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Research Article

Susceptibility to butternut canker disease in field trials is influenced by disease level and hybridization

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Abstract

Butternut (*Juglans cinerea*) has been decimated due to butternut canker disease (BCD). To date, much of the observed disease resistance in butternut occurs in hybrids with Japanese walnut (*Juglans ailantifolia*), a non-native species which was introduced to the US and naturally hybridizes with butternut. We screened a US range-wide collection of butternut and hybrid butternut, including over 300 open-pollinated families, for susceptibility to BCD. Trees were planted in Indiana, US, in two areas (with high or low disease levels) and allowed to become naturally infected. After 10 years, severity of disease symptoms was assessed using a five-point rating scale. We used logistic regression to evaluate factors, like ancestry (butternut or hybrid butternut), on the probability of a tree being rated as resistant. Across all sites, ancestry was a significant predictor of resistance. Our results also demonstrate heritability of disease susceptibility on sites where there was sufficient disease pressure for trees to display disease symptoms. While the most resistant families are hybrid, some butternut families do show modest levels of resistance based on estimated breeding values for each family. Variation in disease level across experimental plantings highlights the importance of considering site influence on disease development. Identifying sites less conducive to disease development may aid in butternut conservation and restoration.

Key words: *Juglans cinerea*, *Juglans cinera* × *Juglans ailantifolia*, *Ophiognomonia clavigignenti-juglandacearum*, disease resistance, quantitative genetics

1 Introduction

Butternut (*Juglans cinerea*) is native to eastern North America, where it occurs in mixed hardwood forests and riparian areas (Ostry et al. 2003). The species was historically a minor component of forests and not a dominant species for any cover type. Its range stretches across most of the eastern US and northward into southeastern Canada. Butternut's demise, in some locations, over the last half century is attributed largely to butternut canker disease (BCD), but silviculture practices leading to a lack of sunlight may also have impaired its regeneration. The disease has led to a greater than 50% reduction in the number of butternut trees in the US alone, including mortality of 77% of butternut in the southeast (USDA Forest Service 1995; Morin et al. 2018), although this estimate is likely conservative.

Butternut canker disease was first observed in the late 1960s in southwestern Wisconsin (Renlund 1971) and over the next three decades symptoms were reported throughout the range. Symptoms include multiple cankers on branches and trunks, which can lead to branch and trunk girdling and ultimately tree death (Tisserat and Kuntz 1984). Removal of the outer bark reveals elliptical-shaped cankers, which are characteristic of the disease (Tisserat and Kuntz 1984). Other symptoms include a black sooty appearance on the outer bark and bark cracking. In some areas, high levels of disease incidence, upwards of 80%, were reported (Prey and Kuntz 1982). The disease is caused by the fungus *Ophiognomonia clavigignenti-juglandacearum* (Oc-j) (formerly *Sirococus clavigignenti-juglandacearum*) (Nair et al. 1979; Broders and Boland 2011).

Ophiognomonia clavigignenti-juglandacearum is found throughout the range of butternut, but only in its asexual state (Anderson and LaMadeleine 1978; Broders and Boland 2011). The fungus is transmitted by rain splash, through the air, and via insect vectors, so distance from inoculum source may influence dispersal (Tisserat and Kuntz 1983; Broders et al. 2015). While the origin of *O. clavigignenti-juglandacearum* is unknown, it is considered invasive and was likely introduced or emerged multiple times in North America (Broders et al. 2012, 2015). A decade after the disease was first recognized,

16 of 24 US states reported declining butternut, with two southern states (North Carolina and South Carolina) reporting no butternut remaining (Anderson and LaMadeleine 1978).

Symptoms of the disease can generally be found wherever butternut occurs, but not all trees succumb from infections. Due to the marked decline in the species, butternut is considered endangered on the IUCN Red List of threatened species (Stritch and Barstow 2019) and under the federal Species at Risk Act in Canada (Environment Canada 2010). In the US, designations are on a state-by-state basis where it is rated from critically imperiled to vulnerable in 22 states (NatureServe Explorer 2025). Site conditions may influence disease incidence and severity. Dry, upland sites were shown to be more suitable for butternut growth and survival, while trees growing in riparian habitats were observed to have more cankers (Boraks and Broders 2014; LaBonte et al. 2015; Morin et al. 2018).

Japanese walnut (*Juglans ailantifolia*) was widely planted in the eastern US over the last two centuries (Bixby 1919). Butternut and Japanese walnut readily hybridize in nature (Hoban et al. 2009). While Japanese walnut and hybrid butternut are not immune to infection (Broders and Boland 2010), both have shown higher levels of resistance to BCD compared to butternut (Orchard et al. 1982; Brennan et al. 2020). For this reason, survival of butternut cannot be attributed to favorable environmental conditions alone. Instead, some populations may be sustained by the presence of hybrid butternut (*J. cinerea* × *J. ailantifolia*), including complex backcrosses, that are naturally distributed across the species' range (Hoban et al. 2009; Boraks and Broders 2014). Even with higher reported levels of resistance, variation in disease susceptibility is observed within both species groups (LaBonte et al. 2015; Brennan et al. 2020). Although the mechanism(s) for variation in Oc-j virulence is unknown, it seems to be a factor in the level of damage in trees (Brennan et al. 2020) and could be important to consider during resistance breeding efforts. Due to the higher demonstrated level of resistance in hybrid butternut, the use of naturally existing hybrids was proposed as a strategy for restoring butternut to eastern forests (Pike et al. 2021).

In the recent past, resistance to BCD was evaluated in butternut and hybrid butternut families by artificial inoculation, a process whereby a liquid spore suspension or plug of mycelium are inserted into a hole in the bark (Orchard et al. 1982; Brennan et al. 2020). Resistance of artificially inoculated trees was then evaluated by measuring the size of cankers formed from inoculations (Ostry and Moore 2008; Brennan et al. 2020). Artificial inoculation bypasses the bark, which may be one of the first lines of defense against the pathogen, so new methods for augmenting natural disease pressure while not relying on natural infection alone are being explored. Resistance of natural populations or non-inoculated trees was assessed by observing crown dieback, canker frequency, and extent of bole damage (Boraks and Broders 2014; Williams et al. 2020). Resistance was also determined based on the size and number of cankers, by assigning a disease rating to trees, and by assessing tree vigor and live canopy proportions (Brennan et al. 2020; Williams et al. 2020). Reassessment of the same trees is critical to document changes in tree health and disease over time (Williams et al. 2020).

Efforts to identify and breed disease resistant butternut have been ongoing for decades (Orchard et al. 1982; Ostry et al. 2003; Michler et al. 2006; Brennan et al. 2020). Despite this work, no single screening or inoculation method is considered the "gold standard" for evaluating resistance to BCD. In other pest and pathosystems, a multifaceted approach is often employed that includes the establishment of multi-year monitoring plots for on-the-ground surveys to identify surviving and/or apparently disease-free or low disease severity trees, sometimes referred to as "lingering trees", followed by screening of clones or progeny in relatively controlled conditions for disease resistance (Sniezko 2006; Sniezko and Koch 2017; Pike et al. 2021). In the latter case, resistance of the parent tree is based on the success or survival of the progeny and is an example of classical selective breeding (Sniezko and Liu 2022). Based on the absence of clear, qualitative resistance in any butternut family (including hybrid butternut), resistance is likely polygenetic, i.e., quantitative. As a result, resistance is defined along a continuum from more resistant to more susceptible. This is not uncommon, particularly for invasive or non-native pathogens that have not co-evolved with the tree hosts they infect (Jacobs et al. 2013; Ennos 2015).

Quantitative resistance of forest trees to novel pathogens has been documented, even though complete immunity is rare or undocumented (Sniezko and Koch 2017; Pike et al. 2021). In some cases, polygenic resistance can be amplified through breeding of lingering or surviving trees to stack genes across multiple loci for desirable traits. Another strategy involves hybridization with a related, resistant species. For example, a backcross hybrid breeding program crossing American chestnut (*Castanea dentata*) with Chinese chestnut (*Castanea mollissima*) was employed (Burnham 1988) to aid in the restoration of American chestnut from chestnut blight. Unlike American chestnut, where hybridization with the non-native species was the intent (Burnham 1988), it was not intentional in initial butternut conservation and breeding (Hoban et al. 2009) but occurred because the resistant non-native species had already been introduced and hybridized with the native species. Furthermore, surviving genotypes from across each species range are necessary to screen for resistance and to develop seed-zone appropriate populations for different areas. This is particularly important for species with broad geographic ranges, such as butternut (Sniezko et al. 2011; Sniezko and Koch 2017).

In this study, we screened a range-wide collection of open-pollinated butternut and hybrid butternut families for resistance to BCD as well as open-pollinated progeny of trees maintained within managed plantings. Seed was collected from wild trees, landscape trees, nut orchards, germplasm repositories, and earlier resistance tests. These plantings contained material sourced from additional geographic areas, including a collection that began in the 1990s by the USDA Forest Service in Minnesota (Ostry 1997). Trees were planted on three sites in two geographically distinct areas, that differed in disease levels (one with high disease incidence and one with low disease incidence). Specifically, we aimed to: (1)

Table 1. The three butternut plantings screened for butternut canker disease resistance.

Planting	Location	Year of Origin	Species/genotypes	Number of trees	Number of accessions
Plot 44	SEPAC	2010	JC, JXC	686	171
Plot 51	Martell	2010	JC, JXC, JN	1456	230
Plot 53	Martell	2011	JC, JA, JXC, JN	1306	111

Note: The number of trees at time of planting does not account for subsequent mortality; JC: butternut; JXC: hybrid butternut; JA: Japanese walnut; JN: black walnut.

identify factors associated with disease resistance; (2) determine whether disease resistance is heritable, and (3) identify the most disease resistant families.

2 Materials and methods

2.1 Plantings

Three butternut plantings were established with one year-old open pollinated seedlings in Indiana as part of the Hardwood Tree Improvement and Regeneration Center's butternut restoration program. Two plantings were established in 2010 (plot 51: 40.43076, −87.03685 and plot 44: 39.04152, −85.53865) and one planting was established in 2011, with additions in 2012 and 2013 (plot 53: 40.42432, −87.04267). Plots 51 and 53 are located at Martell Forest in West Lafayette, IN, while plot 44 is located at the Southeastern Purdue Agriculture Center (SEPAC) in Butlerville, IN. Material originated from across the range of butternut (plots 51 and 44) and from previously established butternut plantings in Indiana and Minnesota (plot 53) (Table 1). Seed collected from wild growing trees was meant to represent butternut populations remaining on the landscape at the time, since BCD is generally present throughout the range and has already killed large numbers of susceptible trees.

Plantings contain a mix of butternut and hybrid butternut families, as well as black walnut (*Juglans nigra*) (plots 51 and 53) and Japanese walnut (plot 53) (total number of families across all three sites is 339). A subset of butternut and hybrid butternut families, included in our study, had been previously screened for disease susceptibility (Ostry and Moore 2008; Brennan et al. 2020). At the time plantings were established, species determinations were made based on seed morphology and other morphological features (Farlee et al. 2010), as modern genotypic tools were not yet available. Due to the nature in which plantings were established and differences in the number of available seed per family, not all families are represented at each site, although plots 44 and 51 contain the greatest overlap of families and were considered sister plantings, having been established at the same time with many of the same families (Table 1). Plots 44 and 51 share 140 families out of 143 families present within at least two plantings. Progeny from 24 families are present within all three plantings.

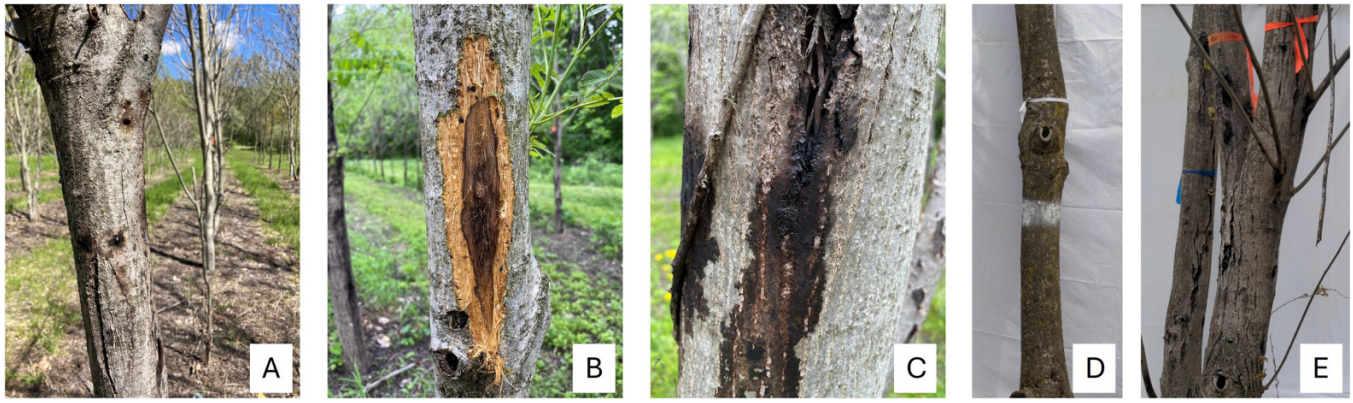
2.2 Assessing disease incidence and severity

At approximately 10 years following planting establishment, trees were rated for BCD susceptibility (plot 44: January 2021; plot 51: December 2020; plot 53: November–December 2021). At the same time, walnut witches' broom (WWB) incidence (presence or absence of WWB symptoms), growth (including diameter at breast height, DBH, and merchantable height), and mortality were recorded. For BCD ratings, teams of at least two people assessed disease severity during leaf off (typically between December and January). Tree stems and branches were visually scanned for symptoms of BCD, including evidence of healed or healing cankers, bark staining, and cracks in the external bark (see Fig. 1). Raters individually assessed each tree using a five-point scale, where 1 is most resistant and 5 is most susceptible, and then came to a consensus rating as a group. Due to the consensus process, ratings could be assigned a final value that was split between two rating levels (e.g., if one rater selected "2" and the other selected "3" and then the two raters mutually agreed upon an intermediate rating of "2.5"). A tree with a rating of one is very resistant with zero or few cankers present on the stem and/or branches, while a five is a very susceptible tree with many cankers present on the stem and/or branches. A tree with a rating of 3 has intermediate resistance or susceptibility. Ideally, trees with ratings of 2.5 or below would be those selected for resistance for HTIRC's operational breeding program. Missing trees or trees where disease symptoms could not be assessed (e.g., due to extensive decay) were not rated, and thus not included in subsequent analyses unless otherwise noted.

2.3 Classifying trees by hybrid ancestry

Trees were classified into one of four species groups: black walnut (*J. nigra*, JN), butternut (JC), hybrid butternut (JXC, including first generation and backcrosses), and Japanese walnut (JA). To classify trees, the leaves from a representative subset of each family were collected, and after freeze-drying, the dried leaf tissue was ground and DNA extracted using CTAB methods (Doyle and Doyle 1987), with minor modification to increase the quality of DNA, and using a 96-deep-well plate to process DNA extraction. Fifty ng/μL of DNA was used for genotyping. For genotyping, we used two methods: a recently developed interspecific 30 single-nucleotide polymorphism (SNP) MassARRAY assay, iPLEX Gold platform (Genome Québec), described in Williams et al. under review, and targeted sequencing. For targeted sequencing, in-house libraries were prepared following the methods described by Poland et al. (2012) and submitted to the Purdue University Genomics Core for sequencing. For SNP calling, we used the pipeline described in Ebrahimi (2022). Morphology was used to assign hybrid status when DNA data were unavailable. SNPs obtained from MassARRAY and targeted sequencing were used for ancestry analysis with fastStructure (Rej et al. 2014), as described in Ebrahimi (2022). This information was used to determine the species of families selected as most resistant based on the quantitative genetics analysis (see 2.5) and for

Fig. 1. Symptoms of butternut canker disease. (A) Cracking and bark exudate (staining) associated with disease symptoms during leaf off; (B) outer bark is removed to reveal diamond-shaped phloem canker distinctive of the disease; (C) staining observed on the outer bark; (D) apparently healthy tree without canker symptoms; (E) bark cracking and sloughing associated with more advanced canker symptoms. Photo credit: (A) James McKenna, USDA-FS; (B and C) Jonathan Shimizu, Purdue University; (D and E) James Warren, USDA-FS.



subsequent analysis (see section 2.4 below). For our purposes, genotyped trees with greater than 95% predicted butternut DNA were classified as butternut, while trees with less than 5% predicted butternut DNA were classified as Japanese walnut. All other trees were considered hybrid butternut, including first generation (F1) and backcrosses. No black walnut trees were genotyped for this study.

2.4 Influence of site, growth, species, and age on disease resistance

Binary logistic regression (R package: stats) was used to model the probability of resistance as a function of species, growth (DBH and merchantable height), and environment (plot) (R Core Team 2024). In addition, a logistic regression model was run for each plot (excluding plot as a variable) to examine site-specific effects on resistance. For plot 53, the model also included year planted, since trees were planted across three years, from 2011 to 2013. Across all models, a tree was classified as resistant ($P(Y = 1)$) if it had a rating below 3 and a tree was classified as susceptible ($P(Y = 0)$) if it had a rating of 3 or above. The assumption of linearity was tested for models containing numerical predictor variables (DBH and merchantable height). For models containing multiple predictor variables, multicollinearity was tested using variance inflation factors (VIF) available in the package car (Fox and Weisberg 2019). The Wald test was used to evaluate the significance of predictors. The area under the receiver operating characteristic curve (AUC) was also calculated for each model (R package: "Deducer") (Fellows 2012). AUC values closer to one suggest the model is better at discriminating compared to models with AUC values closer to 0.5, which indicates models are no better than chance (Hughes and Madden 2003; Turechek and Wilcox 2005).

2.5 Quantitative genetics analysis of BCD susceptibility

Two approaches were used to evaluate susceptibility of open-pollinated butternut families collected across the eastern US. In the first analysis, a linear mixed model using residual maximum likelihood was performed according to Butler et al. (2023) in ASReml-R version 4.2. All families with at least three individuals were included in the analysis, including black walnut families as controls. A total of 329 families met the criteria for the likelihood analysis.

For the likelihood analysis, the severity of BCD at all sites was analyzed in two stages. In the first stage, spatial variation was removed from each of the individual sites using row and column coordinates for each tree and the most parsimonious model based on AIC and BIC criteria as selected using ASRtriala package in R (Gezan et al. 2022). Separate spatial structures and residual error variances for each site, and weights for the performance of each family at each site were determined. These parameters were then combined and analyzed as a multiple environment trial. In the second stage, a predicted value (in terms of BCD rating) was generated for each family at each site and across sites applying weights generated by the software for each genotype and site according to model (1).

$$(1) \qquad \text{predicted.value}_{ijk} \sim \mu + \beta \text{env}_i + \left(g_j + e_{ij}\right) + \epsilon_{ijk}$$

where $\text{predicted.value}_{ijk}$ is the predicted value for the kth observation of the jth genotype in the ith environment; μ is the fixed intercept; β is the fixed effect of the environment; g_j is the random effect of the jth genotype; e_{ij} is the random effect of the interaction between the ith environment and the jth genotype, modeled with a coregionalization structure dependent on env, and ϵ_{ijk} is the residual error term, assumed to be normally distributed with a variance of 1.

In this case, a lower predicted value implies the family is more resistant to BCD. This approach allows the calculation of a predicted value for families that do not occur at each site.

Both generalized and Cullis heritability's were calculated for each genotype. Generalized heritability (2) approximates narrow-sense heritability and is based on the variance components. Cullis heritability (3) was used because of the imbal-

ance and complex residual structure (Cullis et al. 2006).

$$H_{gen} = V_g/V_p \quad (2)$$

where H_{gen} is the generalized heritability; V_g is the genetic variance; V_p is phenotypic variance.

and

$$H_c = 1 - V\,(\text{BLUP difference})\,/2\sigma 2G \quad (3)$$

where H_c is the Cullis heritability, V(BLUP difference) is the predicted error variance associated with the best linear unbiased predictor (BLUP) and 2σ2G is the genetic variance.

The second analysis used a Bayesian ordinal mixed effects model (R package: brms) to estimate heritability and calculate predicted breeding values (Bürkner 2018). Assumptions about distribution of errors were made according to Dickerson (1969). To fit categorical data in a Bayesian ordinal model, BCD ratings were rounded up to the next highest whole number and inverted, where one became five (most resistant) and five became one (most susceptible). Any tree without disease data, where family was not known, or with less than three individuals per family with ratings data, were excluded from the analysis. Black walnut trees were also excluded from the analysis since no black walnut were observed with disease symptoms, and they are generally considered resistant to BCD. In total, 2116 trees from 267 families met these criteria across the three plantings. The Bayesian model was run across all three plantings and for each planting separately and included the row and column position of each tree, as well as the family as factors.

For the model containing all three plots:

$$\text{Resistance} = \text{plot} + \text{row} + \text{col} + (1|\text{family}) \quad (4)$$

and

For the model for each plot:

$$\text{Resistance} = \text{row} + \text{col} + (1|\text{family}) \quad (5)$$

Narrow sense heritability (H^2) was calculated (6), along with the 90% credible interval. Genetic variance (V_g) and environmental variance (V_e) were also calculated with 90% credible intervals.

$$H^2 = V_g/V_p \quad (6)$$

All analyses were conducted in R v4.4.2 (R Core Team 2024) unless otherwise noted.

3 Results

3.1 Disease incidence and severity

At ten years post planting establishment, 31% of planted trees (N = 3133) were marked as dead (including standing dead or tree completely missing) across the three plantings. This included 16% (N = 573) of trees in plot 44, 18% (N = 1254) of trees in plot 51, and 49% (N = 1306) of trees in plot 51. While the precise cause of tree death is unknown, some trees were rogued due to symptoms of WWB prior to BCD assessment, combined with high levels of BCD in two of the three plots (plots 51 and 53).

The relative proportion of trees in each disease rating group was compared visually across each planting (Fig. 2). Trees in plots 51 and 53 had higher disease severities than those in plot 44. Since a disease rating of 1 does not necessarily indicate an absence of disease, we determined the incidence of BCD within each planting for trees that were assigned a disease rating. Disease incidence was highest in plots 53 (90.5%, N = 881) and 51 (78.1%, N = 663) and lowest in plot 44 (10.2%, N = 470).

3.2 Ancestry analysis

SNPs obtained from MassARRAY and targeted sequencing were used by fastSTRUCTURE to estimate a probabilistic degree of admixture and thus assign trees to a given species group (Fig. S1).

3.3 Factors associated with butternut canker disease resistance

Growth parameters (DBH and merchantable height) failed the linearity assumption, and their coefficients were not significant (p < 0.05) in any of the logit models, so they were excluded from subsequent models. Across all plantings, both plot and species group were significant predictors (p < 0.01) (Tables 2 and 3, Fig. S2). A tree was less likely to be resistant (a tree with a rating < 3) if it was in plot 51 or 53 than plot 44 (p < 0.001) (Table 2), although there was no significant difference between the coefficients for plots 51 and 53 (p = 0.49) (Table 3). Similarly, butternut or hybrid butternut were less likely to be resistant than Japanese walnut (p_{JC} < 0.001, p_{JXC} = 0.020) (Table 2) and supported by a significant difference between butternut and hybrid butternut coefficients (p < 0.01) (Table 3). For plot 51, the odds of a butternut being resistant was lower than Japanese walnut (p = 0.004) (Table 2), and there was a significant difference between butternut and hybrid butternut coefficients (p < 0.01) (Table 3). Year of planting and species were both significant in the model for plot 53 (Figs. S3–S5). Both butternut and hybrid butternut were less likely to be resistant compared to Japanese walnut (p_{JC} < 0.001, p_{JXC} = 0.024), while trees planted in 2012 and 2013 were more likely to be resistant compared to trees planted in 2011 (p_{2012} = 0.032, p_{2013} = 0.010) (Table 2). Coefficients for butternut and hybrid butternut in plot 53 were significantly different (p < 0.01), but there was no significant difference between 2012 and 2013 coefficients (p = 0.77) (Table 3). There was no significant effect of species for plot 44. Models across all plots and for plots 51 and 53 had high AUC values (Table 2).

3.4 Heritability of disease susceptibility and estimated breeding values

Both Bayesian and likelihood methods were used to calculate heritability based on disease ratings. For the Bayesian approach, heritability along with the credible interval was calculated across the three planting and for each planting sep-

Fig. 2. Proportion of each rating group by plot. Plots 51 and 53 had high disease incidence, while plot 44 had low disease incidence ($N_{44} = 472$, $N_{51} = 1088$, and $N_{53} = 736$).

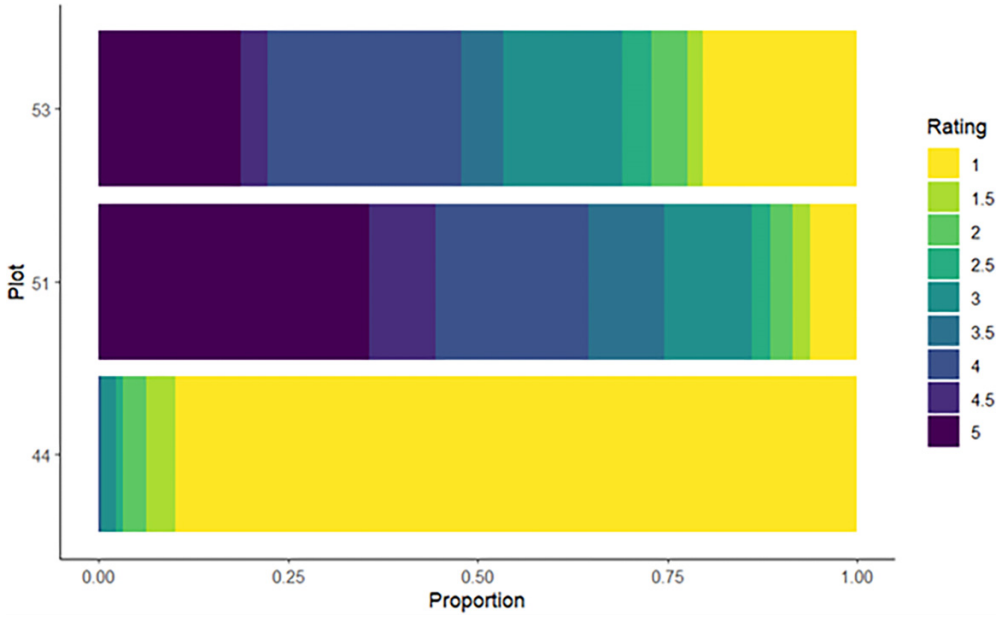


Table 2. Logistic regression model coefficients and test statistics for influences of plot, species, and year on the probability of disease resistance.

Model	Variable	Estimate	SE	*z*-value	sig.	AIC	AUC
All plots	Intercept	8.37	0.83	10.09	< 0.001	1167	0.934
	plot 51	− 6.52	0.34	− 19.20	< 0.001		
	plot 53	− 6.41	0.35	− 18.20	< 0.001		
	species - JC	− 4.90	0.77	− 6.34	< 0.001		
	species - JN	15.67	505.79	0.03	0.975		
	species - JXC	− 1.77	0.76	− 2.32	0.020		
Plot 51	Intercept	0.69	1.22	0.57	0.571	547	0.818
	species - JC	− 3.52	1.23	− 2.85	0.004		
	species - JN	16.87	625.53	0.03	0.979		
	species - JXC	− 0.84	1.24	− 0.68	0.497		
Plot 53	Intercept	2.04	1.06	1.93	0.054	497	0.884
	species - JC	− 6.35	1.11	− 5.73	< 0.001		
	species - JN	14.13	517.75	0.03	0.978		
	species - JXC	− 2.41	1.06	− 2.27	0.024		
	year - 2012	0.79	0.36	2.14	0.032		
	year - 2013	0.84	0.33	2.58	0.010		
Plot 44	Intercept	3.67	0.34	10.87	< 0.001	108	0.526
	species - JXC	0.32	0.79	0.41	0.685		

arately (Table 4). Cullis heritability, which considers spatial variance, was determined for each of the three plantings using the likelihood method (Table 4). Across the three plantings and at the two high disease incidence plantings (plots 51 and 53), resistance was highly heritable ($H^2 = 0.53$–0.71). Heritability of resistance on the low disease incidence planting (plot 44) did not differ significantly from zero ($H^2 = 0.00$–0.11). For the Bayesian analysis, genetic and environmental variance were calculated to examine their effects on the observed heritability (Table S1). Model coefficients for spatial variables (row, column, and plot) are reported in the supplemental materials (Tables S2 and S3).

Breeding values for each family included in the analysis were estimated using the likelihood analysis (Fig. S6, Table S4), and Bayesian model across all plantings (Fig. 3) and for each planting individually (Figs. S7–S9). This allowed for families to be ranked based on their relative resistance. The breeding values of families from known locations were visualized on a map to examine the relationship between relative disease resistance and geographic origin, and to visualize the ori-

Table 3. Comparisons for multiple levels of effects from Wald chi-squared test results for each significant coefficient from Table 4.

Model	Variable	χ^2	df	sig.
All plots	plot	375.9	2	< 0.01
	51 versus 53	0.47	1	0.49
	species	324.2	3	< 0.01
	JC versus JXC	312.6	1	< 0.01
Plot 51	species	138.9	3	< 0.01
	JC versus JXC	135.3	1	< 0.01
Plot 53	species	128.1	3	< 0.01
	JC versus JXC	120.5	1	< 0.01
	year	6.8	2	0.03
	2012 versus 2013	0.09	1	0.77

Table 4. Heritability estimates from Bayesian and likelihood analyses.

Method	Plot	No. families	H^2 (error)
Bayesian	All	267	0.62 (0.58, 0.67)
	44	149	0.11 (0.00, 0.35)
	51	193	0.53 (0.46, 0.61)
	53	85	0.71 (0.63, 0.78)
Likelihood	44	88	0.00 (0.00)
	51	195	0.66 (0.05)
	53	98	0.64 (0.16)

Note: Ninety percent credible interval reported for mean narrow-sense heritability for the Bayesian analyses. Cullis heritability estimates with standard error reported for likelihood analysis.

gin of screened families (Fig. 4). Family locations were determined by the origin of the mother tree, regardless of whether seed was collected from trees in the wild or from within plantings (see Table S5 for the number of families per state). In some cases, seed from the same family was collected from clonal trees planted in multiple locations. The ratings of the five most resistant hybrid butternut families and the three most resistant butternut families (confirmed by genotyping) are reported in Fig. 5.

4 Discussion

4.1 Disease incidence and severity

We found variation in disease incidence and severity across the three field trial sites consistent with previous studies (Boraks and Broders 2014; LaBonte et al. 2015; Sambaraju et al. 2018). This variation was particularly pronounced between the two planting locations, Martell Forest in West Lafayette, IN (plots 51 and 53; north central Indiana) and SEPAC in Butlerville, IN (plot 44; southeastern Indiana). While both locations are surrounded by natural forest and agricultural lands and contained a similar composition of planted hardwood trees, including butternut, black walnut, and red oak, BCD was much more pronounced at Martell Forest. This could be due to higher inoculum levels at Martell Forest versus SEPAC (Jones et al. 2014). While inoculum levels were not assessed as part of this study, other butternut plantings at Martell Forest were previously inoculated with *O. clavigignenti-juglandacearum* (Brennan et al. 2020) potentially increasing the amount of inoculum present in the area. Environmental variation may also be contributing to the observed differences in disease development and tree survival. For example, LaBonte et al. (2015) demonstrated that drier, upland sites were associated with a higher likelihood of butternut survival across a naturally occurring butternut stand in Wisconsin. Similarly, an analysis of butternut abundance and volume by Morin et al. (2018) found dry, upland sites to be the most favorable conditions for butternut growth and survival.

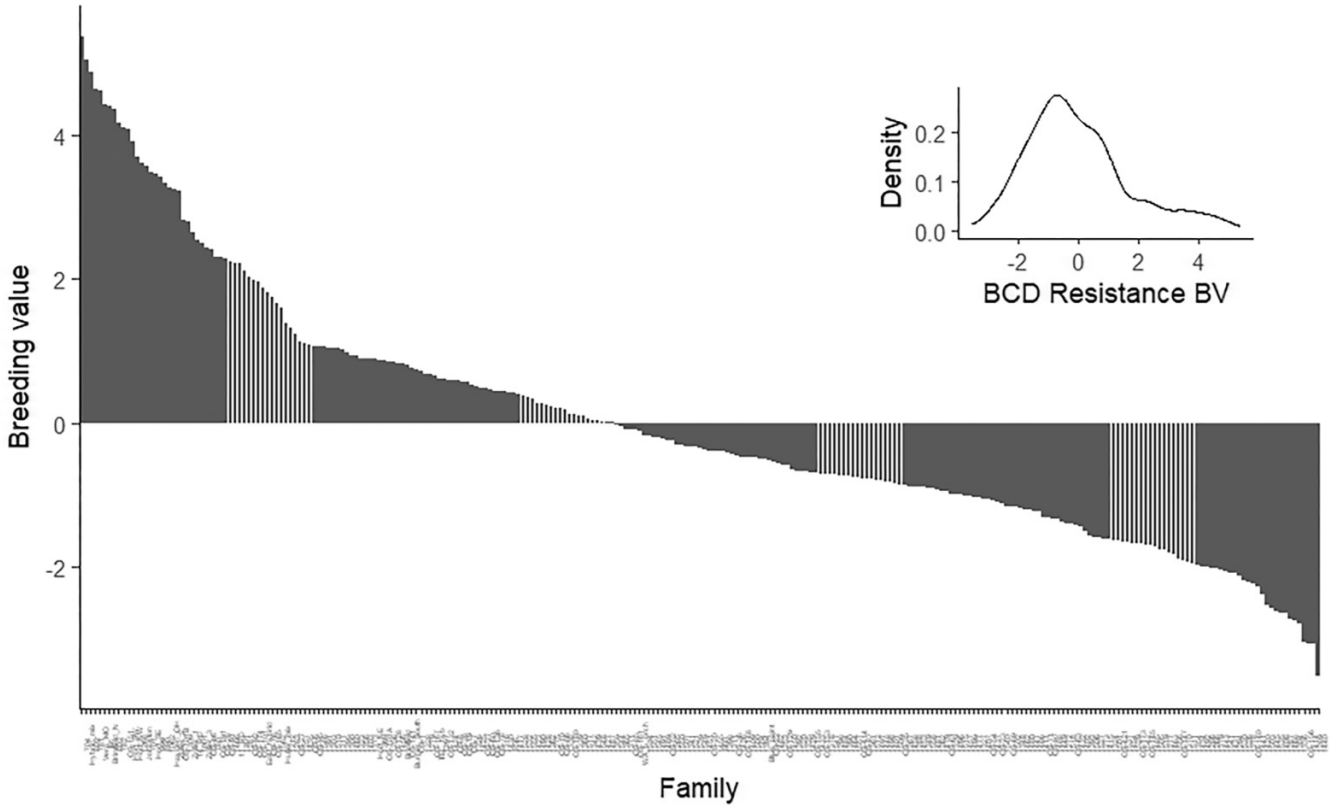
With the diversity of genetic material screened and other non-quantified differences between the planting sites (e.g., related to microclimate), it is not possible to determine exactly which factors contributed to the observed differences in disease incidence and severity in this study (Chellemi and Britton 1992; Dillon and Meentemeyer 2019). However, the observed variation highlights the importance of regular long-term monitoring of disease incidence and severity as part of butternut resistance screening programs. We demonstrated that genetically similar plantings, established at the same time (e.g., plots 44 and 51) can have drastically different levels of disease, which can influence field trial outcomes (Xu et al. 2022; Burns et al. 2023).

4.2 Factors associated with butternut canker disease resistance

Genetic ancestry (species group) was significantly associated with the probability of resistance in the logistic regression model that included all sites and in the models for plots 51 and 53 (high disease sites). Consistent with what is known about species susceptibility to BCD, Japanese walnut had a higher probability of being resistant than hybrid butternut or butternut. Interpretation of log of odds can be complicated, especially for non-numerical variables, so we used a Wald test to evaluate the significance of group predictors coefficients (Hosmer and Lemeshow 2000). Coefficients differed significantly between hybrid butternut and butternut in the model that included all plots and for the models for plots 51 and 53, which indicates different probabilities of disease resistance across species groups. This supports what was reported previously in the literature (e.g., Ostry and Moore 2007; Brennan et al. 2020). In general, Japanese walnut is more resistant than hybrid butternut and hybrid butternut is more resistant than butternut. However, on the low disease site (plot 44) ancestry was not a significant predictor of resistance. This finding highlights the importance of site quality on butternut establishment because butternuts from susceptible families may thrive if site conditions are not conducive for disease development or pathogen or infection levels are below some threshold (Holdenrieder et al. 2004; Goud et al. 2011).

We were able to evaluate the influence of planting year on the probability of resistance at one high disease site at Martell Forest (plot 53). Our results indicate that trees planted in 2012 and 2013 had a higher probability of resistance than trees planted in 2011. This could point to age or size-related differences in disease susceptibility (Simamora et al. 2017;

Fig. 3. Breeding value for each family based on the Bayesian model across all three plots. Positive breeding values indicate higher level of disease resistance, while negative value indicates higher susceptibility. Inset: histogram of breeding values.



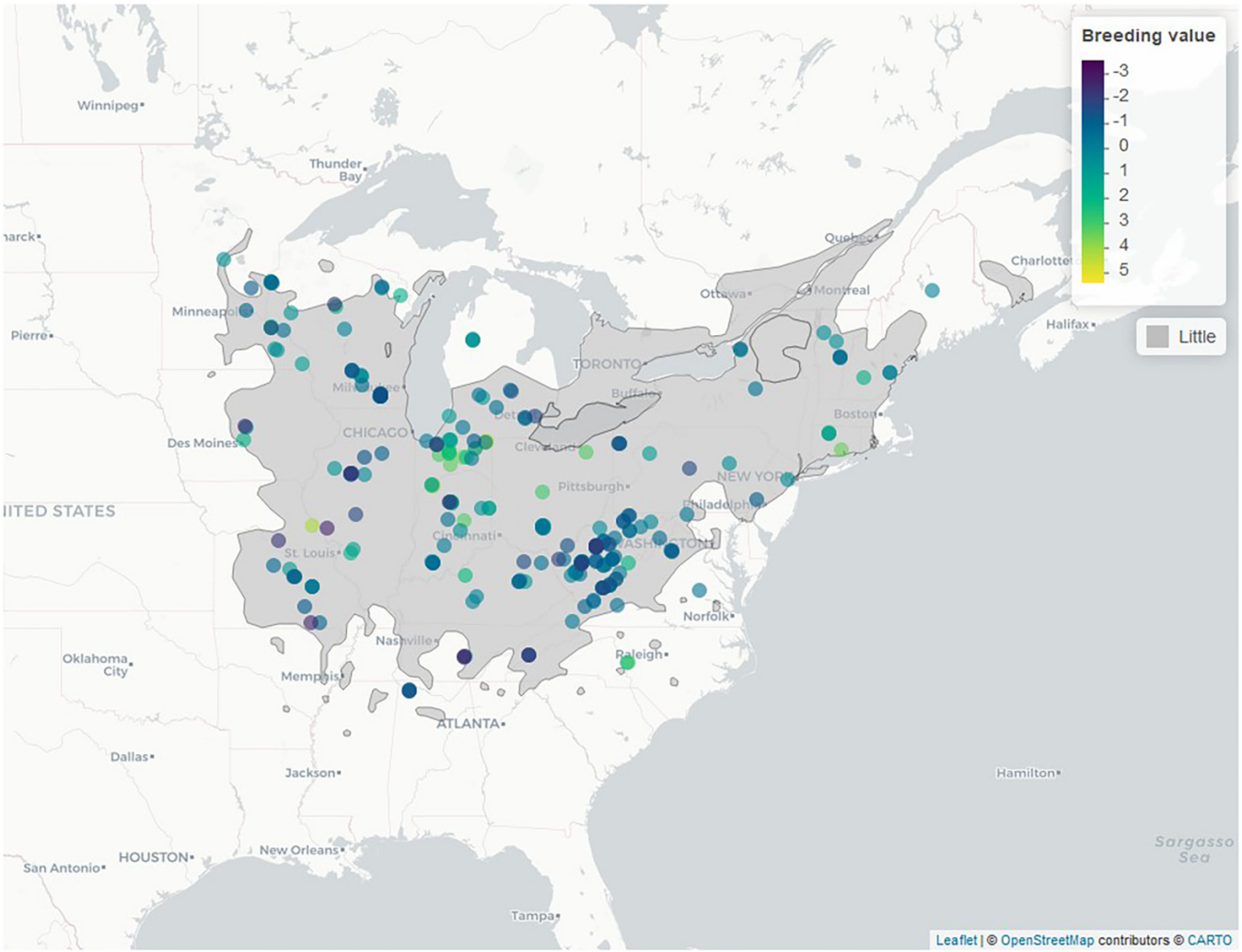
Sambaraju et al. 2018; López-Moral et al. 2022) or a difference in the genotypic composition of trees planted in 2012 and 2013 versus 2011. Hybrid butternut made up a larger proportion of the trees planted in 2012 and 2013 compared to 2011 (Fig. S4), which could contribute either directly (hybrids are generally more resistant to BCD) or indirectly (through influences on amount of inoculum present in the site) to the observed levels of disease susceptibility in those cohorts (Holdenrieder et al. 2004). It could also indicate that the disease needs time to build up on individual trees after planting.

4.3 Heritability of butternut canker disease susceptibility

For the first time, we report the field performance of a US range-wide collection of butternut and hybrid butternut, which are naturalized across the range of butternut (Hoban et al. 2009). Our study included observing over 300 open-pollinated butternut and hybrid butternut families for susceptibility to latent levels of inoculum. A previous study demonstrated weak heritability of disease-related traits in butternut (LaBonte et al. 2015). In contrast, we identified high levels of heritability across field trial plots (Bayesian model, $H^2 = 0.62$), with high heritability observed within plots with high disease incidence and severity (both likelihood and Bayesian models for plots 51 and 53, $H^2 > 0.50$). Heritability in plot 44 was low, likely because disease symptoms were not expressed due to low disease levels. The trees in LaBonte et al. (2015) were identified as butternut and came from one 15-ha hardwood woodlot, while our study contained a mix of butternut and hybrid butternut families, from diverse geographic sources, including 22 US states, planted within common gardens. These differences, particularly the presence of hybrid butternut, likely explain the observed differences in heritability of disease susceptibility in our study compared to LaBonte et al. (2015) and support an earlier study from our group that demonstrated genetic gains in 36 half-sib butternut and hybrid butternut families (Brennan et al. 2020). Furthermore, the low heritability in plot 44 (low disease site) indicates that selecting putatively resistant individuals within a low disease incidence and severity environment is unreliable. Another potential contributing factor could be variation in the virulence of naturally occurring Oc-j isolates. While this was not evaluated as part of the current study, both pathogen load and virulence could be considerations in future resistance screening efforts.

Our study confirmed that the most resistant butternut families are hybrid (McKenna et al. 2011; Brennan et al. 2020), which supports plans to use hybrid butternut as a key part of future butternut restoration efforts (Pike et al. 2021). More than half of the top 20 most resistant families had seed sourced from plantings versus the wild based on the Bayesian analysis across all plots. This could indicate butternut canker resistance can increase through cross pollination of trees in germplasm conservation seed orchards (Carson and Carson 1989).

Fig. 4. Locations of mother trees. Dot color indicates predicted breeding value from the Bayesian model across all plots. Positive breeding value is more resistant family; negative breeding value is more susceptible family. Butternut distribution in grey. Figures was created using the *mapview* R package (Appelhans et al. 2023) and assembled using butternut distribution data from the Atlas of United States trees (Little and Viereck 1971).



The material in our study has a regional bias: maternal parents were acquired from 22 US states, but approximately half of the families were from one of three states: Indiana (63 families), West Virginia (74 families), and Wisconsin (39 families). Fourteen of the top 20 families based on the Bayesian analysis across all plantings were from Indiana (Table 5). Previous studies in other forest tree systems have demonstrated variation in disease resistance associated with geographic origin (Hamilton et al. 2013; Štochlová et al. 2016; Yong et al. 2019). While we observed higher levels of disease resistance in families from the midwest, families originating in the northeast (particularly CT) and the south (namely NC) also had high levels of disease resistance. Geographic patterns should be viewed with caution considering the unbalanced geographic distribution of families in our study as well as the source of material (whether from the wild or plantings). Still, additive genetic variation was high enough to produce significant heritability's and a wide range of breeding values across the range (Figs. 3 and 4; Table 4). Future studies could identify plus-trees from local seed collection zones, or using other available metrics to develop seed orchards that are both locally adapted and have more favorable resistance (Potter and Hargrove 2012; Pike et al. 2020).

In general, hybrid butternut families had higher levels of disease resistance, but some butternut families had modest levels of resistance (Fig. 5) and included individuals that would be classified as resistant based on our current criteria (BCD rating <3). This is consistent with the finding of Brennan et al. (2020), which identified butternut families that performed better than some hybrid butternut families in terms of disease resistance. Even moderate levels of resistance in butternut may be sufficient to achieve success, if paired with selection of sites that are less favorable for disease development and/or more favorable for butternut growth and survival (LaBonte et al. 2015; Morin et al. 2018). However, further testing of the families in our resistance screening pro-

Fig. 5. Proportion of each rating group for the five most resistant hybrid butternut families (704, Hybrid mix, 802, 781, and Vera MO) and the top three butternut families (OS-76, OS-6, and 1300) that were confirmed with genotyping. N indicates number of trees per family that were rated. Rating of 1 is most resistant and 5 is most susceptible.

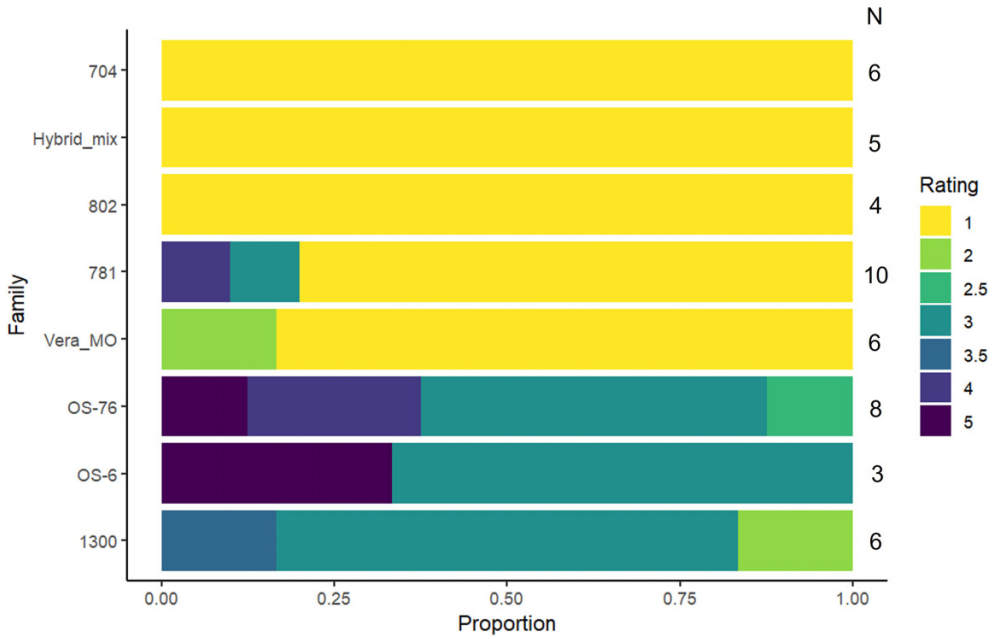


Table 5. Top 20 most resistant families based on a rank of breeding values (from most resistant to most susceptible) from the Bayesian and likelihood analyses across all plots.

Family	Bayesian rank	Likelihood rank	Species	State	Seed source
704	1	16	JXC	IN	planting
Hybrid mix	2	23	JXC	NA	unknown
802	3	15	JXC	IN	planting
781	4	24	JXC	IN	planting
Vera MO	5	21	JXC	MO	planting
Kc	6	18	JXC	IN	wild
Brimfield N	7	36	JXC	IN	wild
803	8	17	JXC	IN	planting
782	9	28	JXC	IN	planting
OS-151	10	32	JXC	NC	planting
Hort SW	11	26	JXC	IN	planting
Hort NW	12	28	JXC	IN	planting
1268	13	4	JXC	CT	wild
Johnston	14	48	JXC	IN	wild
701	15	38	JXC	IN	planting
Hort SE	16	23	JXC	IN	planting
999	17	42	JXC	IN	planting
890	18	41	JXC	OH	planting
1062	19	56	JXC	IN	planting
Hayden OH	20	61	JXC	OH	wild
OS-76	41	176	JC	NA	planting
OS-6	50	207	JC	MN	planting
1300	58	52	JC	IN	wild

Note: Species confirmed with genotyping. State is where mother originated and seed source, whether seed was collected from the wild or plantings. Top three butternut families confirmed with genotyping are also included.

gram, including studies with confirmed infection, are needed to confirm the results of this study and to determine the durability of resistance, that is whether resistant trees continue to stay resistant or succumb to the disease after repeated exposure to the pathogen or to different pathogen strains (Sniezko and Koch 2017).

5 Conclusion

The most disease resistant families in our study were composed of trees that were classified as hybrids with Japanese walnut, but some butternut families exhibited modest levels of disease resistance that may be suitable for butternut restoration in the future. Selection of suitable sites for restoration and resistance screening will likely be a key consideration going forward. Since disease susceptibility is heritable, resistance could be amplified with additional selection and breeding. Site had a considerable influence on disease incidence and severity and the expression of resistance among maternal families. Slight variations in the year trees were planted affected the probability of disease resistance. Future studies could examine the impact of tree age or number of years post-planting on disease susceptibility in the context of resistance screening programs, to identify the optimal time for disease screening. Repeated assessment of disease incidence and severity, over time may be needed since disease pressure and tree responses to infection are dynamic. This will be especially important if disease screening continues to rely on natural infections to select resistant trees. Further refinement of optimal site conditions and silviculture is also warranted to maximize restoration success in the future.

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Supplementary material

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