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Eight-year blight (*Cryphonectria parasitica*) resistance of backcross-generation American chestnuts (*Castanea dentata*) planted in the southeastern United States

Stacy L. Clark[a,*], Scott E. Schlarbaum[b], Arnold M. Saxton[c], Richard Baird[d]

[a] Southern Research Station, U.S. Department of Agriculture Forest Service, 2431 Joe Johnson Dr., Rm 274 Ellington Plant Science, Knoxville, TN 37996-4563, USA
[b] Department of Forestry, Wildlife, and Fisheries, The University of Tennessee, 2431 Joe Johnson Dr., Rm 274 Ellington Plant Science, Knoxville, TN 37996-4563, USA
[c] Animal Science Department, The University of Tennessee, 208C Brehm Animal Science Building, Knoxville, TN 37996-4574, USA
[d] Department of Biochemistry, Molecular Biology, Entomology, and Plant Pathology, Mississippi State University, Box 9655, Mississippi State, MS 39762, USA

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ABSTRACT

The loss of American chestnut (*Castanea dentata*) in eastern North America to chestnut blight, a disease caused by the fungal pathogen (*Cryphonectria parasitica*), has devastated ecological and utilitarian processes and functions. A backcross breeding approach has been developed to confer disease resistance to hybrid seedlings, and forest reintroduction trials will provide important information on performance and durability of resistance in real-world forest conditions. Three plantings were established in 2009 in mesic, even-aged regeneration harvests (site index averaged 23 m for *Quercus rubra*) and were examined for eight-year blight resistance. These plantings are the first forest field trials to test blight resistance of the most advanced breeding generation currently available, the third generation of the third backcross (BC_3F_3), against less advanced breeding generations (BC_1F_3, BC_2F_3), disease-resistant Chinese chestnut (*C. mollissima*), and disease-susceptible American chestnut. We also examined if *C. parasitica* infection was related to tree size and growth. The pathogen infected 36 percent of trees across locations by year 8, but 31 percent of trees died prior to detection of infection. Non-pathogen related mortality was probably due to factors that are typical of hardwood plantings, including repeated deer browsing and native and non-native pest damage. The BC_3F_3 generation exhibited resistance more similar to the Chinese chestnut than the American chestnut, but exhibited significantly lower resistance than Chinese chestnut at the location with the highest blight incidence; genetic family differences among BC_3F_3 progeny were significant at this location. Interactions between planting location and breeding generation affected resistance rankings, suggesting additional or longer-term testing is needed to determine resistance of a particular breeding line across a variety of sites. Probability of disease incidence was positively related to ground-line diameter (GLD), but this relationship depended on location and breeding type. At two locations, American chestnut had 50 percent probability of *C. parasitica* infection when GLD was approximately 70 mm, and the BC_3F_3 had 50 percent probability when GLD was between 93 and 126 mm. The Chinese chestnut maintained low probability of disease incidence (< 35 percent) across all GLD sizes, regardless of location. While a relatively high level of disease resistance was associated with the most advanced breeding generation, BC_3F_3, the plantings are too young to determine durable blight resistance.

1. Introduction

Nonnative diseases have altered ecosystem functions and processes in forests on every continent and have led to loss of biodiversity, ecosystem services, and economic markets (Lutts, 2004; Ellison et al., 2005; Holmes et al., 2009; Lovett et al., 2016). The American chestnut [*Castanea dentata* (Marsh.) Borkh.] was a keystone species across much of its former range in eastern North America until it was decimated by an Asian fungus, causal agent *Cryphonectria parasitica* (Murrill) Barr, reducing the species to remnant understory sprouts by the 1950s (Berry, 1959). The American chestnut provided ecosystem services such as mast production, carbon storage, and insect diversity (Opler, 1979; Diamond et al., 2000; Jacobs et al., 2009) and was important for tannin production, rot-resistant lumber, and as a commodity product and food

* Corresponding author.
E-mail addresses: stacyclark@fs.fed.us (S.L. Clark), tenntip@utk.edu (S.E. Schlarbaum), asaxton@utk.edu (A.M. Saxton), reb58@msstate.edu (R. Baird).

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source for Appalachian subsistence farmers (Ashe, 1911; Buttrick, 1915; Ziegler, 1920; Lutts, 2004).

Mitigation of resident exotic pests has often involved intraspecific or interspecific breeding for disease resistance, which can take many decades in tree species (Schlarbaum, 1999; Sniezko, 2006), and breeding for disease resistance in American chestnut, has been evolving for over 100 years (Van Fleet, 1914; Burnham et al., 1986; Anagnostakis, 2012). Asian *Castanea* Mill. species have moderate to high levels of resistance, with Chinese chestnut (*C. mollissima* Blume) exhibiting the highest resistance (Clapper, 1954). Remnant wild American chestnut and germplasm from early breeding programs of the United States Department of Agriculture (USDA), Connecticut Agricultural Experiment Station (CAES), and The American Chestnut Foundation (TACF) have been used to produce hybrid seedlings using a backcross breeding method (Burnham et al., 1986; Anagnostakis, 2001, 2012). Backcross breeding introgresses blight resistance from Chinese chestnut into a predominately American chestnut genome to produce trees that contain Asian genes for disease resistance, yet retain desirable American chestnut phenotype. After multiple breeding generations, including backcrossing and intercrossing, and selections for phenotype and blight resistance, the BC_3F_3 generation (third generation of the third backcross) was predicted to have sufficient resistance for forest test plantings (Burnham et al., 1986; Hebard, 2001, 2006; Anagnostakis, 2012).

The first forest reintroduction trials of BC_3F_3 progeny were established in 2009 (Clark et al., 2014a, 2016), and these tests can be used to confirm blight resistance found in orchard inoculation tests to further refine the breeding program (Hebard, 2005, 2006; Anagnostakis, 2012). Field trials may yield different pathogen resistance outcomes than orchard inoculation tests, because *C. parasitica* is naturally invading the site (i.e., no inoculations) requiring longer time periods for the fungus to infect the host. Additionally, blight infection and development are affected by environmental conditions and management practices, as found in native American chestnut growing in silvicultural clearcuts (Griffin et al., 1991) and American chestnut planted on surface mine restoration areas (Bauman et al. 2014; Skousen et al., 2018). Blight resistance of BC_3F_3 progeny was lower than an intermediate level in orchard inoculation tests (Steiner et al., 2017), and field performance of less advanced generations seems to vary depending on site conditions and management treatments (Skousen et al., 2013; Gilland and McCarthy, 2014; Pinchot et al., 2017; Thomas-Van Gundy et al., 2017). Long-term field testing across a diversity of site types will be necessary to accurately quantify blight resistance of experimental material (Griffin, 2000).

There have been a number of reintroduction studies of advanced hybrid generation chestnuts on mine reclamation sites (Bauman et al., 2014; Gilland and McCarthy, 2014; Skousen et al., 2018) and old fields (Schlarbaum et al., 1994; Anagnostakis and Pinchot, 2014). In contrast, there have been few studies using advanced hybrid seedlings in forested sites (Anagnostakis and Pinchot, 2014; Clark et al., 2016; Pinchot et al., 2017; Thomas-Van Gundy et al., 2017) that represent a large land type available for reintroduction (Jacobs et al., 2013; Clark et al., 2014b). The first reintroduction trials using BC_3F_3 seedlings (Clark et al., 2014a, 2016) were established to examine survival, growth, blight resistance, and competitive ability over time when planted in silviculturally treated forests in the Southern and Eastern regions of USDA Forest Service, National Forest System. *Cryphonectria parasitica* can survive and sporulate on remnant chestnut sprouts, and oak (*Quercus* L.) and red maple (*Acer rubrum* L.) stems (Fulton, 1912; Baird, 1991; Torsello et al., 1994), providing natural sources of inoculum. Blight resistance of BC_3F_3 was similar to Chinese chestnut in the fourth growing season of these forest reintroduction trials, but blight incidence was relatively low (Clark et al., 2016). To our knowledge, our study (Clark et al., 2016) represents the oldest study examining blight resistance of the BC_3F_3 generation planted in forest reintroduction trials. Our primary objective of this study was to quantify blight resistance of genetic families of the BC_3F_3 breeding generation planted in three forest reintroduction test sites over eight growing seasons. We had a secondary objective to examine natural pathogen infection, specifically to determine if the first infection could be predicted from previous tree growth or size.

2. Methods

2.1. Experimental material and study areas

The study was previously described in detail (Clark et al., 2016), but relevant information will be briefly summarized to meet current objectives. TACF's open-pollinated orchards in Meadowview, VA produced putative half-sibling BC_1F_3, BC_2F_3, and BC_3F_3 progeny from BC_1F_2, BC_2F_2, and BC_3F_2 mother trees, respectively (Hebard, 2006). American chestnut and Chinese chestnut were also obtained from TACF, with the latter being from controlled pollinations or isolated trees. American chestnuts were collected from remnant mother trees in northeastern VA which were assumed to be pure American chestnut due to distance from Asian seed sources and morphology of collected nuts. Hereafter, 'genetic family' refers to seedlings derived from nuts collected from a single open-pollinated or controlled–pollinated mother tree located at the TACF orchard or in a wild population. We abbreviated B_3F_3 family names as previously described (see Table 6 in Clark et al., 2016), and the remaining family names are consistent with those assigned by TACF (Hebard, 2012). We used three American chestnut families (GMNEW, PL1S, and Towers1), one Chinese chestnut family (AD), two B_1F_3 families (NB1 and NB34), two B_2F_3 families (SA330 and SA417), and five B_3F_3 families (D1, D2, D3, D4, and D5). The term 'generation/species' hereafter refers to the backcross breeding generation (BC_1F_3, BC_2F_3, or BC_3F_3), pure American chestnut, or pure Chinese chestnut that the progeny represent. Seedlings were grown by genetic family in a commercial tree nursery for one year using advanced nursery protocols to optimize overall size (Kormanik et al., 1994; Clark et al., 2012). Seedlings from each family were visually assessed and equally divided into two size classes (small or large) based primarily on size of the root collar (Clark et al., 2000, 2016).

Three forest stands managed by the Southern Region of the USDA Forest Service were selected as study locations, which are hereafter referenced by their USA postal code abbreviations for their corresponding state (NC, TN, or VA). A commercial shelterwood-with-reserve regeneration harvest that left a residual basal area of 2.3–4.6 $m^2\ ha^{-1}$ of overstory trees (trees greater than 14 cm in diameter at breast height) was implemented just prior to planting on each site. Stump sprouts of all species excluding oak, hickory (*Carya* Nutt.), cherry (*Prunus* L.) or native chestnut were treated with herbicide (triclopyr) to control stump sprout competition at the time of planting, and competitors were again treated using a basal bark herbicide release (triclopyr) in the early growing season of year 5. Accidental herbicide damage occurred at the VA planting that affected approximately 40 percent of trees in year 5. Damage included primarily stunted growth, and possibly death in two cases. No herbicide damage was detected at the NC or TN locations. Seedlings were planted on a 2.5 m by 2.5 m spacing in February or March 2009.

2.2. Field measurements and laboratory procedures

Tree height (1 cm) and ground-line diameter (0.1 mm) were measured annually in years 1–8 after planting in the dormant season (October-March) after bud set was complete using a standard height pole and a digital or dial caliper, respectively. Seedlings were evaluated annually (years 1–8) after planting for natural occurrences of *C. parasitica* infection (presence or absence) during the late growing season (August-September). Blight disease symptoms were identified as an ellipsoid–shaped canker on the stem that was sometimes accompanied by bark discoloration, fissuring, cankering or orange stromata

protruding through the bark surface (Berry, 1959; Griffin, 1986). In years 5–8, we measured the height on the tree's stem at the lowest point of *C. parasitica* infection, and we ranked cankers according to disease severity symptoms as follows: 4 = no visual evidence of disease symptoms; 3 = cankers were in early phases of development or were exhibiting signs of resistance, including a lack of orange stromata and usually accompanied by slight swelling at the site of infection; 2 = cankers had abundant orange stromata with a portion of the tree killed above the point of infection; and 1 = trees were assumed to have died from *C. parasitica* infection.

To confirm the cankers were result of *C. parasitica* infections, two bark samples (5 × 5 mm) were removed along margins on borders of questionable cankers, which primarily consisted of bark wounds with no visible stromata or probable cankers that appeared to be in the early stages of development. Generally, cankers accompanied by abundant stromata were not sampled. A total of 325 wounds were sampled. The bark samples were placed into microtiter plates so that they could later be identified as to specific cankers and locations on a tree. All samples were stored at 4 °C in the laboratory until processed using isolation procedures (Baird, 1991). The bark samples were surface disinfected in 0.525% w/v sodium hypochlorite solution for 10 min, rinsed in sterile distilled water and cultured onto glucose-yeast extract medium (GYE) in 10 × 1.5 cm Petri plates for 10-d at room temperature exposed to fluorescent light and diffused sunlight. Colonies were then transferred to potato dextrose agar (PDA, Difco Lawrence, KS). Colony morphology was verified after 10-d incubation at 20 °C under fluorescent light. When isolate morphologies were uncertain, molecular sequencing was conducted using ITS primers and sequencing methods as described previously (Baird, 2014). If needed, canker rankings were changed based on assay results. Changes were generally from a ranking of 4 (no blight) to 3 (early blight symptoms) or vice versa.

2.3. Experimental design and statistical analyses

In years 5–8, a yearly resistance ranking was assigned to each tree using the canker ranking described above, and the highest canker ranking was used for trees with multiple cankers. Although we did not measure canker lengths, this qualitative ranking system closely resembled that of the ranking system used following stem inoculations (Hebard, 2005). If the tree died-back completely from *C. parasitica* infection and produced a new sprout, a yearly resistance ranking value of 2 was recorded for the first and second year of the new sprout, but a value of 4 was assigned thereafter until new pathogen infection occurred. If the tree died before infection occurred, yearly resistance ranking was recorded as missing data for the year of mortality and each year thereafter.

In subsequent data analysis, 'year' refers to the number of growing seasons after planting (1–8), with year 9 used in analysis if the tree was alive in year 8 and/or pathogen infection was never detected, as described below. We produced an overall resistance value by summing the number of years the tree lived before being infected (1–9) with the number of years the tree lived before dying from blight infection (1–9). If a tree lived all 8 years and was never infected, an overall resistance of 18 was recorded (i.e., 9 + 9). A tree was assigned an overall resistance of 17 if it was infected in year 8 and lived all eight years (i.e., 8 + 9). If the tree died and evidence of blight was never recorded, overall resistance was recorded as missing data. Overall resistance ranged from 2 (tree died in year 1 from pathogen infection in year 1) to 18 (tree did not develop blight symptoms and lived all eight years).

We used a 5% alpha level to denote statistical significance for all tests and SAS was used to conduct all statistical analyses (SAS, 2012). Trees that died by from causes other than blight were not analyzed. Data were analyzed using a resolvable incomplete block design with single tree plots and a nested, factorial treatment arrangement. This design was used to accommodate the relatively high number of family by size class treatment combinations that occurred at each location (20–24). Incomplete blocks were used to control for environmental variation that changed rapidly, thus requiring blocks with fewer experimental units than the number of treatments. Incomplete blocks were grouped together within a location to form complete replications of each treatment combination (i.e., resolvable). Each incomplete block consisted of six trees that represented six different treatment combinations at each location. Treatment combinations within each incomplete block were arranged using Proc Optex in program SAS (2012). Planting location was treated as a fixed effect. Family was treated as a fixed effect nested within the fixed effect of breeding generation/species and location. All American and BC_3F_3 families could not be planted at each location, due to lack of available material (Clark et al., 2016). Seedling size class was a fixed effect cross-classified with generation/species and family.

We conducted analyses of variance to determine treatment effects on dependent variables: yearly resistance ranking for years 5–8, overall resistance, and height of the first blight canker. For yearly resistance ranking, year was included as a repeated measure, and we used an autoregressive covariance structure (Littell et al., 1998). Normality assumption of residuals was assumed if the Kolmogorov–Smirnov D-statistic was < 0.10; however, the Kolmogorov–Smirnov D-statistic was 0.23 for yearly resistance ranking. Since yearly resistance is essentially already rank-transformed, data were analyzed using the above linear models to produce non-parametric results (Conover and Inman, 1981). For overall resistance, normality was acceptable with Shapiro-Wilke estimates of 0.92 after a square transformation. Height to the first blight canker did not violate normality assumptions with a Shapiro-Wilke estimate of 0.98. For all models, homogeneity of variance assumptions were tested by examining plots of residual versus predicted values. Unequal variance was added to the overall resistance model by using the 'Group' option in the 'Repeated' statement, and denominator degrees of freedom were adjusted using the Kenward–Roger method. A likelihood ratio test was used to test whether the unequal variance model was justified. We computed comparisons among least-square means using Tukey's mean separation and macros (DAWG, 2011) were used to more easily identify differences by assigning associated letters to the means. Means were reported with the associated standard error (e.g., $\bar{x} \pm$ SE). The 'Slice' option in the 'Lsmeans' statement was used to test simple effects within interactions when significant.

Generalized linear mixed models (GLMM) with repeated measures (Proc GLIMMIX) was used to determine if fixed treatment effects (generation/species and planting location) and growth covariates (total stem height and total GLD; annual height growth and annual GLD growth of prior growing seasons) could predict the probability of the first *C. parasitica* infection. Negative height or GLD growth values due to stem dieback were entered as zero. Year after planted was included as a repeated measure with an autoregressive covariance structure. We specified a binary response distribution and modelled on event = 1 (blight symptoms present). Overdispersion of the residuals was checked using a Pearson chi-square test, and the model had a value approximating 1, indicating lack of overdispersion. We selected variables to include in the final GLMM by first testing candidate predictor variables in logistic regression models without repeated measures (Proc Logistic), and using model building techniques of Hosmer and Lemeshow (2000) and Menard (2010) to select the most parsimonious model. GLMMs often have convergence issues when too many effects are included in the model (Bolker et al., 2009), so selection techniques had to be conducted using logistic regression without the repeated measures effects before terms were entered into the GLMM. We first conducted univariate tests on candidate variables using logistic regression, and variables were entered into a preliminary model if the univariate test was significant at $P < 0.20$. We tested for multicollinearity, linearity, and interactions, and we compared several candidate models. The final model included variables and interactions that were significant ($P < 0.05$), and had the lowest corrected Akaike information criterion (AICc) value.

Table 1
Raw means of percent of trees with disease symptoms of *Cryphonectria parasitica* at each location by year and across all locations. Trees were excluded that died before evidence of blight infection was recorded. In parenthesis, top number refers to sample size used to compute the raw mean and bottom number refers to trees excluded from analysis because of mortality from unknown causes.

Year	NC	TN	VA	All
5	19 (290/54)	7 (320/124)	20 (294/75)	15 (904/253)
6	37 (281/63)	13 (311/133)	27 (277/92)	25 (869/288)
7	44 (277/67)	12 (306/138)	31 (252/117)	29 (835/322)
8	54 (262/82)	17 (304/140)	40 (237/132)	36 (803/354)

3. Results

3.1. Overall disease incidence

Cryphonectria parasitica infected 15 percent of trees in year 5 and 36 percent of trees in year 8 across all locations, excluding trees that died before they contracted the pathogen (Table 1). The NC location had the highest blight incidence across most years (19–54 percent of trees), and the TN location had the lowest (7–17 percent of trees). The 1 percent decrease in blight at the TN location from year 6–7 was due to a single Chinese chestnut tree infected in year 6 that was no longer infected in year 7 (assays conducted for both years). In year 5, 22 percent of trees were dead from causes that could not be linked to pathogen infection, increasing to 31 percent in year 8.

3.2. Yearly resistance ranking

All main treatment effects were significant for yearly resistance ranking of *C. parasitica*, and the interaction between generation/species and year were significant (Table 2 and Fig. 1). The BC_3F_3 generation was not significantly different from Chinese chestnut in years 5–7 but had a significantly lower ranking in year 8. The BC_3F_3 generation had a higher yearly resistance ranking than American chestnut all 4 years. The interaction between generation/species and location was also significant (Table 2 and Fig. 2). The TN location had no differences among generation/species. At the NC location, where blight was most prevalent, the BC_3F_3 generation had a higher yearly resistant ranking than American chestnut, but a lower ranking than Chinese chestnut. In VA, the BC_3F_3 generation was not significantly different from the Chinese chestnut, both of which had a higher yearly resistant ranking than American chestnut. At all locations, the three breeding generations (BC_1F_3, BC_2F_3, and BC_3F_3) did not differ in yearly resistance rankings.

Table 2
General linear model for yearly resistance ranking to *Cryphonectria parasitica* for years 5–8 after planting. NDF and DDF denote numerator and denominator degrees of freedom, respectively. Italicized P-values are significant at $P < 0.05$.

Source of variation	NNF	DDF	*F*	*P*
Location	2	820	33.07	*< 0.0001*
Generation	4	820	26.16	*< 0.00001*
Location × generation	8	820	3.83	*0.0002*
Size	1	820	10.09	*0.0016*
Location × size	2	820	0.02	0.9831
Generation × size	4	820	3.07	*0.0159*
Family (location generation)	17	820	2.85	*0.0001*
Size × family (location generation)	17	820	1.37	0.1446
Location × generation × size	8	820	0.84	0.5701
Year	3	2311	61.24	*< 0.0001*
Year × location	6	2311	8.34	*< 0.0001*
Year × generation	12	2311	3.68	*< 0.0001*
Year × location × generation	24	2311	1.4	0.0923
Year × family (location generation)	51	2311	1.31	0.0725
Year × size	3	2311	2.67	*0.0458*
Year × location × size	6	2311	0.53	0.7821
Year × generation × size	12	2311	1.01	0.4336
Year × size × family (location generation)	51	2311	1.12	0.2565
Year × location × generation × size	24	2311	0.89	0.6207

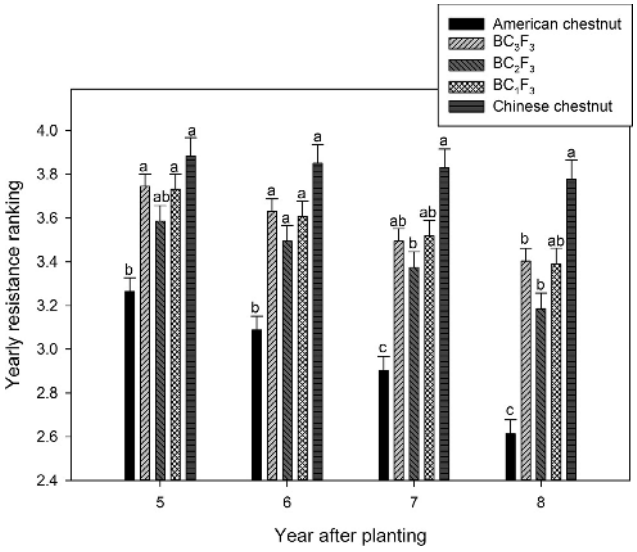

Fig. 1. Least-squares means and associated standard errors of yearly resistance ranking to *Cryphonectria parasitica* by year after planting and generation/species. Bars with the same letter are not significantly different within a year. Yearly resistance ranking ranged from 1 (tree died from blight infection) to 4 (no blight infection).

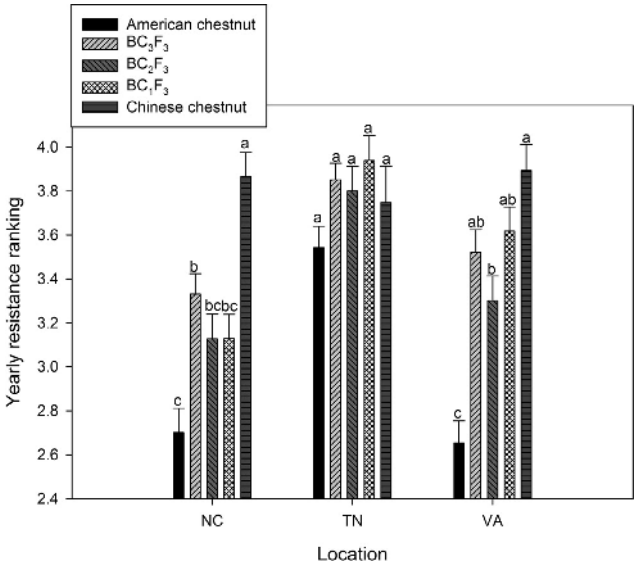

Fig. 2. Least-squares means and associated standard errors of yearly resistance ranking to *Cryphonectria parasitica* by location and generation/species. Bars with the same letter are not significantly different within a location. Yearly resistance ranged from 1 (tree died from blight infection) to 4 (no blight infection).

Family differences in yearly resistance ranking were not significant at the TN location (Table 3). At the NC location, family D4 was the only BC_3F_3 family that had a lower ranking than Chinese chestnut, and all three BC_3F_3 families were not significantly different than American chestnut family TOWERS1. At the VA location, all three BC_3F_3 families were not significantly different than the Chinese chestnut and American chestnut family GMNEW. The BC_3F_3 families were not significantly different than any families in the BC_1F_3 or BC_2F_3 generations, regardless of location.

The interactions between seedling size class with year and seedling

Table 3
Least-squares means of yearly resistance ranking (YR) and overall resistance (OR) to *Cryphonectria parasitica* for each genetic family at each location. Family means followed by the same lowercase letter are not significantly different within a location.

Generation	Family	NC		TN		VA	
		YR	OR	YR	OR	YR	OR
American	GMNEW	2.5 a	13.7 d	3.7 a	17.1 a	2.9 ab	14.7 bc
American	PL1S			3.4 a	16.3 a	2.4 a	13.1 c
American	TOWERS1	2.9 abc	15.1 bcd				
B1F3	NB1	2.9 abc	14.8 bcd	3.9 a	17.7 a	3.6 bc	16.7 ab
B1F3	NB35	3.4 bcd	16.5 abcd	4.0 a	17.9 a	3.6 bc	16.7 ab
B2F3	SA330	3.0 abc	15.1 bcd	3.7 a	17.3 a	3.0 ab	14.7 bc
B2F3	SA417	3.2 abcd	16.2 abcd	3.9 a	17.7 a	3.7 bc	17.1 ab
B3F3	D1			3.8 a	17.4 a		
B3F3	D2	3.7 cd	17.1 ab	3.8 a	17.4 a	3.4 bc	16.0 abc
B3F3	D3			3.9 a	17.4 a		
B3F3	D4	2.8 ab	15.0 cd	4.0 a	17.8 a	3.6 bc	16.7 ab
B3F3	D5	3.5 bcd	16.9 abc	3.8 a	17.3 a	3.5 bc	16.7 ab
Chinese	Chinese	3.9 d	17.5 a	3.7 a	17.4 a	3.9 c	17.7 a

size class with generation/species were significant (Table 2). Small-size class seedlings were not different than large size seedlings, except in year 8 when small-size class trees had a higher yearly resistance ranking (3.4 ± 0.04) than large-size class trees (3.2 ± 0.05). The American chestnut large-size class seedlings had a lower yearly resistance ranking (2.7 ± 0.09) than small-size class seedlings (3.2 ± 0.08), but none of the other generations/species had differences between size classes.

3.3. Overall resistance

All main treatments were significant for overall resistance to *C. parasitica* (Table 4). The interaction between location and generation was also significant, and the interaction between family and seedling size class was bordering on significance. The TN location had no differences in overall resistance among generation/species (Fig. 3). The BC_3F_3 generation was not significantly different than Chinese chestnut at the VA location, but was significantly less resistant than Chinese chestnut at the NC location. The American chestnut had lower overall resistance than the BC_3F_3 generation, and the BC_3F_3 generation was similar to the other two breeding generations (BC_1F_3 and BC_2F_3) at the NC and VA locations. Overall resistance differences among families followed similar trends as yearly resistance ranking with one exception (Table 3). In VA, BC_3F_3 family D2 had similar overall resistance as both American chestnut families (compared to only family GMNEW for yearly resistance) and to the Chinese chestnut family. Small size seedlings had higher overall resistance (16.8 ± 0.14) than large size seedlings (16.3 ± 0.15).

3.4. Height of first pathogen canker

The only treatment that affected height of the first canker was planting location ($F_{2, 46} = 9.99$, $P = 0.0002$). None of the other treatments or their interactions were significant or approaching significance ($F \leq 1.77$, $P < 0.1817$). The NC location seedlings had cankers that were higher on the stem (94 cm ± 7.3) than the VA (37 cm ± 13.1) or TN (33 cm ± 14.5) locations.

Table 4
Linear model for overall resistance to *Cryphonectria parasitica*. NDF denotes numerator degrees of freedom, and DDF denotes denominator degrees of freedom adjusted using the Kenward–Roger method. Italicized P-values are significant at $P < 0.05$.

Source of variation	NDF	DDF	*F*	*P*
Location	2	92	34.90	*< 0.0001*
Generation	4	228	26.59	*< 0.0001*
Location × generation	8	213	4.32	*< 0.0001*
Size	1	472	10.06	*< 0.0001*
Location × size	2	332	0.31	0.7311
Generation × size	4	227	1.92	0.1080
Family (location generation)	17	265	2.92	*< 0.0001*
Size × family (location generation)	17	264	1.6	0.0643
Location × generation × size	8	212	1.01	0.4291

3.5. Probability of first blight symptoms

The most parsimonious GLMM model to predict probability of the first disease incidence included location ($F_{2,1064} = 18.69$. $P < 0.0001$), generation/species ($F_{4,1064} = 9.65$, $P < 0.0001$), their interaction ($F_{8,1064} = 2.19$, $P = 0.0261$), and GLD one year prior to blight infection ($F_{1,4156} = 202.10$, $P < 0.0001$). The GLD was logarithmic transformed (base 10) to meet linearity assumptions. Inclusion of other covariates (e.g., total height, height growth, GLD growth of other prior growing seasons) did not improve the logistic regression model and these candidate variables were not included in the final GLMM. At all locations, GLD was positively related to probability of blight incidence for all generations/species (Fig. 4). American chestnut had the highest disease probability predictions across the range of GLDs in NC and VA, obtaining 50 percent disease probability at approximately 70 mm GLD (Fig. 4). The BC_3F_3 generation had the next highest predictions, obtaining 50 percent disease probability at 93 and 126 mm at the NC and VA locations, respectively. The Chinese chestnut had the lowest probabilities for blight infection across the range of GLDs at the NC and VA locations. At the TN location, the Chinese chestnut had the highest probabilities of infection across the range of GLDs, followed by the American chestnut; the BC_3F_3 generation had nearly identical predications to the BC_2F_3 generation, and the BC_1F_3 had the lowest probabilities. However, probabilities at the TN location were relatively low compared to the other two locations (< 40 percent).

4. Discussion

Reintroduction trials represent a culmination of over 100 years of research and breeding program development to produce a blight-resistant chestnut, and we provide preliminary evidence of success and challenges in achieving restoration goals. Our results, coupled with recent orchard inoculation tests (Steiner et al., 2017), suggest that the TACF breeding program was successful in transferring resistant genes to backcross progeny while maintaining desirable morphological and physiological traits of the American chestnut (Diskin et al., 2006; Knapp et al., 2014; Clark et al., 2016). Results are too preliminary, however, to make inferences on durable disease resistance.

Relatively low levels of blight were reported during the first four years in this study (Clark et al., 2016), but after eight years, natural disease incidence was sufficiently high to conduct preliminary

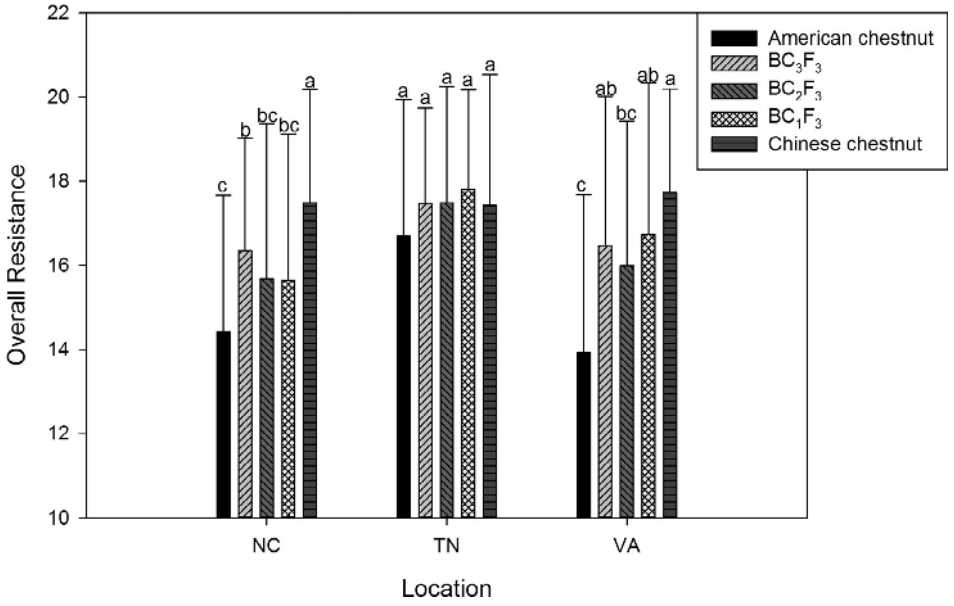

Fig. 3. Least-squares means and associated standard errors of overall resistance to *Cryphonectria parasitica* by location and generation/species. Bars with the same letter are not significantly different within a location. Overall resistance ranged from 2 (least resistant) to 18 (most resistant).

comparisons of pathogen resistance among backcross populations and controls (American and Chinese chestnut). Over time, the differences between Chinese chestnut and the other chestnut types became more pronounced, but differences among the hybrids remained relatively small (Fig. 1). Examined collectively across all locations, BC_3F_3 trees were more similar to Chinese chestnut than American chestnut trees according to both measures for blight resistance. These results differ from orchard inoculation tests that indicated BC_3F_3 progeny from TACF had resistance more similar to the American chestnut than Chinese chestnut (Steiner et al., 2017). Discrepancies between orchard and field trials were expected given that orchard tests have traditionally used inoculations of virulent *C. parasitica* strains (Hebard, 2012), and natural infections in our study were from unknown strains that will probably differ in virulence and compatibility (Anagnostakis et al., 1986; MacDonald and Fulbright, 1991; Double et al., 2014). Additionally, field tests will inevitably contain fewer families and smaller sample sizes than orchard tests, and natural infection in field tests will delay expression of resistance traits. Orchard inoculations typically occur when the trees are 2–5 years old (Anagnostakis, 2012; Hebard, 2012), whereas natural infection in various field plantings were below 25 percent in the first five years (Bauman et al., 2014; Clark et al., 2016; Thomas-Van Gundy et al., 2017). Previous studies have shown over 80 percent blight incidence in native American chestnut sprouts after 10 years (Griffin, 1989; Griffin et al., 1991).

Virulence of the fungus and resistance expression of the host will vary depending on tree size, longevity, health, and environmental factors, but relationships between the environment, time, and disease development have been largely understudied in American chestnut (Roane et al., 1986; Griffin, 2000; Griffin et al., 1991). Planting location affected blight incidence and survival among BC_3F_3 and parental species at forest reintroduction trials in West Virginia, presumably due to differences in soil texture and root rot from *Phytophthora cinnamomi* Rands. that stressed trees at one site (Thomas-Van Gundy et al., 2017). Soil preparation treatments that impacted competition and growth affected differences in blight incidence among American chestnut and hybrids in a surface mine restoration area (Bauman et al., 2014). In our study, site differences still occurred even among locations with similar productivity and treatments, indicating resistance testing in forest conditions will be inherently complex. BC_3F_3 seedlings had less overall resistance than the Chinese chestnut at one of three locations (Figs. 2 and 3), which may be because increased disease pressure (Table 1) allowed for expression of differences in resistance at this planting. This difference was not significant in year 4 when blight incidence was relatively low (Clark et al., 2016). Inferential diagnosis of mechanisms controlling the differences in disease incidence among locations are beyond the scope of this study. Exploratory analysis of relationships between edaphic conditions and blight resistance over time might yield some insight into interaction among environmental factors, host, and pathogen, but this has not yet been conducted.

We conducted competition control treatments prior to and several years after planting (see Section 2) in our plots to improve growth and survival, and potentially increase expression of resistance (Griffin, 1986). Competition control will increase growth of trees and decrease the time to bark fissuring and canker development (Griffin et al., 1991; Bauman et al., 2014). Previous research indicates that mesic sites with competition control, such as those in this study, afforded the best survival, perhaps due to reduced drought stress, improved growth, and less virulent blight strains (i.e., hypovirulent strains) (Griffin, 1986; Griffin et al., 1991, 2006). However, the competition control in year 5 caused accidental herbicide damage to some trees at the VA location. The herbicide damage probably stressed trees which can decrease resistance (Griffin, 1986). The herbicide release was applied to the entire plot and did not favor one breeding generation/species; therefore, damage probably weakened and reduced overall disease resistance of generation/species similarly. Results at the VA location were similar to those of the NC location, providing some antidotal evidence that the herbicide damage did not bias results.

The two types of disease-resistant systems, yearly resistance ranking versus overall resistance, yielded similar results despite differences in methodology. The advantages of the overall resistance system is that detailed observations of disease symptoms were not needed, only annual binary data were needed (presence or absence of disease symptoms). The overall resistance system combined two mechanisms of resistance, including time to initial blight infection, a measure of resistance to infection, and length of time the tree lived after blight infection, a measure of tolerance to disease. Both measures are important in determining durable resistance (Bingham et al., 1971). The yearly resistance ranking provides advantages in that it is a qualitative measure of reaction to the pathogen, similar to a ranking system used after inoculations (Graves, 1950; Hebard, 2005), and, therefore, may provide better comparisons to orchard tests than the overall resistance metric.

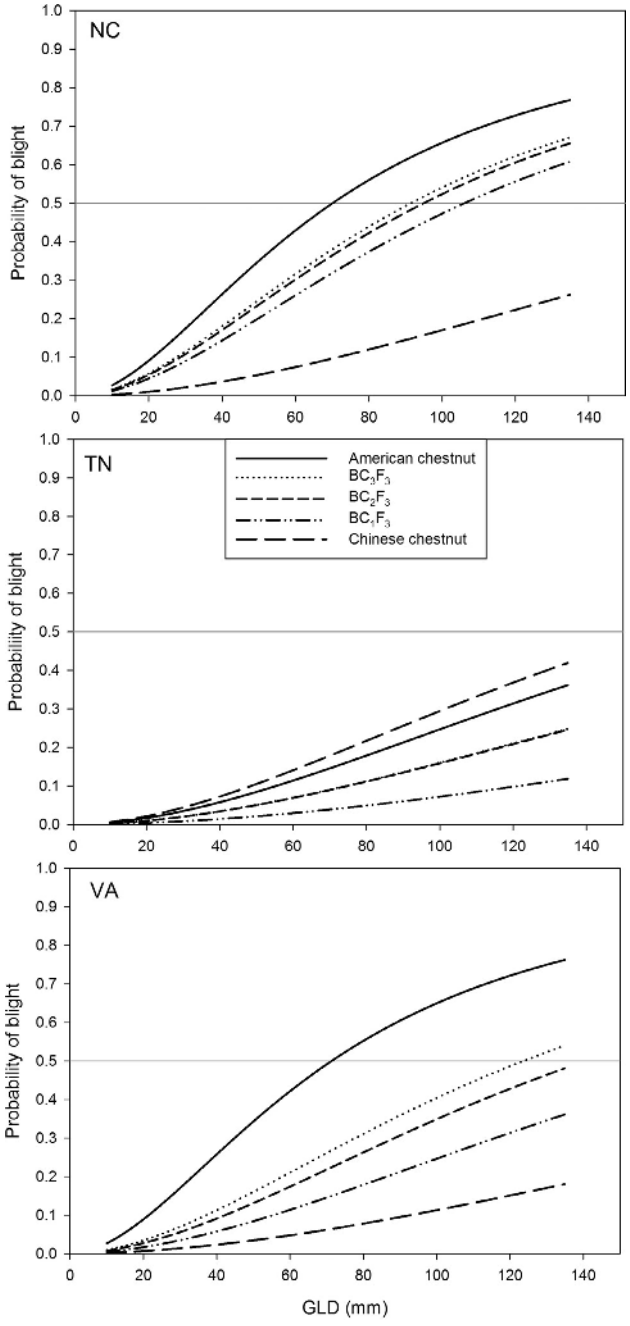

Fig. 4. Predicted probability of *Cryphonectria parasitica* infection by ground-line diameter (GLD) for each breeding generation/species at three locations. A reference line of 50 percent probability is shown.

The only significant difference among BC_3F_3 families were between families D2 and D4 at the NC location (Table 3), where D2 had higher yearly and overall resistance than D4. Family D2 exhibited relatively high blight resistance in orchard inoculation tests (Hebard, 2012; Jared Westbrook, The American Chestnut Foundation, personal communication) and in a forest reintroduction trial in West Virginia (Thomas Van-Gundy et al., 2017) providing limited evidence of agreement among orchard and field resistance scores. This family also had shorter heights in this study (Clark et al., 2016) and in two West Virginia plantings (Thomas-Van Gundy et al., 2017). Limited evidence of stability in family rankings over time was found at the NC location; families D2 and D5 exhibited the highest blight resistance rankings in year 4 (Clark et al., 2016) and in year 8 (Table 3). However, American family GMNEW was significantly more blight resistant than TOWERS1 in year 4, but these differences decreased by year 8 and were no longer significant. Unfortunately, the D1 and D3 families were only planted at one location (TN), where blight was relatively low, and assessment of family differences in blight resistance at this location are premature.

We did not find significant differences among American families, but some differences were relatively large. For example a difference of 0.4 in yearly resistance and 1.4 in overall resistance between families GMNEW and TOWERS1 at the NC location and similar differences between the GMNEW and PL1S families at the VA location were not statistically significant, but may represent important biological differences to programs breeding for low levels of resistance in American chestnut (Griffin, 2000; Griffin et al., 2006). However, more long-term data are needed because blight resistance in American chestnut are largely affected by virulence of blight strains that can change over time (Griffin et al., 2006).

Canker development will be affected by tree size (MacDonald and Thor, 1967; Paillet, 1984) but few studies have empirically examined this relationship, particularly for hybrid seedlings. Our results indicate that larger size class trees at the time of planting will have slightly higher disease incidence than small size class trees over time, probably because larger seedlings are more competitive, grow larger (Dey et al., 2008), and develop bark fissures more quickly, allowing easier infection into the cambium (Roane et al., 1986). Trees of any size, however, could be infected (data not shown). Our study shows that the first pathogen infection is also dependent on breeding generation/species in addition to tree size, with American chestnut trees developing disease symptoms at smaller sizes than backcross generation or Chinese chestnut trees (Fig. 4). Location of planting also affected the probability of the first blight infection, which was probably a function of blight pressure. At the TN location where blight incidence remained below 20 percent, trees had less than a 50 percent predicted probability of developing a canker, regardless of size or breeding generation/species.

Height of initial canker infection was not affected by any treatment except planting location, suggesting trees will develop initial blight infection at similar stem heights regardless of breeding type and initial seedling size. At the NC location, pathogen infection tended to occur higher on the stem (94 cm) than the other two locations (approximately 35 cm). These results cannot be easily explained and detailed investigations on natural canker development on planted trees, particularly hybrids, do not currently exist. MacDonald and Thor (1967) found that blight lesions occurred at approximately 60 cm on native American chestnut sprouts, with the majority occurring within the first 100 cm. Differences in course-scale topo-edaphic conditions may have contributed to differences in canker development among locations. Infection and disease development are affected by moisture and temperature (Griffin, 1986; Rigling and Prospero, 2017) that probably differed among locations at various canopy strata.

A challenge to this study was correct identification and confirmation of cause of canker formation by *C. parasitica* because infection was natural, not artificial, as is conducted in orchard inoculation tests. We overcame this challenge by prudent and annual observations of trees which was challenging in newly regenerated stands where dense vegetation may hinder view of the entire stem's bole and branches. Additionally, infections and canker formations are not always easily recognizable when damage from falling branches, insects, and mammal herbivory can damage cambium causing a wounding reaction that can sometimes have similar appearance to blight symptoms (e.g., swelling and bark discoloration) (Bauman et al., 2014). Additionally, we collected samples from hundreds of potential infections to confirm our observations, but this required substantial time and resources that may be limited in large-scale reintroduction trials.

5. Forest management implications

The breeding program successfully introgressed pathogen-resistance to the BC_3F_3 backcross generation, but resistance was not as high as the

Asian parent, particularly where disease pressure was most severe (e.g., NC location). Results varied by location, even though locations were selected that were similar in productivity and within the same broad geographic region, and TACF American chestnut parents used in the breeding program were from a relatively small geographic range (Hebard, 2001). Complex interactions between the plant host, environment, and *C. parasitica* suggest that future testing should be widespread and breeding selections should be based on many sites, not just a few as was conducted in this study.

Over 100 years of breeding developments in American chestnut have culminated to produce the first reintroduction trials using hybrid seedlings (Burnham et al., 1986; Anagnostakis, 2012; Clark et al., 2016), and our results indicate more work might be needed to improve resistance, mirroring results from orchard inoculation tests (Steiner et al., 2017). Additional orchard selections, progeny tests, and field testing are required before restoration efforts that involve substantial resources and infrastructure should begin (Steiner et al., 2017; Clark et al., 2014b). A transgenic chestnut is currently being developed, and will require similar rigorous testing as hybrid seedlings prior to investment of restoration activities (Newhouse et al., 2014).

If surviving backcross seedlings can maintain resistance to *C. parasitica*, many will be competitive and a part of the next stand, owing to their relatively fast growth rate in open conditions (Jacobs et al., 2009; Clark et al., 2016; Pinchot et al., 2017), but models suggest that pathogen-resistant chestnut populations will disperse relatively slowly, requiring multiple generations (Gustafson et al., 2017). Expression of resistance in hybrid seedlings can also change over time, but this is not well understood. Studies of pure American chestnut indicate disease incidence and tolerance were affected by weather conditions, canopy conditions, and blight strains, all of which are dynamic (Griffin et al., 2006).

Challenges other than blight will also impede chestnut restoration efforts. Although not a major deterrent to success in these plantings, root rot from *Phytophthora cinnamomi* has impacted other restoration test plots and early tests using pure American chestnut (Rhoades et al., 2003; Clark et al., 2014a; Pinchot et al., 2017). Other impacts in this study included deer (*Odocoileus virginianus*) browse, cicada damage, Asiatic oak weevil (*Cyrtepistomus castaneus*) (Case et al., 2017), herbicide damage, and the Asian gall wasp (*Dryocosmus kuriphilus*), contributing, in part, to the 31 percent mortality that was not related to *C. parasitica* (Clark et al., 2014a, 2016). Nonnative pests will continue to plague forests of the United States for the foreseeable future unless importation of nonnative plants are severely limited (Lovett et al., 2016); therefore, the American chestnut restoration program, like any breeding or genetics program, will require long-term, dedicated partnerships, funding, and infrastructure to achieve success (Clark et al., 2014b; Thomas et al., 2014; Wheeler et al., 2015; Sneizko and Koch, 2017).

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