptation to host and environment could be accelerated by ongoing meiotic recombination. Many fungi that were historically considered asexual (Crous et al. 2006; Sarr et al. 2014) are now known to undergo meiosis at least periodically (Taylor et al. 2016). Both mating types exist in *M. phaseolina* (Nagel et al. 2018), but their ratios and the extent of admixture are unknown in extant populations. Gene flow could also occur through horizontal transfer of giant *Starship* transposable elements, which carry accessory genes to recipients (Gluck-Thaler et al. 2022; Urquhart et al. 2024). Parasexual recombination can happen during anastomosis of vegetatively compatible hyphae (Pereira et al. 2018; Sybuia et al. 2022), but allo-recognition systems usually prevent anastomosis between genetically distinct strains (Gonçalves and Glass 2020; Pereira et al. 2018; Sybuia et al. 2022). Because of this, parasexual recombination would be less likely to produce novel genotypes than meiotic recombination, which could occur between diverse genotypes (Bennett and Turgeon 2016). Exploring the potential for meiosis

in *Macrophomina* spp. is key to understanding the evolutionary potential of this genus.

To fill these knowledge gaps, the objectives of this work were to evaluate the evidence for host specialization, geographic adaptation, and recombination using a global survey of *Macrophomina* isolates from diverse geographic, temporal, and host sources. This investigation is critical to the global efforts to control diseases caused by *Macrophomina* spp., which are increasing along with abiotic stresses induced by climate change, and for gaining a broad understanding of the factors shaping fungal evolution on a global scale. To our knowledge, this is the first global genomic diversity study for any genus or species in the *Botryosphaeriaceae*.

Results

A global genomic survey revealed 14 genotypic clusters of *Macrophomina* spp.

Whole-genome shotgun sequences were obtained for 463 *Macrophomina* spp. isolates (435 newly sequenced and 28 retrieved from NCBI GenBank; Supplementary Table S1). These isolates were derived from 23 countries, 91 plant hosts, and soil. Collection years ranged between 1927 and 2021 and therefore included many isolates described as *M. phaseolina* by morphology alone and/or before 2014, when additional species were described within the genus *Macrophomina*. The most common

hosts in our study reflected the most frequently researched hosts of *Macrophomina* spp. identified by Pennerman et al. (2024) (Supplementary Fig. S1).

High-quality, bi-allelic single-nucleotide polymorphisms (SNPs) with presence in at least 90% of isolates were identified using reference genomes of strains Mp11-12 (from strawberry [Burkhardt et al. 2019a]; 578,310 SNPs; average of 8.07 SNPs/kb for scaffolds with SNP loci), AL-1 (from alfalfa [Burkhardt et al. 2019a]; 588,334 SNPs; 9.39 SNPs/kb), and mp117 (from soybean field soil [Gluck-Thaler et al. 2022]; 584,184 SNPs; 10.32 SNPs/kb). Using these SNPs, 14 population clusters were identified by a combination of admixture models, reticulate phylogenetic trees, and principal component analyses (Fig. 1; Supplementary Figs. S2 and S3). The cluster assignments were largely congruent among references. The only discrepancies were related to cluster assignment of isolates F285 (at least 10% membership by Q value in clusters VIII, IX, XIII, and XIV) and F385 (clusters II, III, IX, and XI). Cluster assignments in figures throughout this work are shown using the Mp11-12 reference, unless otherwise noted. Two clusters contained isolates that were not described as *M. phaseolina*: cluster I contained *M. euphorbiicola* and *M. pseudophaseolina* (12 isolates total), and cluster II contained eight *M. tecta*, one putative *M. vaccinii* isolate, and two *M. phaseolina* isolates (identified by multi-gene phylogenies shown below; cluster II = 11 isolates total). The remaining 12 clusters contained isolates described as *M. phaseolina*.
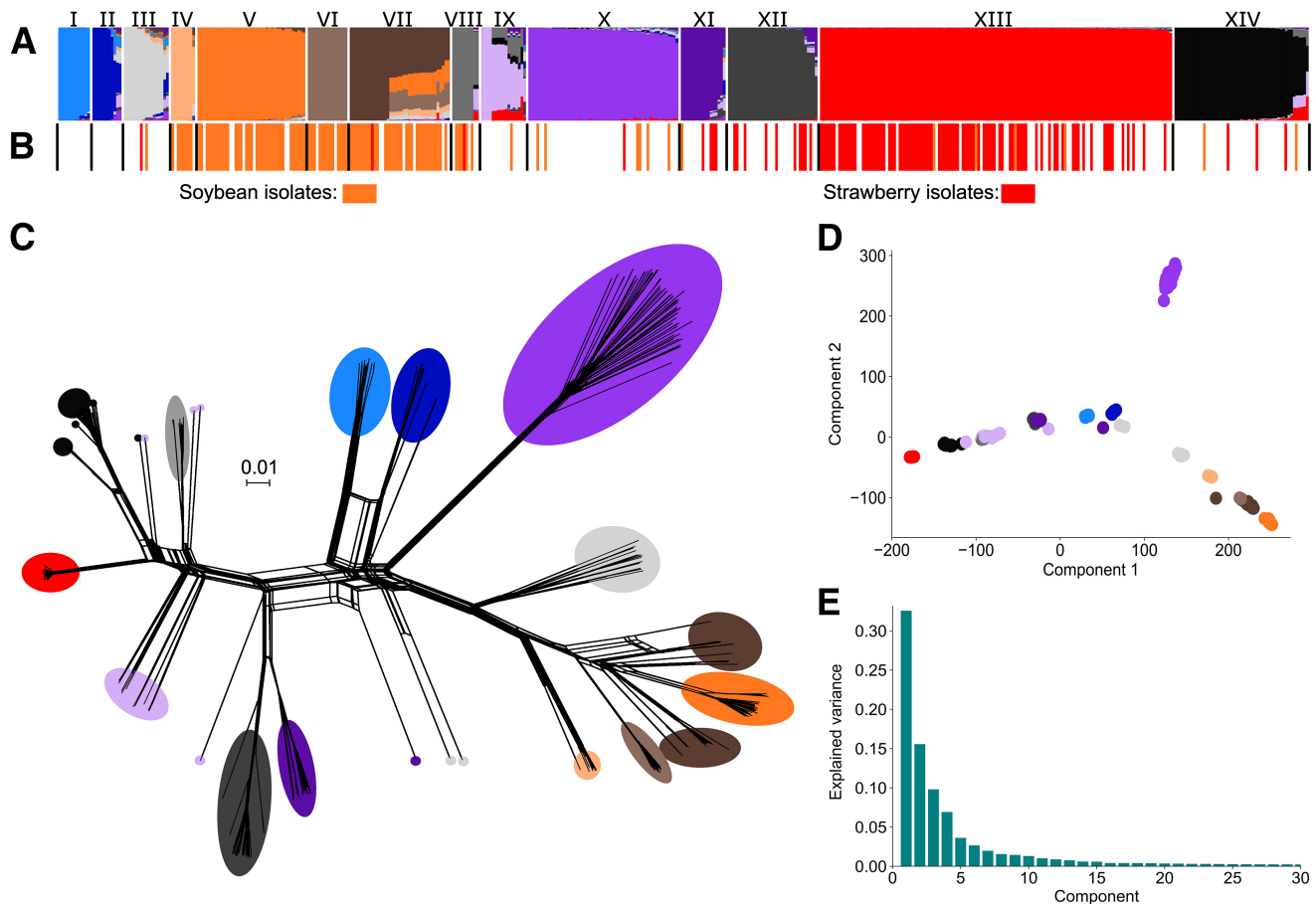
Fig. 1. Grouping of *Macrophomina* spp. isolates with Mp11-12-based single-nucleotide polymorphisms. **A,** The admixture plot was generated with sNMF at K = 14. From left to right, the clusters are labeled with Roman numerals and color-coded. **B,** Colored bars indicate the isolation source for each isolate in the above admixture plot, where strawberry is in red, soybean or soybean field soil is in orange, and cluster borders are in black. The **C,** reticulate phylogenetic tree and **D,** principal component analysis plot use the same colors as the admixture plot to indicate the major cluster assignment. **E,** The Scree plot shows the eigenvalues for the first 30 principal components.

Pangenomic gene content was structured by the 14 population clusters

A pangenomic analysis of gene content was conducted to evaluate the strength of the cluster designations and the genetic diversity within *Macrophomina* spp. A total of 113,290 homologs were identified across all genome assemblies. These homologs comprised 10,984 "super-core" homologs (present in all 463 *Macrophomina* spp. isolates), 18,866 "core" homologs (present in at least 95% of the isolates), and 45,233 "accessory" homologs (present in at least two isolates but fewer than 95% of all isolates;

Fig. 2A to C). Hierarchical clustering based on core and accessory homolog presence/absence variation yielded groups that were congruent with those identified by admixture and principal component analyses (Fig. 2D). There were 38,207 singletons, and the pangenome appeared slightly open when these genes were included. When singletons were excluded, the pangenome structure was clearly closed (Fig. 2E). The rarefaction observed for highly sampled clusters (e.g., V, VII, X, XII, XIII, and XIV) suggested that continued sampling would have corroborated our finding of a closed pangenome structure (Fig. 2F).
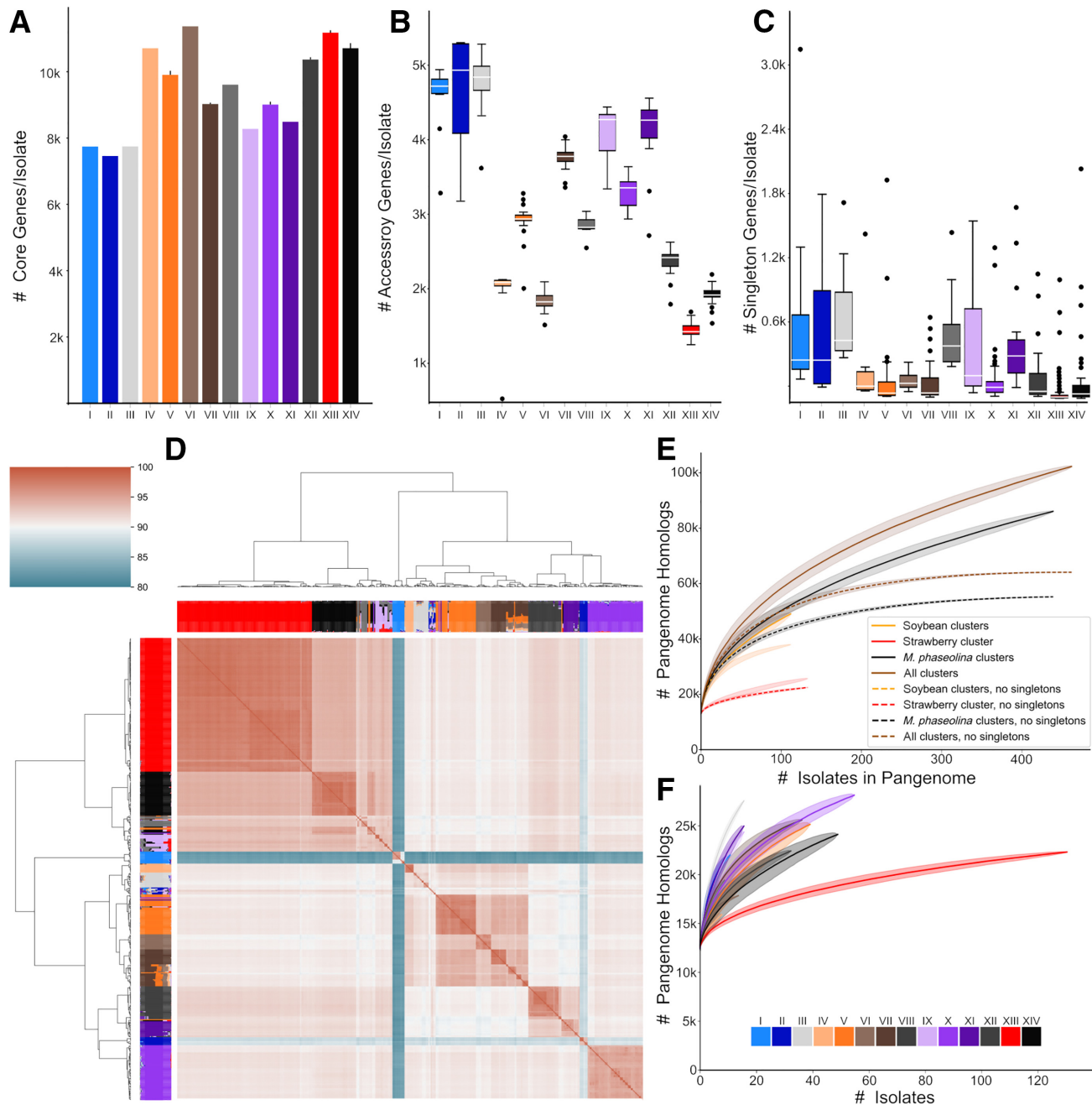
Fig. 2. Homolog presence/absence variation by population cluster. **A,** Bar plot depicting the average number of core gene homologs (present in >95% of isolates) for each population cluster. Error bars represent one standard deviation above and below the mean. **B,** Boxplot depicting the number of accessory gene homologs (present in more than two isolates, less than 95%) for each population cluster. **C,** The number of singletons (genes present in a single isolate) for isolates in each population cluster. **D,** A hierarchically clustered heatmap of core and accessory homolog presence/absence variation. Color intensity within the heatmap indicates the percentage of shared homologs for each pair of isolates. The corresponding Mp11-12 SNP-based admixture plot is displayed between the dendrogram and heatmap. **E,** Rarefaction plot of homologs among all isolates or in selected groups, with and without singletons. **F,** Rarefaction plot of gene homologs for each population cluster. In both E and F, shaded regions surrounding the means represent one standard deviation above and below the mean with 1,000 randomized replications.

Strawberry-derived isolates from *M. phaseolina* cluster XIII were specialized for virulence in strawberry and associated with recent outbreaks of strawberry crown rot disease

Significantly more isolates from strawberry (85 out of 106) were in cluster XIII than would be expected by random chance (χ^2 $P < 0.001$). We hypothesized that cluster XIII isolates were specialized for virulence in strawberry and tested this with virulence assays of 41 isolates representing eight *M. phaseolina* clusters. Five of the six tested isolates from the strawberry-associated cluster XIII consistently caused disease in the susceptible strawberry cultivar Monterey, as indicated by the area under the disease progress curve and measured plant and fruit biomass (Fig. 3). No isolate from another cluster caused disease on strawberry plants, but almost all isolates could be recovered from asymptomatic plants at the end of the 7-week growth period (Fig. 3D). This indicates that the potential for asymptomatic, endophytic growth on strawberry is common in *M. phaseolina*. Isolates from clusters other than XIII were not derived from strawberry, and there may be potential for some strawberry-derived isolates in other clades to cause disease on this host. Nevertheless, these data indicate that not all strains of *M. phaseolina* are specialized for virulence on strawberry.

The cluster XIII host-specialized strains appeared to have emerged recently and were responsible for most new reports of *M. phaseolina* causing disease in strawberry. Consistent with recent emergence, isolates in cluster XIII had low genetic diversity, as evidenced by their high fraction of gene conservation and low anchor distance divergence (Fig. 2D and F; Supplementary Fig. S4). Our dataset included only three isolates of XIII before it was first recovered from strawberry: chrysanthemum in 1934 from Missouri (F103), slash pine in 1983 from Paraguay (F287), and pepper in 2000 from Australia (F322). From 2001 onward, cluster XIII isolates were associated with the nearly simultaneous first reports of highly destructive disease in strawberry in Florida (Mertely et al. 2005), Spain (Avilés et al. 2008), California (Koike 2008), Mexico, and Australia (Golzar et al. 2007; Hutton et al. 2013) (Supplementary Fig. S5).

Clusters IV, V, VI, VII, and VIII were associated with the soybean host

Most isolates from soybean or soil from soybean fields were assigned to five clusters: IV (7 soybean and soybean field isolates/9 total), V (34 isolates/41 total), VI (13 isolates/15 total), VII (31 isolates/38 total), and VIII (5 isolates/7 total; collectively 90/110). These distributions were significantly different from what would be expected under the null hypothesis of no association between the soybean host and isolate genotype (χ^2 $P < 0.001$). The 110 soybean isolates were derived from the United States (56 isolates), Paraguay (42 isolates), Australia (5 isolates), and Denmark (1 isolate), indicating that this trend appears to be a general phenomenon not limited to specific geographic areas. We hypothesize that the genotype × soybean host association results from the increased virulence of these population clusters on soybean, as it was for strawberry.

Evidence of isolate genotype × host associations was not observed for other clusters, suggesting that some populations contain generalists

Strawberry and soybean were the only hosts that were sampled sufficiently to enable robust associations between isolate genotype and host origin. However, the available data did not reveal additional isolate genotype × host associations and suggest that some clusters have generalist populations. For example, common bean ($n = 31$) and muskmelon ($n = 15$) were among the most highly sampled hosts after strawberry and soybean, and their isolates were derived from nine and five clusters, respectively. Some clusters contained isolates from a wide diversity of hosts, such as cluster III, with 12 hosts out of 17 isolates sampled, or cluster X, with 26 hosts out of 57 isolates sampled. These data suggest that true generalist populations exist alongside specialist populations in *Macrophomina* spp.

Lack of genotypic association with geographic location suggests that *Macrophomina* spp. were frequently trafficked between countries

Overall, SNP-based genotype clustering was not associated with the geographic origins of isolates ($P = 0.616$ after 10,000 permutations), as indicated by a Mantel test with the latitudes, longitudes, and Q values of 415 isolates with known sub-country regions. Similarly, analysis of molecular variance identified more variation of Mp11-12 SNP alleles within countries (90.44%) than between countries (9.56%; $P < 0.001$) or between host isolation sources (15.14%; $P < 0.001$). The average calculated fixation index (Hendrick's corrected G_{ST}, G'_{ST}) among the genotype clusters ($G'_{ST} = 0.439$) was higher than when grouping isolates by country of origin ($G'_{ST} = 0.155$; Supplementary Fig. S6), indicating that genetic diversity was lower when isolates were grouped by genotype than by geographic origin.

Similarly, most clusters were found in each of the three well-sampled continents. All SNP-based clusters were found in North America, from which most isolates were derived from the United States ($n = 220$ isolates from the United States out of 230 total from North America). Clusters VI and VIII were exclusively found in the United States; these were the only clusters to be found only in one country or on one continent. The second-most sampled continent was South America ($n = 86$ isolates), which contained all clusters except VI, VIII, and XIV. Australia was the third-most extensively sampled continent ($n = 83$), and it collectively contained all clusters except IV, VI, and VIII (Fig. 4A). This indicates that isolates from each genotypic cluster are widely dispersed across continents rather than found specifically within certain regions. The weak influence of geography on population structure was visualized with minimum spanning trees generated from the Mp11-12 SNP data, which show that isolates were more clearly defined by genotype than by host country (Fig. 4B and C).

Recombination appears to be ongoing in *Macrophomina* spp., including between *M. phaseolina* and *M. tecta*, and could be the result of meiosis

Evidence of apparently recent and ongoing recombination was revealed by fractional ancestries (quantified by Q values) and linkage disequilibrium (LD) decay. Most isolates (361 out of 463) had high (>95%) fractional ancestries, meaning that most of their SNP genotype calls corresponded to their assigned cluster (Supplementary Table S1). However, there were also 57 isolates with less than 75% of their fractional ancestry attributed to their assigned cluster and 30 isolates with less than 50% of their fractional ancestry represented by their assigned cluster. In LD decay plots, the r^2 values declined to around 0.15, after which they tended to remain level, similar to what has been shown for populations with ongoing recombination (Supplementary Fig. S7) (Feurtey et al. 2023). There was evidence suggesting that hybridization occurred between *M. tecta* (cluster II) and *M. phaseolina*, as several cluster II isolates had high ancestry fractions for *M. phaseolina* clusters (Fig. 1A; Table 1). Evidence suggesting hybridization between cluster I (*M. pseudophaseolina* and *M. euphorbiicola*) and *M. phaseolina* clusters was less apparent, with Q values for the assigned cluster among cluster I isolates being at least 90.4% for the Mp11-12 reference. This suggests that recombination could be occurring between *M. tecta* and *M. phaseolina* but may not be occurring between species in cluster I and *M. phaseolina*.

Fig. 3. Virulence of *Macrophomina phaseolina* isolates in strawberry (cultivar Monterey). Boxplots depict **A,** area under the disease progress curve (AUDPC); **B,** aboveground plant fresh weight at the end of the experiment; and **C,** total fruit weight over the entire experiment. Plots depict the pooled results from two experiments, each with 10 biological replicates per treatment. Asterisks indicate a significant ($P < 0.001$) difference from the water control by a Mann–Whitney U test. **D,** Bar plot depicting the percent recovery of *M. phaseolina* from all 20 plants per treatment.
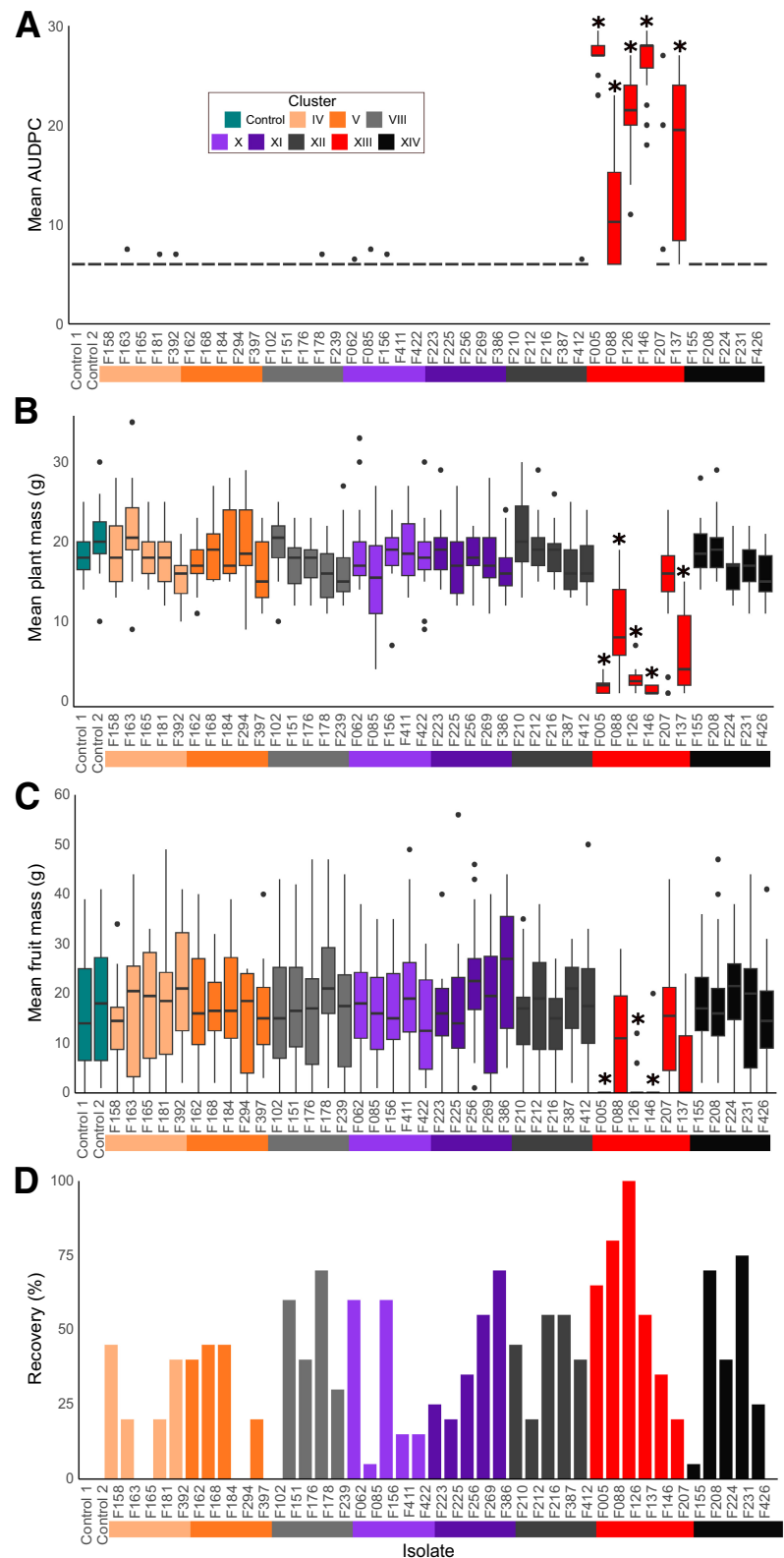
A heterothallic mode of sexual reproduction for *M. phaseolina* was previously reported by Nagel et al. (2018). This was confirmed here for *M. phaseolina* and newly determined for *M. pseudophaseolina*, *M. euphorbiicola*, and *M. tecta*. Across all isolates, there were nearly equal numbers of each mating type (mating type 1 = 238, mating type 2 = 225), but mating type distribution was significantly different from the expected 1:1 ratio for nine clusters ($\chi^2 P = 0.0442$; Table 1). Five clusters had equal mating type ratios ($\chi^2 P = 0.427$; Table 1). Although admixed isolates overall had partial memberships in all clusters (Supplementary Table S2), four of these clusters, II, III, VII, and IX, also had relatively low average Q values corresponding to the main genotype for all references (Supplementary Table S4). This suggests that isolates in these populations were more prone to "outcrossing" with isolates from other clusters. These data indicate that the genetic potential for meiosis exists in *Macrophomina* spp. and is likely to contribute to recombination in these species.

Table 1. Mating type ratios of *Macrophomina* spp. genotype clusters

Cluster[a]	MAT1-1-1[b]	MAT1-2-1[c]	$\chi^2 P$ value[d]
I	5	7	0.564
II	7	4	0.545
III	15	2	0.0036
IV	8	1	0.0442
V	40	1	<0.0001
VI	0	15	0.0003
VII	19	19	1
VIII	10	0	0.0016
IX	8	9	1
X	25	32	0.427
XI	16	1	0.0007
XII	34	0	<0.0001
XIII	0	134	<0.0001
XIV	51	0	<0.0001

[a] The population cluster as defined by the highest percentage assignment using the Mp11-12 reference.
[b] The number of isolates in each cluster with the *MAT1-1-1* gene, indicating a *MAT1-1* locus.
[c] The number of isolates in each cluster with the *MAT1-2-1* gene, indicating a *MAT1-2* locus.
[d] The P value for a chi-square (χ^2) test with the null hypothesis being equal counts of the mating types for each cluster.

Genomic evidence supports the delineation of currently described *Macrophomina* spp., which can be differentiated by new species-specific primers

Reference-independent, whole-genome comparisons of anchor nucleotide distances (ANDIs) supported the circumscription of *M. phaseolina*, *M. pseudophaseolina*, *M. euphorbiicola*, and *M. tecta*. The minimum ANDI for any two isolates of different species (=0.0113) was greater than the maximum ANDI for any two isolates of the same species (=0.009; Supplementary Fig. S4). There was just one isolate (F289) that did not fall into any of these species, as its pairwise ANDI values were all ≥0.0113, above the threshold observed for within-species
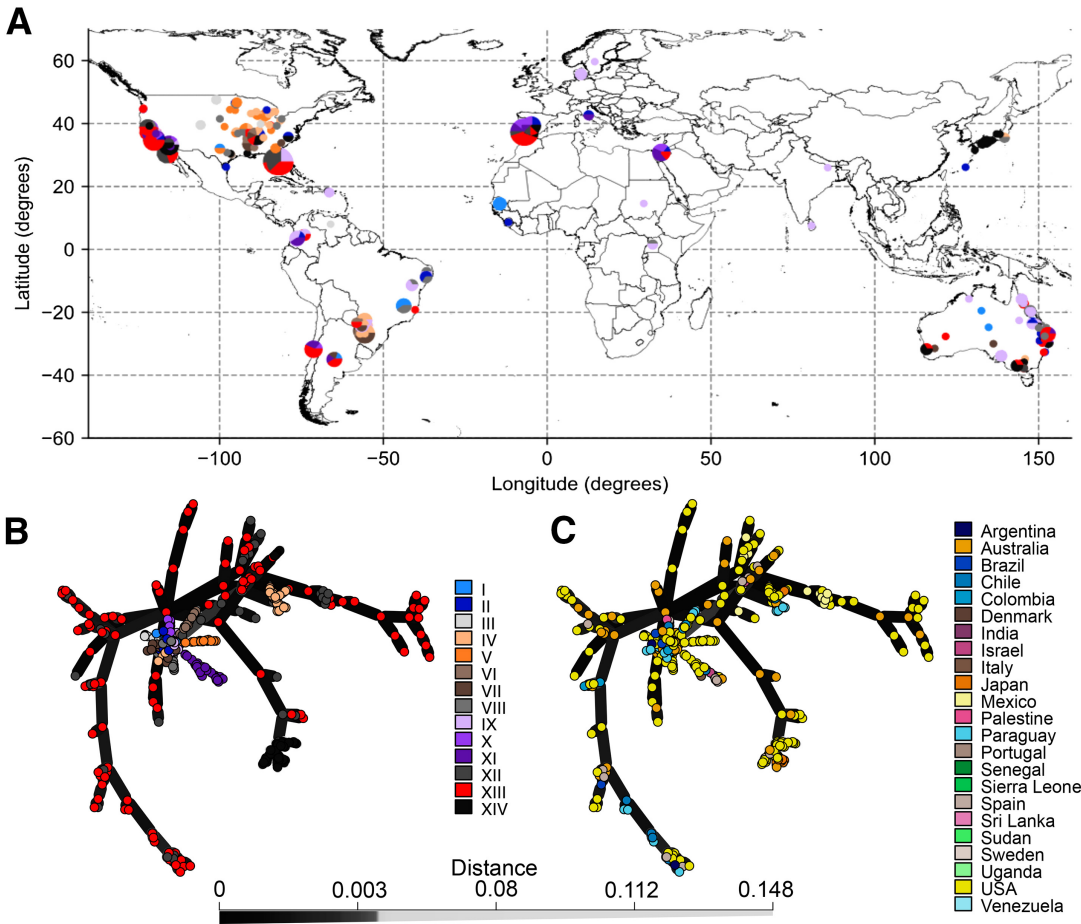
Fig. 4. Geographic distribution of *Macrophomina* spp. genotype clusters. **A,** Isolates without a known state, province, or locality were assigned longitudes and latitudes at the midpoints of their respective sub-country region (Supplementary Table S1). Pie charts scaled to the number of isolates recovered are shown for locations that had multiple isolates. The color code for the pie charts is the same as provided for panel B of this figure. Minimum spanning trees for isolates in this study colored by **B,** genotype cluster or **C,** the country of origin.

comparisons. A multilocus tree based on a concatenation of four commonly used phylogenetic markers showed that F289 was most similar to the type strain of *M. vaccinii* (Supplementary Figs. S8 and S9). Morphological comparisons between F289 and selected *M. phaseolina* strains showed that pycnidia or conidia sizes overlapped with those reported for both *M. phaseolina* and *M. vaccinii* (Zhao et al. 2019) (data not shown), indicating that these characteristics could not clearly differentiate these species. We considered F289 to be a putative *M. vaccinii* isolate but could not confirm this with genomic comparison with known strains of this species.

A series of species-specific qPCR assays were designed and validated to enable rapid identification of the species analyzed in this study. The qPCR assays were first validated in silico to target an identified locus that was present in each tested species (*M. phaseolina*, *M. pseudophaseolina*, *M. euphorbiicola*, and *M. tecta*) but absent in off-target organisms in *Botryosphaeriaceae* and the nr/nt database of GenBank. Each target was tested in vitro on a panel of 97 isolates and found to generate the predicted results for all assays, with one exception: the *M. tecta* assay also amplified F289, the one isolate that could not be classified to species. Additionally, the *M. phaseolina* assay did not amplify an individual *M. phaseolina* isolate that was highly admixed with *M. tecta*, as expected (Supplementary Tables S1, S2, and S3). These results highlight the limitations of species boundaries in the genus *Macrophomina*, which may be blurred by inter-specific hybridization.

We also designed a conventional PCR assay that generates a 507-bp amplicon, here called the "mspp locus," that can be sequenced and aligned to known references. This locus is within a predicted fatty acid synthase beta subunit, annotated as gene "M11_12_v1_07558" in Burkhardt et al. (2019b). The mspp PCR assay was validated with representatives of each *Macrophomina* sp.; the amplicons were sequenced and yielded the expected result (Supplementary Fig. S10). Alignment of sequences at the mspp locus can differentiate all *Macrophomina* species and the population cluster XIII with a high degree of accuracy (Table 2; Fig. 5). The mspp locus provided a similar amount of phylogenetic information as the standard approach of sequencing and concatenating alignments of internal transcribed spacer (ITS), *TEF1-α*, *actin*, and *beta-tubulin* sequences, and it is more informative than any of these standard reference genes on their own (Fig. 5; Supplementary Fig. S9). Notably, the ITS

sequence was not sufficient to delineate *Macrophomina* spp., as multiple species are represented by single ITS haplotypes (Supplementary Fig. S8).

Discussion

This detailed look at the factors structuring genetic diversity in *Macrophomina* spp. has highlighted new avenues for research that were previously overlooked. This work shows that in *M. phaseolina*, long touted as being a broad-host-range generalist, host specialization exists, and therefore, not all isolates are virulent on all hosts. Research into the diversity of symbiotic relationships between *Macrophomina* spp. and host plants is necessary to fully elucidate these organisms' potential for asymptomatic infection and its influence on geographic distribution. Lastly, the new methods developed to discriminate *Macrophomina* spp. enable broader searches for the rare species in this genus and should help to elucidate their distributions and host ranges.

The hypothesis that *M. phaseolina* is a generalist, broad-host-range pathogen (reviewed in Pennerman et al. 2024) implies that the sub-specific genotype is irrelevant, as any isolate could cause disease in any host. Our data provide a contradiction to this by demonstrating that a specific lineage, cluster XIII, is specialized for virulence on strawberry and that specific clusters are associated with soybean (Gladieux et al. 2018; Mercier et al. 2019; Resende et al. 1994). *Macrophomina phaseolina* is best characterized as both a poly-specialist, with multiple populations specialized for different hosts, and a generalist, with populations containing isolates derived from many hosts, no obvious specialization, and a capacity for asymptomatic infection.

Although specialized for virulence on strawberry, these isolates are likely to retain the ability to cause disease in other hosts, as has been shown for other host specialized pathogens, such as *Verticillium dahliae* (Resende et al. 1994), *Botrytis cinerea* (Mercier et al. 2019, 2021), and *Magnaporthe oryzae* (Gladieux et al. 2018). Lineages of *M. oryzae* are specialized for pathogenicity in specific grasses; when sampling for *M. oryzae* from natural blast infections, the host-specialized strain is almost always recovered (Ascari et al. 2024; Gladieux et al. 2018), similar to what we observed for strawberry and soybean. However, *M. oryzae* isolates can cause disease in grasses other than the one they are specialized in and can sometimes be recovered from other species (Gladieux et al. 2018; Rahnama et al. 2023). We

Table 2. Primers and probes designed for detection and differentiation of *Macrophomina* spp.

Primer ID[a]	Sequence (5′ to 3′)[b]	Targeted species	Annealing temp (°C)	Amplicon size (bp)
mp_F	TAGCAGCTGCACTTCCCAAA	*M. phaseolina*	61	78
mp_R	CTTCTCCTATTTCTGGTGCC			
mp_P	/6-FAM/CTACGGGCG/ZEN/CCGATGAGGTC/IABkFQ/			
me_F	CACATTTCATGCGCTACGTC	*M. euphorbiicola*	64	192
me_R	CAACGCTGGTCGAGATGAT			
me_P	/6-FAM/ACGTGCTGC/ZEN/TGGTACGCGCC/IABkFQ/			
ms_F	TTCCAGAAGTTTGCTCTGGC	*M. pseudophaseolina*	66	175
ms_R	CCTCCGTAGATGAGCACTTT			
ms_P	/6-FAM/CCCGCGTCGGTTGCTCTGGA/BHQ-1/			
mt_F	ACTCTACAACCCAACGCCAA	*M. tecta*	62	203
mt_R	CATCGTCATCAATGGGCTC			
mt_P	/6-FAM/AAGCGCGAA/ZEN/ACCCGCGCGAA/IABkFQ/			
mspp_F	GCCACATTGATGCCTTGAAG	*Macrophomina* spp. (to sequence)	62	507
mspp_R	GGTAGCGAAACTTGAGCAAG			

[a] Primer IDs with an _F denote a forward primer; _R, reverse; _P, probe.
[b] The primer or probe sequence used in each assay. Fluorophores and quenchers in probes are delineated with a slash (/) and contain non-IUPAC characters.

also observed a small percentage of strawberry- and soybean-derived isolates in other clusters and speculate that isolates in the strawberry- and soybean-associated clusters retain virulence in other hosts. Evaluating the host range of specialized genotypes will be an important avenue of future research.

The emergence of specialized virulence in strawberry appears to be a major factor in the increased frequency and severity of strawberry crown rot disease caused by *M. phaseolina* worldwide. The emergence of this host-specialized strain also coincided with the withdrawal of methyl bromide as a highly effective soil fumigant. Several authors have postulated that *M. phaseolina* emerged in strawberries due to the discontinuation of methyl bromide as a highly effective soil fumigant, partly because epidemics mostly arose in fields that had adopted substitute fumigants (Hutton et al. 2013; Koike 2008; Zveibil et al. 2012). This hypothesis is not universally supported by the data: side-by-side comparisons often do not show that methyl bromide controlled *M. phaseolina* significantly more than replacement fumigants (Chamorro et al. 2016; Hutton et al. 2013;

Koike et al. 2013; Zveibil et al. 2012). Furthermore, virulent isolates have been recovered from strawberry nurseries in which methyl bromide continues to be used (Burkhardt et al. 2019a). Our data show that the emergence of a host-specialized strain was a crucial factor for explaining new reports and the surging destructiveness of this pathogen in strawberry in multiple countries.

The strawberry-derived isolates from clusters XI, XII, and XIV were not tested. Some isolates in these three clusters were found to be virulent in previous studies and are associated with strawberry crown rot disease in Argentina (Baino et al. 2011), Australia (Herrington 2018), Chile (Sánchez et al. 2013), Israel (Zveibil and Freeman 2005), and Italy (Gerin et al. 2018). Thus, it is likely that other isolates of *M. phaseolina* could cause disease on strawberry, potentially under different environmental conditions and/or in other cultivars than were tested in our study.

The lack of geographic influence on population structure was surprising, given that many pathogens have population structures influenced by geography, even aerially dispersed fungi
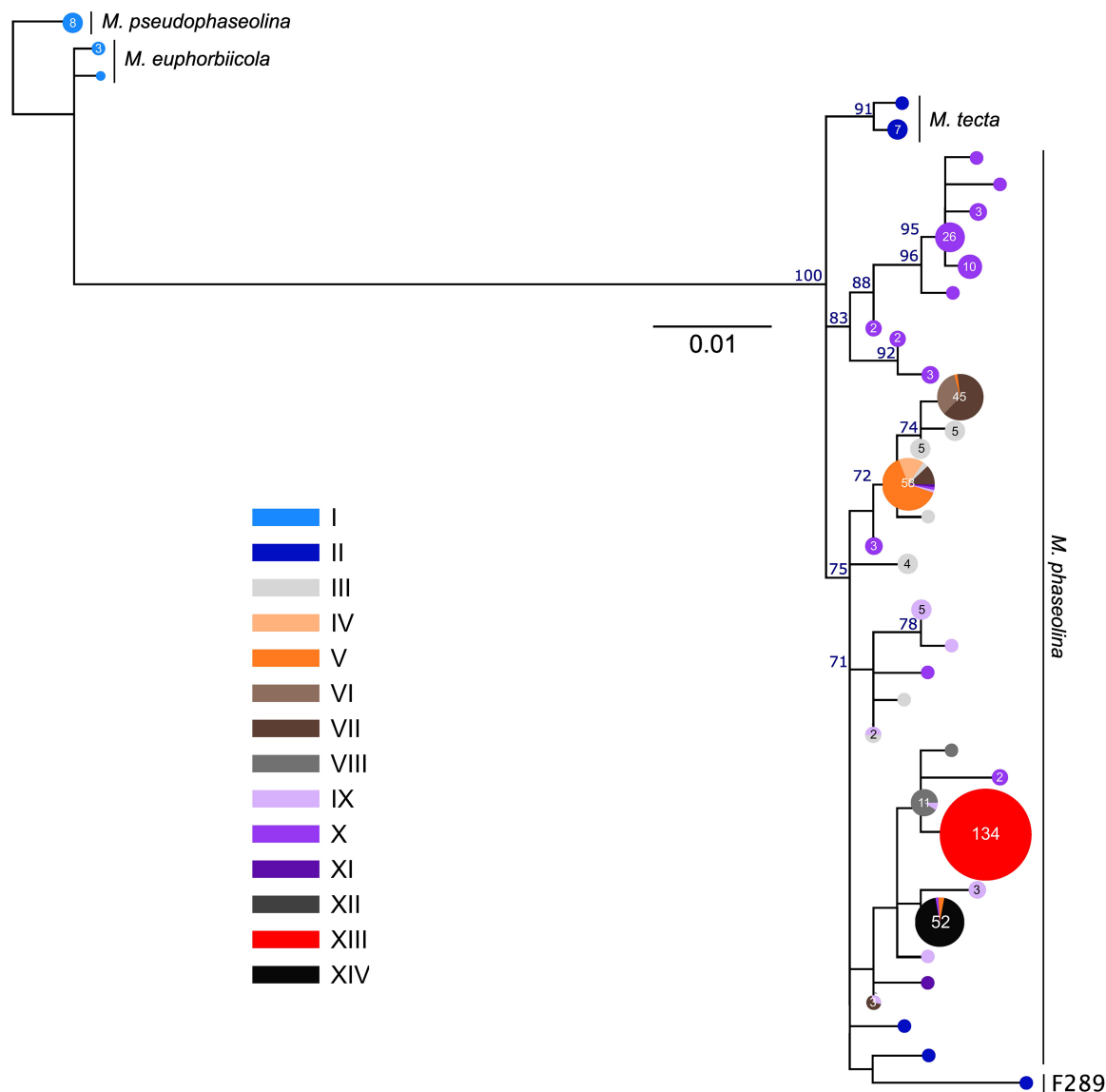
Fig. 5. Phylogenetic tree built from sequences at the approximately 500-bp locus targeted by the "mspp" PCR primers. The phylogenetic tree was calculated by RAxML-NG version 1.2.1 with the GTR + G model of evolution and 1,000 bootstrap replicates. Only bootstrap values greater than 70 are shown. Species *Macrophomina euphorbiicola*, *M. pseudophaseolina*, *M. tecta*, and *M. phaseolina* are distinguishable and indicated by text annotations. All isolates from the strawberry-associated cluster XIII had identical sequences that were distinct from other *Macrophomina* spp. isolates. The sequences used are provided in Supplementary Information S3.

(Ali et al. 2014; Feurtey et al. 2023; Sotiropoulos et al. 2022). However, many *Botryosphaeriaceae* pathogens are known to asymptomatically colonize plant tissues (Slippers and Wingfield 2007), and this niche has been considered a reason populations of *Botryosphaeria dothidea* are not structured by geography (Marsberg et al. 2017). The potential for asymptomatic growth on plant tissues or seeds by *M. phaseolina* is known for some hosts (Fuhlbohm et al. 2012, 2013; González and Tello 2011; Jamal et al. 2022; Koike et al. 2016; Moslemi et al. 2024) but is a relatively understudied phenomenon. In support of this hypothesis, our data showed that most *M. phaseolina* isolates could initiate asymptomatic infections in strawberry (Fig. 3D). Similarly, we found that cluster XIII isolates of *M. phaseolina* could cause asymptomatic infections in a broad range of Fabaceae and Brassicaceae cover crops (Qin et al. 2025). Asymptomatic infections on plants or seeds are likely to play a much larger role in *M. phaseolina*'s life cycle than was previously appreciated and warrant greater attention in future research.

The currently described *Macrophomina* species were supported by reference-free whole-genome comparisons (Supplementary Fig. S4) and phylogenies of core genes (Supplementary Fig. S9). However, admixture between species indicates that the boundaries in *Macrophomina* spp. are fluid and could complicate future efforts at specific detection. This led to challenges in designing qPCR assays with 100% analytical specificity, as an admixed isolate with *M. phaseolina* and *M. tecta* ancestry did not amplify with either assay. However, our species-specific assays performed well overall and were the first of their kind for *M. pseudophaseolina*, *euphorbiicola*, and *tecta*. The *M. phaseolina* assay provides a slight improvement to the existing qPCR assay (Babu et al. 2011) by including a TaqMan probe in the design, which enables multiplexing with other targets. The existing *M. phaseolina* assay by Babu et al. (2011) was in silico predicted to correctly amplify all *M. phaseolina* isolates and not other *Macrophomina* species. Our new *M. phaseolina* assay also has an intentionally broader target than the Burkhardt et al. (2018) assay, which is only predicted to amplify all isolates in cluster XIII, except F219.

Materials and Methods

Global collection and sequencing of *Macrophomina* spp. isolates

Effort was made to collect isolates from diverse hosts, geographic locations, and times. Isolates were drawn from public culture collections (e.g., the Queensland Plant Pathology Herbarium, Westerdijk Institute, NARO Genebank, and the National Reference Repository Library), private collections of individual investigators, and recently published works (Burkhardt et al. 2019a; Weiland 2022). A standardized naming system was applied to 463 isolates received, ranging from F001 to F518. Metadata for each isolate were largely provided by the collectors and are listed in Supplementary Table S1. Longitudes and latitudes were assigned to isolates based on the midpoint of known states, provinces, or localities.

DNA extraction and sequencing

All isolates were single-hyphal-tipped to ensure genetic uniformity. The tissue used for DNA extraction was grown on potato dextrose agar, potato dextrose broth, or 10% V8 juice broth and was sometimes lyophilized. DNA extractions were performed with the OmniPrep for Fungus kit (G-Biosciences, St. Louis, MO, U.S.A.) per the manufacturer's instructions, with an added step of treatment by the Invitrogen RNase A/T1 Cocktail (Fisher Scientific, Waltham, MA, U.S.A.) at the final step and incubating for 60 min at 45°C followed by 30 min at room temperature. Most DNA extracts were then further purified by

AMPure XP beads (Beckman Coulter, Brea, CA, U.S.A.) to improve the quality of DNA extracts before library preparation. Libraries were prepared with the DNA KAPA Hyper Prep Kit (Kapa Biosystems-Roche, Basel, Switzerland) and sequenced on an Illumina NovaSeq6000 with 150-bp paired-end reads. For isolates from Brazil, DNA was extracted by the method described in Kjærbølling et al. (2018) and sequenced on an Illumina NextSeq2000 at the Max Planck Genome Center in Cologne, Germany. Additional reads were retrieved from NCBI GenBank for 28 isolates; accession numbers are available in Supplementary Table S1. Estimated genomic sequencing coverage was typically between 72 and 108×, whereas a small number of isolates had coverage between 26 and 64× (Supplementary Table S1).

Sequence read processing and mapping

Unless specified, libraries, modules, and codes were used with their default parameters. Sequencing reads were preprocessed by removing contaminants, unique molecular identifiers, adaptors, and ambiguous and low-quality nucleotides using HTStream version 1.3.3 (https://github.com/s4hts/HTStream). BBMap version 35.85 (https://sourceforge.net/projects/bbmap/) was used to limit the total number of bases to a maximum of 5.5 Gb (approximately 100× coverage) with a minimum read length of 100 bases for each isolate. Cleaned reads were mapped to the assembled genomes of *M. phaseolina* strains Mp11-12 (F001), AL-1 (F003), and mp117 (F509) (Burkhardt et al. 2018, 2019b; Gluck-Thaler et al. 2022) using the Burrows-Wheeler Aligner version 0.7.17 (Li and Durbin 2009), and alignments were subsequently processed with SAMtools version 1.16.1 (Danecek et al. 2021). BEDtools version 2.30.0 (Quinlan and Hall 2010) converted the resulting BAM files into BED files. SAMtools flagstat evaluated mapping efficiency. The BED files of mapped read coverages were used to determine if the strawberry *M. phaseolina* detection locus (Burkhardt et al. 2018) and the general *M. phaseolina* detection locus (Babu et al. 2011) were present among the isolates by counting at least five sequencing reads mapped to a total of at least 95% of the length of the reference locus.

Genome assembly and annotation

Cleaned reads were de novo assembled into genomes with SPAdes version 3.15.5 (Prjibelski et al. 2020), and genome assembly quality was evaluated using QUAST version 5.2.0 (Gurevich et al. 2013) and BUSCO version 5.4.5 (Manni et al. 2021) with the dothideomycetes_odb10 database (version 2019-11-20), which contained 3,786 benchmarking universal single-copy homologs. SPAdes assemblies were processed through the NCBI Foreign Contamination Screen to remove sequencing adapters and prokaryotic contaminants before submission to NCBI WGS (Astashyn et al. 2024).

Protein-coding genes were annotated from these assemblies with GeneMark-ES version 4.71 (Ter-Hovhannisyan et al. 2008) with the fungus parameter. Proteins with fewer than 100 amino acids were excluded from analyses. These putative proteins were consolidated into a pan-proteome with CD-Hit version 4.8.1 (Fu et al. 2012) with the reciprocal sequence identity threshold and length difference thresholds at 0.8. "Super-core" homologs were represented by every isolate included in the current study. "Core" homologs included super-core homologs and those that were present in at least 95% of isolates. "Accessory" homologs were present in at least two isolates and up to 95% of isolates. "Singleton" sequences were only identified in one isolate.

Variant calling and population genomic analyses

Biallelic SNPs with a minimum coverage of five reads for each isolate, a minimum base quality of 20 for each isolate, and a minimum allele frequency of 5% overall among all

Macrophomina spp. isolates were identified with Freebayes version 1.3.6 (Garrison and Marth 2012). SNP loci missing from more than 10% of used *Macrophomina* spp. isolates were excluded. Admixture was analyzed with the unpruned loci with sNMF version 1.2 (Frichot et al. 2014) with K ranging from 2 to 20. A final K of 14 was chosen in accordance with the elbow method for clustering algorithms. SNP intra-scaffold LD correlations were calculated with VCFtools (Danecek et al. 2011) and the unpruned loci. Fixation indices were computed with R package vcfR (Knaus and Grünwald 2017). The R package poppr was used to generate minimum spanning trees and to conduct analyses of molecular variance (Kamvar et al. 2014).

Sequences corresponding to the *MAT1-1-1* and *MAT1-2-1* genes were extracted from full mating type loci published by Nagel et al. (2018), and then BLASTn searches of these queries against each reference assembly were used to determine which gene was present in each isolate. Taxonomic assignments were verified by retrieval of reference nucleic sequences of ITS (reference NCBI accessions KF951717.1, KF951791.1, OR052068.1, MK687450.1, and NR_175162.1), *TEF1-α* (KF952088.1, KF952153.1, OR030468.1, MK687426.1, and MW592271.1), *actin* (KF951857.1, KF951918.1, MH712508.1, MK687442.1, and MW592058.1), and *beta-tubulin* (KF952178.1, KF952233.1, MF457658.1, MK687434.1, and MW592300.1) genes or genomic regions. These reference sequences represented phylogenetic markers from *M. phaseolina* CPC 21420, *M. pseudophaseolina* CPC 21417, *M. tecta* BRIP:70781, *M. vaccinii* CGMCC3.19503, and *M. euphorbiicola* strains CMM4134, McBr87, and MFLU 23-0004. The reference sequences were used in BLAST searches (Camacho et al. 2009) against our assembled genomes to identify the corresponding sequence in each isolate. The ITS, *TEF1-α*, *actin*, and *beta-tubulin* sequences were aligned by MUSCLE (Edgar 2004), and RAxML-NG version 1.2.1 (Kozlov et al. 2019) was used to generate phylogenetic trees with the GTR + G evolution model and 1,000 bootstrap replicates. Phylograms were visualized with the R package ggtree (Xu et al. 2022). The software andi (Haubold et al. 2015) was also used to estimate isolate relatedness with the assembled genomes. Reticulate phylogenetic trees were generated using SplitsTree version 4.19.1 (Huson and Bryant 2006) (NeighborNet function). Other figures were made with Inkscape 1.3, Python 3.11.4 libraries matplotlib and sklearn, and R 2023.03.1. Heatmaps were generated using Ward's method parameter in the Python Seaborn library and using data that were one-hot encoded. GIS shapefiles were retrieved from the IPUMS database (Ruggles et al. 2024).

Development of species-specific qPCR assays and a PCR protocol for a phylogenetically informative locus

Phylogenetically informative sequences commonly shared among all isolates were identified using the BEDtools suite (Quinlan and Hall 2010) to compare BED files of read coverages with the Mp11-12 genomic reference. Species-specific target candidates for qPCR assays were identified by searching the CD-HIT-determined homologs for those that were specific to the target *Macrophomina* spp. Putative species-specific homologs were further verified by BLAST searches of the locus against all genome assemblies and NCBI nr/nt and wgs sequence databases.

Species-specific TaqMan qPCR assays were designed for *M. phaseolina*, *M. pseudophaseolina*, *M. euphorbiicola*, and *M. tecta*. All assays used a 20-μl reaction mixture containing 1× PerfeCTa qPCR ToughMix (Quantabio, Beverly, MA, U.S.A.), 0.25 μM of forward and reverse primers (Table 2), 0.15 μM of TaqMan probe (Table 2), and approximately 15 ng of DNA. DNA for these reactions was isolated by using the OmniPrep

kit as described above or by using the Quick-DNA Miniprep kit (Zymo Research, Irvine, CA, U.S.A.). Reactions were run in a LightCycler II (Roche, San Francisco, CA, U.S.A.) with an initial denaturation step of 95°C for 2 min, followed by 40 cycles of 15 s at 95°C and 30 s at the annealing temperatures provided in Table 2. These qPCR assays were tested on 97 isolates that included at least two representatives of each species and at least five representatives of each *M. phaseolina* population cluster (Supplementary Table S3). All qPCR assays were predicted to yield exactly zero or one amplicon depending on the isolate (Supplementary Table S1).

An additional protocol was developed for PCR amplification of a single, phylogenetically informative locus present in all *Macrophomina* spp. that can be sequenced to determine species assignment. This assay was run with a 50-μl reaction mixture containing 0.5 μM of each primer (Table 2), 1× Promega GoTaq Master Mix (#M7123; Promega, Fitchburg, WI, U.S.A.), and 2 μl of DNA isolated using the Quick-DNA Fecal-Soil Microbe kit or the Quick-DNA Miniprep kit (Zymo Research). Reactions were run in a Bio-Rad C1000 Touch (Bio-Rad Laboratories, Hercules, CA, U.S.A.) with an initial denaturation step of 95°C for 5 min, followed by 40 cycles of 95°C for 30 s, 62°C for 30 s, and 72°C for 1 min, then a final 72°C extension for 5 min. PCR products were purified using the GeneJet PCR Purification Kit (Thermo Fisher Scientific, Waltham, MA, U.S.A.), and the 507-bp bands were visualized with GelRed Nucleic Acid Stain (Biotium, Fremont, CA, U.S.A.) on a 1.5% agarose gel for 40 min at 110V. Purified amplicons were sequenced with forward and reverse primers by Eton Biosciences (San Diego, CA, U.S.A.).

Virulence assays in strawberry

United States-derived isolates were selected at random from most *M. phaseolina* clusters: IV, V, VIII, X, XI, XII, XIII, and XIV. Isolates were grown in the dark for 1 week on potato dextrose agar amended with 50 mg/ml kanamycin, 100 mg/ml streptomycin, and 12.5 mg/ml chlortetracycline. One 5-mm-diameter plug of each culture was transferred to 200 ml of sterile 10% V8 broth and incubated at 25°C and 200 rpm in the dark for 3 days. The liquid cultures were then blended in a Waring blender for 7 s and allowed to settle for 5 min in a 50-ml conical tube before the volumes of settled mycelia were quantified. Suspensions were diluted to 8% mycelia by volume with sterile deionized water.

Bare-root, frozen strawberry crowns (cultivar Monterey) from a low-elevation nursery were planted in potting soil (Sunshine Mix #1; Nutrien Ag Solutions, Loveland, CO, U.S.A.) and grown at 25/20°C day/night temperatures in a greenhouse with no supplemental lighting for 1 week to break dormancy. After dormancy was broken, the roots were rinsed with tap water and submerged in a fungal inoculum for 10 min. The negative control was submerged in 10% V8 broth for 10 min. Plants were then repotted in 10.8 × 10.8 × 12.4 cm pots and watered with the respective leftover inoculum. Inoculated plants were then grown at approximately 28/22°C in a greenhouse with day lengths between 14 and 14.6 h in Salinas, California. For the course of the experiments, plants were watered as needed and fertilized weekly using Peter's Professional 20-20-20 fertilizer (ICL Fertilizers, St. Louis, MO, U.S.A.). Ten biological replicates for each fungal isolate were established per experiment, and the experiment was conducted twice.

Ordinal disease ratings were conducted weekly for 7 weeks on a 1 to 5 scale, where 1 = asymptomatic, 2 = stunting, 3 = wilting of less than 50% of the foliage, 4 = wilting of more than 50% of the foliage, and 5 = plant death. All peduncles bearing fruit and flowers were removed and weighed at 5 and 7 weeks postinoculation. After removing peduncles, fruit, and flowers for separate measurements at 7 weeks postinoculation, whole plants were removed from the soil, roots were trimmed flush

with the crown, and aboveground fresh weight was recorded. From each plant, two cross-sections of the crown were excised and their cortical tissues removed (as per Henry et al. 2019). These crown stele tissues were surface sterilized in 1% sodium hypochlorite solution for 2 min, then plated on potato dextrose agar amended with kanamycin, streptomycin, and tetracycline as described above. Plates were incubated at 30°C for 5 to 7 days, and the presence of *M. phaseolina* was determined by the morphology of fungal growth emanating from these tissues. Data were analyzed by checking for normality with the Shapiro-Wilk and Levene's tests. The Wilcoxon test identified statistical significance at $\alpha = 0.05$.

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